

**EFFECTS OF SELECTED CARBON AND NITROGEN
RATIO IN BIOFLOC REARING SYSTEM FOR NILE
TILAPIA (*OREOCHROMIS NILOTICUS*) AND ASIAN
STINGING CATFISH (*HETEROPNEUSTES FOSSILIS*)**

A

Thesis

Submitted to the

*West Bengal University of Animal and Fishery Sciences
in partial fulfillment of the requirements for the Degree of*

Doctor of Philosophy

in

AQUATIC ANIMAL HEALTH

by

PRASENJIT MALI



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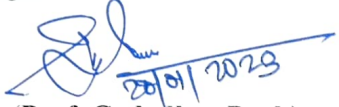
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CERTIFICATE

This is to certify that the work recorded in the thesis entitled “**Effects of selected carbon and nitrogen ratio in biofloc rearing system for Nile Tilapia (*Oreochromis niloticus*) and Asian stinging catfish (*Heteropneustes fossilis*)**” submitted by **PRASENJIT MALI**, Registration No. 1055 of 1998-1999, in partial fulfilment of the requirement for the degree of **Doctor of Philosophy (Aquatic Animal Health)** in the Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, is the faithful and bonafide research work carried out under my supervision and guidance. The results of the investigation reported in this thesis have not so far been submitted for any other degree or diploma. The assistance and help received during the course of the investigation have been duly acknowledged.

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The research work embodied in this Thesis entitled “**Effects of selected carbon and nitrogen ratio in biofloc rearing system for Nile Tilapia (*Oreochromis niloticus*) and Asian stinging catfish (*Heteropneustes fossilis*)**” has been carried out by me for the degree of **Doctor of Philosophy (Aquatic Animal Health)** in the Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, India. The manuscript has been subjected to plagiarism check by “**Ouriginal software**”. The percentage of plagiarism identified in the entire Thesis is 4% (Introduction - 0%, Review of Literature - 4%, Materials and Methods - 0%, Results and Discussion - 0%, Summary and Conclusion - 0%). The work submitted for consideration of award of Ph.D. is original.

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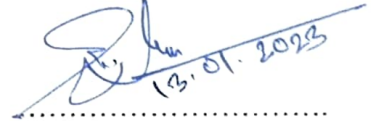
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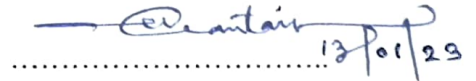
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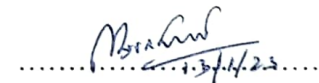
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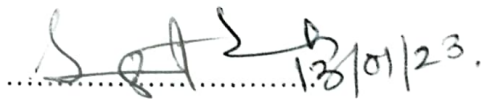
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LIST OF ABBREVIATIONS

µg/g	Microgram per gram
µl	Microlitre
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
APHA	Americal Public Health Association
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BAL	Belize aquaculture farm of central America
BFT	Biofloc technology
BV	Biofloc vloume
BW	Body weight
C/N or C:N	Carbon to nitrogen ratio
CAT	Catalase
CD	Degeneration of cytoplasm
CF	Condition factor
CFU	Colony forming unit
CH	Cellular hypertrophy
cm	Centimeter
CO ₂	Carbon dioxide
COVID-19	Coronavirus disease 2019
CuSO ₄	Copper sulphate
CV	Vacuoles in cytoplasm
d	day / days
DE	Degeneration of epithelium
DL	Damage in lamina propria
dl	Decilitre
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DoF	Director of Fisheries
DPX	Dibutyl phthalate xylene
DWG	Daily weight gain
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agricultural Organization
FBL	Final body length
FBM	Final body mass
FBW	Final body weight
FCO	Fermented Organic Carbon
FCR	Feed conversion ratio
FER	Feed efficiency ratio
FeSO ₄	Ferrous sulphate
FWG	Final weight gain
GIFT	Genetically Improved Farmed Tilapia

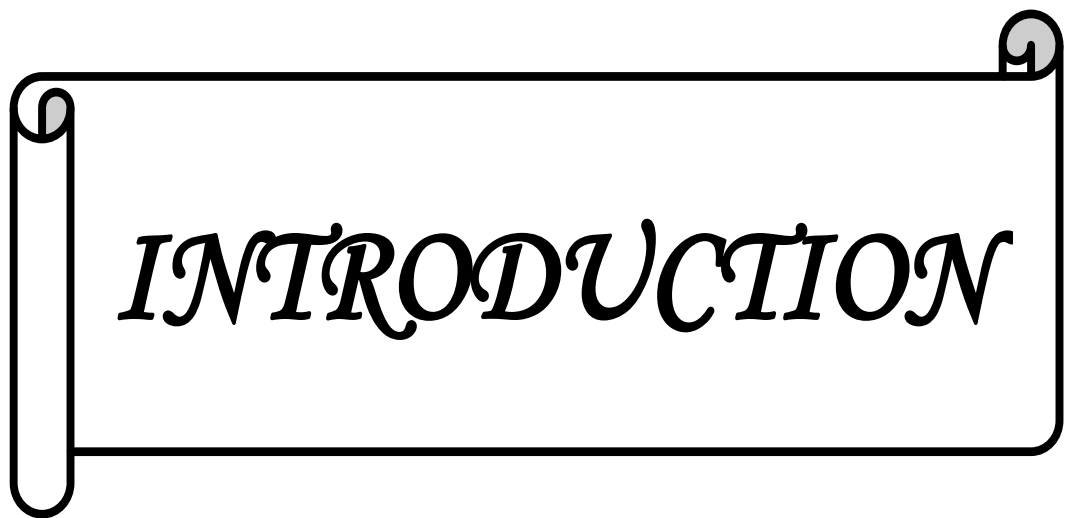
gm	Gram
GSH	Glutathione peroxidase
GSI	Gastrosomatic index
h	Hour
H&E	haematoxylin and eosin
H ₂ O ₂	Hydrogen peroxide
H ₂ S	Hydrogen sulfide
H ₂ SO ₄	Sulphuric acid
ha	Hector
Hb	Hemoglobin
HBSS	Hank's balanced salt solution
HCL	Hydrochloric acid
HDPE	High density polyethylene
HP	Horse power
HSI	Hepatosomatic index
Ht	Hematocrit
IBL	Initial body length
IBM	Initial body mass
IBW	Initial body weight
ICAR	Indian Council of Agricultural Research
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IMC	Indian major carp
IU/l	International units per litre
IUCN	International Union for Conservation of Nature
K ₂ Cr ₂ O ₇	Potassium dichromate
kg	Kilogram
KH ₂ PO ₄	Monopotassium phosphate
KVK	Krishi Vigyan Kendra
l	Litre
LB	Loss of brush border structure
LPM	Liter per minute
LV	Loss of absorptive vacuole
m	Meter
M	Molarity
MAMPs	Microbe-associated molecular patterns
MD	Mucinous degeneration
MDA	Malondialdehyde
mEq	Milliequivalent
min	Minute
mg	milligram
ml	Millilitre
mM	MilliMolar
MMC	Melanomacrophage centres
MPO	Myeloperoxidase
MT	Million tones
N	Normality
N ₂ H ₄	Hydrazine
NA	Necrotized area
Na ₂ CO ₃	Sodium carbonate
Na ₂ SO ₄	Sodium sulphate

NaOH	Sodium hydroxide
NEDD	N-(1-naphthyl) ethylene diamine dihydrochloride
ng	Nanogram
NKM16	Neel Kranti Mission, 2016
NO ₂ -N	Nitrite nitrogen
NO ₃ -N	Nitrate nitrogen
OD	Optical density
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate buffered saline
PER	Protein efficiency ratio
PHB	Poly-β-hydroxybutyrate
ppm	Parts per million
ProPO	Prophenoloxidase
RBC	Red blood cells
RBT	Respiratory burst test
ROS	Reactive oxygen species
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
SGR	Specific growth rate
SLP	Swollen lamina propria
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences
SR	Survival rate
TA	Total alkalinity
TAN	Total ammonia nitrogen
TDS	Total dissolved solids
TEC	Total erythrocyte count
TFI	Total feed intake
THC	Total heterotrophic count
TL	Total length
TLC	Total leucocyte count
TMB	Tetramethyl benzidine hydrochloride
TP	Total protein
TSS	Total suspended solids
USD	United States Dollar
w	Weight
WBC	White blood cells
WG	Weight gain
WHO	World Health Organization
ZEAH	Zero exchange autotrophic heterotrophic systems
ZnSO ₄	Zinc sulphate

ABSTRACT

The biofloc technology (BFT) has gained popularity in aquaculture sector during last decade due to its ability towards bio-security, growth, feed utilization, health and cost effectiveness. To understand the present scenario of BFT in West Bengal, a preliminary survey was conducted first with the help of randomly selected 110 biofloc farmers from 13 districts. *Oreochromis niloticus* (Nile tilapia) was found highest reared species followed by others including *Heteropneustes fossilis* (Singhi). Majorities were failed to achieve expected profit due to mortalities. Only, 13.6% farmers were found satisfied. Synchronizing carbon to nitrogen (C:N) ratios with microbial dynamics was found the major gap. In this study the effects of different C:N ratios in jaggery based BFT on water quality, growth, feed performances, welfare, body composition of both *O. niloticus* and *H. fossilis* were investigated in a separate 120 d and 180 d trial, respectively. A completely randomized design (312.5 g/m³ in 4000 l) was used in triplicates, where a control group without biofloc and three BFT Tilapia (0.5±0.05 g) groups were reared in different C:N ratios i.e., 15:1(CN15), 20:1(CN20) and 25:1(CN25), respectively. Similarly, Singhi (5±0.05 g) were reared in control and BFT with C:N ratios of 8:1(CN8), 12:1(CN12) and 16:1(CN16) in another randomized design (2.33 kg/m³ in 1500 l) in triplicate. The fish were fed daily with commercial diet at 5-2% of their body weight. BFT groups were found better in all aspects than control. Highest survivability was found in lower C:N ratio groups. Highest final body weight and biomass were recorded as 160.35±2.16 g and 366.31±3.54 kg in CN15 of Tilapia, and 57.65±1.53 g and 38.92±1.21 kg in CN12 of Singhi groups, respectively. Feed performances among BFT groups for both were found insignificantly difference. Biofloc proximate showed higher protein and ash content with increasing C:N ratios. The CN15 followed by CN20, and CN12 followed by CN8 showed better results on assessment of stress and liver biomarkers, hematology, innate immunity, antioxidant properties, histological status of liver and intestines. Decreased dissolve oxygen, pH, ammonia nitrogen, nitrite and nitrate, and increased total suspended solids, floc volume and total heterotrophic count were recorded with increasing C:N ratios. Considering all aspects the C:N ratios of 15:1 and 12:1 were found most suitable for biofloc culture of Tilapia and Singhi, respectively.

CHAPTER - 1



1. INTRODUCTION

Expansion and intensification of aquaculture production is one of the top priorities among aqua culturist to mitigate the demand of earth increasing population which has reached 7.9 billion in 2021. The world fish production was estimated about 178 million metric tonnes (MMT) in 2020 (FAO, 2022) out of which India's contribution was 14.16 MMT (DoF: HFS-2020). There is no doubt that development of sustainable aquaculture is required to offer utmost production devoid of additional utilization of primary resources to avoid damage to the environment and also to achieve the most favorable cost-profit proportion in terms of social and financial aspect, simultaneously. For producing more aquatic crops without much expanding the usage of natural water and land resources as well as developing environment friendly sustainable aquaculture systems to provide reasonable returns for economic support and social sustainability, the biofloc technology had gained attentions during last few years (Ahmad *et al.*, 2017). Biofloc technology is presently known as one of the most attractive approaches in the aquaculture sector which can show an alternative pathway of sustainable aqua farming (Dauda *et al.*, 2018). In addition with maintaining ideal water quality in the fish culture ground, biofloc technology is able to reduce the need of culture water exchange and it also effective to drop the feed conversion ratio of cultured fishes by generating consumable microbial protein particles which is a mixture of bacteria, diatoms, algae, planktons, uneaten food particles and dead organisms. Biofloc technology is an environmentally friendly technique where nutrients can be recycled constantly based on the production of heterotrophic microorganisms.

The technology is based on correcting carbon to nitrogen ratio in fish culture water which sustain the water quality by absorbing nitrogenous substances produced by fishes, boost up culture feasibilities by dropping feed conversion ratio and feed costs simultaneously and finally plays an important role in fish welfare by competing with pathogenic organisms. Higher production of fish could be achieved by this zero or minimal water exchange intensive fish culture method (Emerenciano *et al.*, 2012). A complex interaction between organic matter, different microorganisms and physical substrate always take place in biofloc culture by which the system itself can provide 24 h supply of protein rich feed to the cultured species. The technology offered

enhance growth rate, lower FCR rate by supplying dietary protein feed and reduce supplementary feed costs (Avnimelech, 2009) which generally occupied the major of total production costs in any aquaculture practice (Dauda *et al.*, 2019). Besides that climate change had become the greatest threat to the human civilization during few decades, which resulted many droughts followed by limiting that valuable fresh water resources worldwide (Bakhshi *et al.*, 2016). Avnimelech (2009) projected that 70% of the global population would face water scarcity by 2050. Now a day's biofloc technology had been widely used for culturing numerous shrimp and fish species by microbial immobilization of ammonium with limited water exchange facilities (Ekasari *et al.*, 2015).

Providing alternative occupational area amidst and post COVID-19 pandemic situation has become major challenge at present. The National Fisheries Development Board, Hyderabad has implemented some key projects including biofloc technology for environmental sustainability, better management practices. Government of India approved the “Pradhan Mantri Matsys Sampad Yojana” with an estimated investment of 20,050 crores for up-coming five years with intention to bringing ‘Blue Revolution’. Biofloc technology also has got importance among this nation dream venture to mitigate the critical gaps in fish production in our country (Ranjit Singh, 2020). West Bengal Government also has put special emphasis on biofloc technology with the intention of employment generation during the COVID-19 pandemic situation (West Bengal Budget 2022-23). Azim and Little (2008) reported the biofloc technology could increase the fish production by around 45% over the common aquaculture systems. In reality majorities of the natural water resources are being utilized by both traditional and professional aqua culturist. Person don't have own water area, need huge investment for starting fish culture in pond, whereas setting up of a small biofloc unit on land with minimum investment is quite easy. Biofloc systems have several advantages but the system also has many operational drawbacks which can be overcome by proper management practices successfully. Lot of social media groups are very active to promote biofloc technology by presenting, how this intensified culture technique can be easily performed by ordinary man. Social media group for selling all biofloc equipments are also available and any one can get access there. Lot of private paid trainers throughout the West Bengal are also equally promoting it by their own efforts. As a result people already entered into that field with less study and absurd dream became upset. At this situation a survey has been

conducted to assess the present scenario of fresh water fish culture in biofloc tank in West Bengal. The survey not only uncovers the emerging problems, lacuna associated with that technique, but also would help to find out the possible remedies to mitigate all those critical gaps.

Still more researches are needed to be performed to establish the potential species under such system. Nile tilapia, *Oreochromis niloticus* has characteristics such as being omnivorous, compatibility of the digestive tract to absorb the bioflocs and the ability to tolerate high concentrations of suspended material in the water, resistance to low concentrations of oxygen and stress, and ability to be cultured at high densities that make it a good candidate species for culturing in the BFT system (Hwihy *et al.*, 2021 and Shourbela *et al.*, 2021). However, biofloc technology application for culture of Asian stinging catfish (Shingi), *Heteropneustes fossilis* is yet to be fully demonstrated. No proper documentation of biofloc culture on Singhi in terms of growth performances, body proximate composition, welfare issues along with water quality management have been done till date. Moreover, in general, limited studies have been conducted so far to investigate the potential of biofloc technology in augmenting the welfare of cultured animals, particularly in terms of immunity in India. Histological studies on digestive organs like liver and intestines of biofloc reared fishes are also very rare. All the previous studies related to welfare issues of biofloc reared fishes were performed at the end of the experiment but during the culture period it was not done properly.

Previous studies supported that aquatic organisms grown in a biofloc environment exhibited a higher total antioxidant capacity. The sugar is naturally loaded with antioxidants and vital minerals such as zinc and selenium, which prevent free radical damage and thus strengthen immunity and boost resistance against infections. Molasses is considered as the best carbon source due to its solubility but availability of molasses is not uniform throughout the West Bengal, whereas jaggery is easily available everywhere in West Bengal. Therefore this study needs to address the potential effects of jaggery as a carbon source with different ratio against nitrogen, on water quality, health condition, hematology, innate immune systems of Nile tilapia and Singhi reared in zero water exchange system. However, studies of the influence of the carbon to nitrogen (C:N) ratios of a biofloc system on inorganic nitrogen control, physiological and health parameters in Nile tilapia was done before but for Singhi it was not done. The physiological roles of the immune and antioxidant

systems are critical for fish to control their health and to ensure satisfactory growth performance in different environments. More researches are needed to investigate the effects of different C:N ratios on the growth performance, feeding performances, non-specific immune responses and serum antioxidative abilities of Nile tilapia and Singhi juveniles reared in biofloc systems and also to identify the optimum C:N ratio for Nile tilapia and Singhi culture in biofloc based system in Indian context. So understanding the present scenario of freshwater fish culture of biofloc system and standardization the proper carbon input to immobilize the available nitrogen in biofloc rearing system are urgently needed because the key parameters in successful BFT performance is the proper C:N ratio management (Avnimelech, 1999). Keeping all these points in mind the present studies are performed with the following objectives.

Objectives:

1. To study the present scenario of biofloc tank culture practices of *Oreochromis niloticus*, *Heteropneustes fossilis* and others freshwater finfish in West Bengal.
2. To evaluate the effects of Biofloc Technology (BFT) with different C/N ratios on growth and feeding performances of *Oreochromis niloticus* and *Heteropneustes fossilis*.
3. To investigate the effects of BFT with different C/N ratios on proximate composition of biofloc as well as harvested *Oreochromis niloticus* and *Heteropneustes fossilis*.
4. To study the effects of BFT with different C/N ratios on health issues of *Oreochromis niloticus* and *Heteropneustes fossilis*.
5. To find out the effects of BFT with different C/N ratios on histological alteration in liver and intestine of *Oreochromis niloticus* and *Heteropneustes fossilis*.
6. To assess the effects of BFT with different C/N ratios on water quality parameters during culture of *Oreochromis niloticus* and *Heteropneustes fossilis*.

CHAPTER -2



2. REVIEW OF LITERATURE

2.1. Present status of freshwater aquaculture production

Global fish production was estimated about 179 million tones (MT) in 2018. Out of which, 156 MT were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita. Aquaculture accounted for 46 % of the total fish production and 52 % of fish used for human consumption (FAO, 2020). In 2018, total worldwide capture fisheries production was recorded at 96.4 MT that was raise of 5.4 % from the earlier three years average production (FAO, 2020). Global aquaculture production attained ever time record of an estimated trade value of USD 263.6 billion on 2018 (FAO, 2020). In 2018, inland aquaculture produced 51.3 MT of aquatic animals, contributing of 62.5 % of the world's farmed food fish production, as compared with 57.9 % in 2000. In the period 1961–2017, the average annual growth rate of total food fish consumption increased at 3.1 %, outpacing world annual population growth rate i.e., 1.6 %. The COVID-19 pandemic has affected most countries in the world, with severe impacts on the global economy. FAO had projected that total fish production might expand from 179 MT to 204 MT in between the year from 2018 to 2030. Aquaculture production had been projected to reach 109 MT in 2030, an increase of 26 MT or 32 % over 2018. World food fish consumption in 2030 had been projected to be 18 % higher than in 2018.

Fish production in India was estimated to have reached about 14.16 MT in 2019-20 out of which 3.72 MT and 10.43 MT were obtained from marine and inland fish production sector, respectively. The percentage contribution of fisheries sector in Indian economy in the year 2018-19 was 1.24 % which was near about Rs. 212,915 core. Among inland fisheries production in India in 2019-20, Indian Major Carp contributed the major share of 5.9 MT. The others notable production was Exotic Carp (0.98 MT), Indian Minor Carp (0.48 MT), Catfishes (*Wallago attu* and *Pangasianodon sp.*) (0.43 MT), Murrels (*Ophiocephalus spp.*) (0.2 MT) and other fresh water fishes (2.3 MT) (Hand book on Fisheries Statistics 2020).

West Bengal stood second position in India after Andhra Pradesh for inland fish production with an estimated production of 1.61 MT in 2019-20. Marine production of West Bengal was 0.16 MT in the year 2019-20. In West Bengal the major inland fish production in 2019-20 were obtained from Indian Major Carp

(0.099 MT), Indian Minor Carp (0.005 MT), Exotic carp (0.019 MT), Murrels (0.025 MT), Catfishes (0.004 MT) and other fresh water fishes (0.03 MT) (Hand book on Fisheries Statistics 2020).

2.2. Nile tilapia, *Oreochromis niloticus*

Tilapia belongs to the family ‘Cichlidae’ and is a freshwater inhabitant in ponds, rivers and lakes. Internationally, tilapia is famous as abundant consumed cultured aquatic species. In the past, tilapia was mainly found in African and Middle Eastern provinces but later it was captivated worldwide and became attractive in Asian and American countries. Tilapia continued its rapid augment in global production from last decades (Elangovan *et al.*, 2019). Due to low price and gentle in taste it became an appropriate substitution for costly fishes. Approximately 40% of global freshwater aquaculture production was derived from tilapia production (Scorvo-Filho *et al.*, 2010). The Nile tilapia, *Oreochromis niloticus* was considered as one of the most essential species of fish in tropical and sub-tropical aquaculture (FAO, 2012). Tilapia was seemed to be hardy species which could raise and breed in a broad range of environmental conditions and also could tolerate stress (Siddik *et al.*, 2014). The mono-sex male tilapia was well known for better production and low management necessities (El-Sayed, 2002). Tilapia was also popularly known as ‘aquatic chicken’ as its rate of consumption had increased worldwide (Fitzsimmons, 2005).

Annual worldwide production of cultured tilapia had increased continuously in last few years (Ahsan *et al.*, 2013). Freshwater aquaculture consisted broad variety of systems from different point of view like economical side, infrastructure facility, specie selection and physical aspects. It consisted mostly of domestic ponds and small to average level fish producers that generated a variety of fish in polyculture systems for home and provincial consumption (Little *et al.*, 2016). Tilapia and striped catfish was considered one of the widely recognized freshwater aquaculture species for export and local consumption (Naylor *et al.*, 2021). Nile tilapia (*Oreochromis niloticus*) stood second in finfish production after the carps with a total global production of 3.7 MT worth about 6 billion USD (FAO, 2016). Nile tilapia was produced in more than 80 countries by adopting diverse culture systems starting from extensive to intensive practices (Norman-López and Bjørndal, 2009). Tilapia became a popular fish species for domestic markets in Asia, South America and Africa

(FAO, 2016). Global tilapia market had been expected to reach US\$ 9.2 Billion by 2027, from the tilapia market in 2020 of US\$ 7.9 Billion (DUBLIN, 2021).

2.3. Asian stinging catfish (Shingi), *Heteropneustes fossilis*

The Asian stinging catfish *Heteropneustes fossilis* (Bloch, 1794), belongs to the Heteropneustidae family (Siluriformes) commonly known as the “Shingi” among bengali speaking community in Bangladesh and India (Rahman *et al.*, 2019). It was widely spread in Bangladesh, India, Pakistan, Laos, Myanmar, Nepal, Sri Lanka, and Thailand (Talwar and Jhingran, 1991). Adults lived in ditches, ponds, swamps and in muddy rivers (Halwart and Gupta, 2004). *H. fossilis* was recognized for its delicious taste, high market price, high nutritional value and medicinal properties (Froese *et al.*, 2011 and Chakraborty and Nur, 2012). It was a significant food fish as it preserved high amounts of protein, iron (226 mg/100 g) and calcium (Saha and Guha, 1939) in compare to many other freshwater fishes (Alok *et al.*, 1993). *H. fossilis* was categorized as least concern in worldwide (IUCN, 2020). It possessed an additional respiratory or air breathing organ by which it could survive for few hours out of water. It also could exist in low oxygenated water and in moist mud for an indefinite period (Ali *et al.*, 2014). The stinging catfish was well known for its extremely painful sting to humans where a poison released from a gland on its pectoral fin spine. This species grow to a maximum length of 30 cm or 12 inches and was an important of local farmed fishes and also found in the aquarium trade (Froese *et al.*, 2011). Normally *H. fossilis* was carnivorous, but it might also responds to additional feed like trash fishes, oil cake, rice bran, slaughter domestic waste, compost and also bio-gas slurry mixtures (Dehadrai, 1978). Kohinoor *et al.* (2012) reported that during past few years *H. fossilis* had became progressively endangered species due to shortage of its natural habitats and breeding grounds for ecological alterations, decline natural water areas, massive utilization of pesticides in crop cultivation, industrial waste water discharge, hydrological changes of running water bodies for dam construction and lastly over exploitation by local peoples. Along with the aforesaid reasons, International Union for Conservation of Nature (IUCN) listed *H. fossilis* as one of the threatened species in India and Bangladesh due to climate changes and unscientific fishing practices in open water bodies (Khan *et al.*, 2000). So, to provide protection from extinction, conservation of the natural stocks, development of both traditional and alternative culture skill for *H. fossilis* was urgently required.

2.4. What is Biofloc?

The biofloc was the heterogeneous macro aggregates of suspended microorganisms which constituted consortium of floc forming microorganisms, particles, diatoms, filamentous algae, micro/macro invertebrates, protozoa, zooplankton, colloids, organic polymers, cations, fecal matters, uneaten feed and dead cells (Avnimelech, 2012 and Ekasari *et al.*, 2010). Biofloc system was a promising tool in fish a culture system that was continuing on the way to confirming sustainable aquaculture since last decades (Bossier and Ekasari, 2017 and Dauda *et al.*, 2018). It was an *in-situ* water treatment way (Vinatea *et al.*, 2018), where immobilization process of toxic nitrogenous substances was conducted by incorporation of the external carbon to the culture system to stimulate the development of heterotrophic bacterial community instead of altering the toxic ammonia to nitrites to nitrates (Dauda and Akinwale, 2015). It was an environmental friendly aquaculture system (Emerenciano *et al.*, 2013) where forced growth of heterotrophic bacteria was performed within the culture system by means of least or zero water exchange (Sgnaulin *et al.*, 2018). The biofloc culture technology was known by diverse names by different researchers which include: Zero exchange autotrophic heterotrophic systems (ZEAH) (Wasiolesky *et al.*, 2006), Suspended bacterial-based system or active sludge (Rakocy *et al.*, 2004), Suspended growth systems (Hargreaves, 2006) or microbial floc systems (Avnimelech, 2012). The process involved a stable carbon to nitrogen ratio to stimulate the growth of heterotrophic bacteria that utilized ammonia for its growth and discharged nitrogen gas from the culture system (Dauda *et al.*, 2018). A carbon to nitrogen ratio from 10:1–20:1 were able to stimulate the development of heterotrophic bacteria by converting the toxic ammonia from the culture system to the production of cellular protein (Avnimelech, 2012), which might serve as high-quality protein feed to the cultured aquatic species (Bossier and Ekasari, 2017). Biofloc technology could maintain water quality through uptake of ammonia to produce microbial proteins as an available food for the cultured species and thus it could lower food conversion ratio and minimize the feed cost.

2.5. Principal of Biofloc technology

The principle of this technique was based on the judicious use of nutrient recycling (Ray *et al.*, 2011) which completely depends on the maintenance of carbon nitrogen ratio to culture water (Avnimelech *et al.*, 2012). In the beginning

researchers gained the facts of carbon and nitrogen manipulation for the production of heterotrophic bacteria, which they used as feed in fish and shrimp culture (Avnimelech, 2009). In general proper ratio of the carbon nitrogen (C:N) were managed to stimulate the accumulation of heterotrophic bacteria to create microbial biomass (Avnimelech *et al.*, 1999). The supplemented carbon helped to hold the excreted ammonia from the cultured fishes or shellfishes (Avnimelech *et al.*, 1994) and altered the ammonia present in the water into bacterial biomass (Schneider *et al.*, 2006). In biofloc system the formation of high-quality dense single-cell microbial protein (Crab *et al.*, 2012) might function as bioreactor for controlling water quality (Avnimelech *et al.*, 1989) and simultaneously acted as protein food source for the cultured fishes and shrimps (Avnimelech *et al.*, 1994). Besides that the immobilization of toxic nitrogen species occurred in bioflocs far rapidly as because the microbial biomass production rate of heterotrophic bacteria were found 10 times higher in compare to the autotrophic nitrifying bacteria (Hargreaves, 2006). The basic principle of this technology lay on the flocculation of heterotrophic bacteria and algae within the system (Avnimelech, 2006).

2.6. Role of Microbes and Planktons in Biofloc Systems

A complex interaction existed among the diverse classes of complementary and competitive microorganisms in BFT, and also a range of stimulatory and inhibitory functions lay between algae and bacteria in the system (Pérez-Fuentes *et al.*, 2016). There were three major nitrogen conversion pathways involved in the aquatic system which included photoautotrophic, chemoautotrophic and heterotrophic systems. Photoautotrophic system normally existed in traditional pond systems where conversion of ammonia-nitrogen involved the uptake of ammonia-nitrogen (ammonia-N) by phytoplankton (algae). Chemoautotrophic system also called 'nitrification' where ammonia-nitrogen had been converted to less toxic nitrate-N but did not remove nitrogen from the system (Ebeling *et al.*, 2006). In heterotrophic conversion of ammonia-nitrogen, the microbial community in water shifted from microalgae and chemoautotrophic bacteria to heterotrophic bacteria, with increased of carbon to nitrogen ratio. The amplified carbon to nitrogen ratio improved the metabolic actions of heterotrophic bacteria, which not only could dominate that particular environment superior but also could convert nitrogenous metabolites into consumable biomass (Crab *et al.*, 2012).

Table 2.1. Comparison between the photoautotrophic, chemoautotrophic and heterotrophic microbes.

	<i>Photoautotrophic</i>	<i>Chemoautotrophic</i>	<i>Heterotrophic</i>
<i>Removal of Ammonia-nitrogen</i>	Removed through uptake by phytoplankton	Converted to less toxic nitrate	Converted to consumable body biomass
<i>Dominated community</i>	Dominated by algae	Dominated by nitrifying bacteria	Dominated by heterotrophic bacteria
<i>Oxygen dependence</i>	Dominates at lower dissolved oxygen	Requires higher DO compared to photoautotrophic	Requires maximum amount of oxygen
<i>Role of carbon nitrogen ratio</i>	Less favoured at higher carbon to nitrogen ratio	Supported by intermediate carbon to nitrogen ratio	Most favoured at higher carbon to nitrogen ratio
<i>Light dependence</i>	More active during day time when the photosynthesis is high	Does not depend on photosynthetic activities	More active in limited light

2.7. History of Biofloc technology

Historically French Research Institute for Exploitation of the Sea, Oceanic Center of Pacific first introduced biofloc technology (BFT) with culture of different penaeid species at Ifremer-COP in early 1970s (Avnimelech *et al.*, 1989). Very soon it became a accepted technology for the intensive culture of *Oreochromis niloticus*, *Penaeus monodon*, Pacific white shrimp *Litopenaeus vannamei*, giant freshwater prawn *Macrobrachium rosenbergii* (Prajith and Kurup, 2011), *Fenneropenaeus merguensis* and *Litopenaeus stylirostris* (Aquacop, 1975). At the similar era, Ralston Purina in tie with Aquacop developed a system based on nitrifying bacteria by keeping shrimp in entire darkness, which was applied to *L. stylirostris* and *L. vannamei* in Crystal River (USA) and Tahiti (French Polynesia) for biofloc shrimp culture on profit (Rosenberry, 2010). During the year 1980, Ifremer initiated a French scientific program ‘Ecotron’ to better understand such system. Several studies on BFT were conducted to explain the interrelationships within different fields like water, microbes and shrimp nutritional physiology. Israel and USA (Waddel Mariculture Centre) also started research and development programme in BFT with tilapia and white shrimp in the same period. Commercially BFT was started in 1988 in Sopomer farm in Tahiti (French Polynesia) by using 1000 m² concrete tanks and limited water exchange where a world record production of 20–25 ton/ha/year with two crops was achieved (Aquacop, 1993) and also in “Belize aquaculture farm of central America”

or “BAL” (located at Belize, Central America), where production around 11-26 ton/ha/cycle was achieved in a 1.6 ha lined grow-out ponds.

The scientific concepts of BFT grew concomitantly and independently by two groups of researchers. One group was led by Steve Hopkins of the Waddell Mariculture Center, South Carolina, and the other group was guided by Avnimelech in Israel. In both cases the conventional external biofilter or high water exchange systems in aquaculture was replaced by the concepts of active microbial suspension and heterotrophic feed web to generate an intensive microbial community for degradation and assimilation of organic residues gathering in the pond with limited water exchange condition (Hopkins *et al.*, 1993 and Avnimelech *et al.*, 1994). In spite of the progress of BFT with quite simple operation principles, this technology did not accepted by fish farmers until the 2000’s. Avnimelech (2009) pointed out that high turbidity caused by the bioflocs was the major reason for that hesitation which was against the principle that the aquaculture needs clear pond water. But gradually it was accepted worldwide for the reasons including; increased scarcity of freshwater, implementation of strict policy on the amount of wastewater discharge by developed countries (Boyd, 2003) and massive outbreaks of viral diseases of shrimp which was spread among the adjacent farms used same water source.

During the last few decades successful implementation of BFT was extended in Asia, Latin and Central America and small scale greenhouses in USA, South Korea, Brazil, China, Italy, Indonesia, Australia, India and others (Taw, 2010). Research institutes of several countries already put emphasis on applied fields of Biofloc technology such as culture management, energy kinetics, nutrition, feed managements, microbes identification, economics etc. (Emerenciano *et al.*, 2013). In Belize 13.5 MT shrimp/ ha was achieved by BFT model at the time which was also applied in Indonesia. A combined technology with BFT and partial harvest knowledge was done in Medan, Indonesia and better results were obtained during 2008 and 2009. After that biofloc technology became popular in Bali and Java. In Indonesia intensification of shrimp aquaculture was performed in ponds by biofloc technology covered with high density polyethylene (HDPE) lined and plastic sheet. BFT was initiated in Malaysia in a 1000 ha integrated shrimp farming project at Seitiu, Terengganu by Blue Archipelagio (Emerenciano *et al.*, 2013). Prof. Yoram from Israel, contributed vastly for the modification and promotion of biofloc technology and later the technique developed in Israel were adopted by other countries due to its

numerous advantages. BFT was also known by various names by different researchers like, Active sludge or suspended bacteria-based system (Rakocy *et al.*, 2004), Microbial floc system (Ballester *et al.*, 2010), Single-cell protein production system (Avnimelech *et al.*, 1989), Suspended-growth systems (Hargreaves, 2006), Zero exchange autotrophic-heterotrophic system (ZEAH) (Wasielisky *et al.*, 2006), Aerobic Microbial floc system (Yuvarajan *et al.*, 2018). In India the on farm trials of biofloc culture of *Macrobrachium rosenbergii* were conducted in Cochin University of Science and Technology, Kerala in 2006 (Hari *et al.*, 2006). Even the nursery rearing with the BFT gave a prospect for getting better larval stocking density and fish welfare than standard conventional culture systems (Prajith and Kurup, 2011). Researchers had elaborated this bifloc technology since the said innovative waste management technology to our country till date.

2.8. Social obligation for sustainable aquaculture

The fast growing demand for both capture and cultured fishes compelled the aquaculture industry to adopt more intensification through high stocking density. Subsequently the aquaculture sector intensified the application of artificial feeds which made it most cost-effective and waste producing industry. Alike other intensive food producing sector, commercial fish farming caused major environmental costs. It is needless to say that aquaculture development should be in a sustainable way which is nothing but the management and conservation of the natural resources and the direction of technical and conventional change in suitable approach to guarantee the continued satisfaction of human needs for both present and upcoming generations. Nations achievement for providing food and nutrition security to the population was considered as an important marker of wealth in the developing countries (FAO, 2018). During the last few years, adequate fish for human consumption were obtained in most developing countries by the capture fishery sector (Béné *et al.*, 2016). The space for capture fisheries were declining d by d and undoubtedly in future the capture fishery sector were unable to mitigate the gap of fish demand for the growing world population. Hence adaptation of sustainable aqua farming skills to erase the gap between fish supply and demand had become the major concern of the fisheries experts throughout the world (FAO, 2018). In most of the developing countries, aquaculture had been renowned as a faster way of improving livelihood security and financial development through the series of associated activities (Béné *et al.*, 2016).

However, the aquaculture sector was still to be developed in terms of productivity and quality inputs and culture technologies. The average per capita fish consumption in India was 9 kg/person/year against the global per capita fish consumption of 16 kg/person/year and 20 kg/person/year in developing countries (FAO, 2018). The World Health Organization (WHO) recommended for consuming at least 12 kg fish in a year. Fish was rich in easily digestible high-quality proteins and healthy fats and also a unique source of essential nutrients, including iodine, vitamin D, and calcium and long-chain omega-3 fatty acids. According to FAO (2010) approximately 16% of animal protein consumed by the world's population was originated from fish and over one billion people worldwide depend on fish as their main source of animal protein. Sustainable development should conserve land, water, plant and animal genetic resources and it should be technically correct, economically feasible and socially tolerable and must not degrade environment (FAO, 1991).

Biofloc culture technology was considered as an environment friendly system where *in situ* heterotrophic microorganisms or single cell proteins (SCP) were produced by conversion of uneaten feeds, feces and surplus nutrients. These single cell proteins were considered as nutritious food resources for cultured fish or shrimps in biofloc systems. The biofloc technology were also able to maintain good water quality by immobilization of ammonia (Emerenciano *et al.*, 2017), improved culture feasibility through reducing feed conversion ratio and supplementary feed costs (Avnimelech, 2012), ensured biosecurity by providing probiotic effect (Defoirdt *et al.*, 2004 and Crab *et al.*, 2012). The technology was a zero or nominal water exchange intensive aquafarming systems where high stocking densities could be maintained to gain high fish productivity (Emerenciano *et al.*, 2017). All these special features made BFT economically attractive to aquaculture investors (Avnimelech *et al.*, 2012). BFT was a potential management practice to achieve the 'triple win' goals i.e., higher fish production, attractive fish production systems and resourceful use of energy, water, land along with reduction of Green House Gas emissions (Ogello *et al.*, 2021).

To knock the huge potential for improvement of fisheries and for focusing attention to aquaculture, the Government of India launched the Pradhan Mantri Matsya Sampada Yojana on September 2020. This scheme would ensure a projected investment of Rs 20,050 crore up to the year 2025. The biofloc was one of the most promising innovative aquaculture techniques which might promote fish production and accelerate employment security.

2.9. Prospect of Biofloc culture in India

Biofloc technology (BFT) was considered as one of the promising sector for the new “blue revolution” in aquaculture. The term “Blue revolution” denoted the concept of rapid increase in the fish production and marine product quantity through package programme (Singh, 2020). “Blue revolution” was launched in India during the seventh five-year plan (1985-1990) and since then the Central Government had been sponsoring the Fish Farmers Development Agency (FFDA). It had brought improvement in aquaculture by adopting new techniques for fish breeding, rearing, marketing, export etc. where biofloc had been included recently. The target of Blue Revolution was not only to attain financial wealth of the nation and the fish farmers but also to put thrust on nutritional food security through sustainable aquaculture production through optimum utilization of water resources along with maintaining bio-security and environmental hazards. The Blue Revolution also known as The Neel Kranti Mission, which from onwards 2016 (NKM16) had taken initiation to develop modern world class aquaculture industry in India through multi-dimensional approach to every actions related with improvement of both inland and marine sector and increase the contribution of Indian fisheries in the world export (Singh, 2020). Another key goal of NKM 16 was to doubling the income of the fish farmers with inclusive involvement of the socio-economically weaker sections not compromising with biosecurity. West Bengal Government had taken initiation by introducing Bio-floc in the State and 29 numbers of Bio-floc units were being constructed with involvement of 1.20 crore as model basis (West Bengal Budget 2022-23).

Keeping this in mind the biofloc technology (BFT) might be able to play a key role to achieve the aim of blue revolution in our country. It had been already established that the application of biofloc technology in grow out systems of few aquaculture species could get better net production of 8–43%, compared to the non biofloc systems like traditional water exchange system, clear water system or re-circulating aquaculture system. Biofloc technology (BFT) had been effectively implemented in aquaculture mainly shrimp culture to get economical, environmental and marketing advantages over the conventional culture method. The facility of minimal water exchange and reduced supplementary feed input could make it an inexpensive sustainable future aquaculture technique. Fish culture with high stocking density usually required some waste treatment facilities whereas biofloc itself was a waste treatment system. Biofloc systems also could prevent the transmission of

disease causing organisms to a farm from incoming water. Biofloc systems used a counter intuitive approach which promoted solids and the associated microbial community to accumulate in water in presence of sufficient aeration and continues mixing for maintaining an active floc in suspension so that the water quality could be controlled. Management of biofloc systems was not as simple as it seems; however, to some extent few technical erudition was essential to run the system in positive and productive way (Azim and Little, 2008).

2.10. Different carbon sources used for Biofloc technology

Controlling the carbon nitrogen (C:N) ratio with addition of external organic carbon to aquaculture system was appeared to be one of the prospect intensive management ways to uphold production in low or zero-water exchange culture systems (Crab *et al.*, 2007 and Gao *et al.*, 2012). Carbon sources performed as a substrate for the microbial production but in reality the mode of action differs between different carbon sources (Avnimelech, 1999). Study already established that for every one gram (g) of carbohydrate, the carbon yield was 0.4 g (Megahed, 2010) and 20 g carbohydrates were required for the immobilization of one gram of mineral nitrogen (Avnimelech, 1999). But the availability of low-cost carbon source was a big concern to run this technology all over the world, because, easily available cheap carbon sources were required to minimize the input cost as much as possible in contrast to the net return. Diverse type of carbon sources, such as molasses, glucose, cassava starch, cornmeal, wheat flour, sorghum meal, sugar bagasse, sugar, rice bran, ground bread crumb, glycerol, and anhydrous glucose, were used to maintain C:N ratio, improving nutrient dynamics for enhancing the production of various shellfish and finfish (Yu *et al.*, 2020, Bakhshi *et al.*, 2018a and 2018b, Romano *et al.*, 2018, Dauda *et al.*, 2017 and 2018, De Lima *et al.*, 2018 and García-Ríos *et al.*, 2019). Different studies also showed that the combination of diverse pelletized plant meals (Taw, 2010) that contained high C:N ratio could also be useful to enrich the bioflocs (Azim *et al.*, 2008 and Browdy *et al.*, 2012).

The formation of flocs by different carbon sources were largely depends on their carbon content and degradation rate. Normally, simple sugars such as molasses were degraded quicker than complex sugars such as cassava starch, which, improved water quality by lowering concentrations of ammonia and accelerated the superior growth rate of beneficial microbial biomass (El-Sayed, 2021). Molasses was

considered the most widely used carbon sources in BFT systems including all larval, nursery, and grow-out phases due to its effectiveness in maintain better water quality for the sustainable aquaculture production (El-Sayed, 2021 and Samocha *et al.*, 2007). The BFT system was facilitated with reusing floc water for the intensive culture of detritivores fish and shellfish but the discharge of this nutrient-rich wastewater into the environment might create pollution. The key obstacles in the application of different sugars in BFT were the high investment costs and contest between the carbon sources. The majority of the sugars used in BFT were not used in many countries due to their high economic impact (Ekasari *et al.*, 2016).

Researchers were trying to exploit various sugars with unique features such as simple availability, low price and helpful bacterial assimilation to promote sustainable and commercial biofloc culture (Elayaraja *et al.*, 2020). The sugarcane product jaggery contains several essential components, including minerals (*viz.* Calcium: 40-100 mg, Magnesium: 70-90 mg, Potassium: 1056 mg, Phosphorus: 20-90 mg, Sodium: 19-30 mg, Iron: 10-13 mg, Manganese: 0.2-0.5 mg, Zinc: 0.2- 0.4 mg, Copper: 0.1-0.9 mg and Chloride: 5.3 mg per 100 g of jaggery), vitamins (*viz* Vitamin A: 3.8 mg, Vitamin B1: 0.01 mg, Vitamin B2: 0.06 mg, Vitamin B5: 0.01 mg, Vitamin B6: 0.01 mg, Vitamin C: 7.00 mg, Vitamin D2: 6.50 mg, Vitamin E: 111.30 mg, Vitamin PP: 7.00 mg), protein (0.28%), carbohydrates (Sucrose: 72-76 g, Fructose: 1.5-7 g, Glucose: 1.5-7 g), water 1.5-7 g, and calories 312 mg (Singh *et al.*, 2013), which was used as sweeteners and dietary feed supplements for livestock (Singh, 2001). Jaggery had been used as a new carbon source for shrimp biofloc culture which showed a noteworthy raise in microbial growth, nutritional values of floc content and also gave hopeful results in growth performance and survivality of shrimp similar to the other carbon sources like molasses and sugar (Sakkaravarthi and Sankar, 2015). Elayaraja *et al.* (2020) showed the potential effects of jaggery as carbon source on water quality, growth performance, innate immunity and disease resistance to bacterial infection in Nile tilapia reared in zero water exchange system. Singh *et al.* (2009) reported that jaggery provided the basic energy needed for heterotrophic microbial cells growth, which were the primary component of biofloc. Furthermore, the sugar usually contained antioxidants and vital minerals such as zinc and selenium that might prevent free radical damage and boosted immunity and increased resistance against infections (Nayaka *et al.*, 2009).

2.11. Maintenance of Carbon and Nitrogen ratio (C:N) in Biofloc culture system

The most crucial parameters in successful biofloc farming was the maintenance of proper C:N ratio (Avnimelech, 1999, Zhao *et al.*, 2014, Pérez-Fuentes *et al.*, 2016 and Dauda *et al.*, 2018). In general the biofloc system operated through the incorporation of an external carbon sources to increase the C:N ratio which eventually converted the inorganic nitrogen to microbial biomass (Avnimelech, 1999). Micro-organisms used carbohydrate as energy source and nitrogen for the synthesis of protein to produce new cells (Avnimelech, 1999). Generally, a C:N ratio of 10:1 to 20:1 was suggested for the growth of bioflocs and healthy performance of biofloc culture system (De Schryver *et al.*, 2008, Avnimelech, 2012 and Emerenciano *et al.*, 2012) because, this range of C:N ratio were able to causes a faster removal of nitrogen as well as restoration of new bacterial cells (Zhao *et al.*, 2014). Carbon and Nitrogen ratio maintenance was also require for controlling accumulation of organic nitrogen and production of microbial communities in the biofloc cultured water (Asaduzzaman *et al.*, 2008 and Emerenciano, 2012). In presence of suitable C:N ratio the inorganic nitrogen was converted into organic nitrogen to produce bacterial cells (Aly *et al.*, 2008). During aerobic situations the state of C:N ratio should be more than bacterial body compositions, because carbohydrate was concerned in the respiration process (Emerenciano, 2013). In biofloc farming the practice of minimizing the artificial feed application and increasing C:N of higher than 10:1 by utilizing diverse low-cost locally available carbon sources were very common (Crab, 2010). Utilization of biofloc components would also decrease the amount of protein in the supplementary feed (Avnimelech, 1999 and Hargreaves, 2006). The accumulation of toxic inorganic components like NH_4^+ and NO_2^- were reduced in biofloc due to consumption by the microbial community in the water along with maintenance of high C:N ratio in the biofloc system. By addition of cheap carbohydrate sources like molasses, jaggery or others usually in C:N ration around 12:1–15:1 in the water column, biofloc might suitably converted the toxic nutrients in the water to beneficial food sources for fish or shrimp consumption (Schneider *et al.*, 2005).

Several studies was conducted to study the effects of C:N ratios in the biofloc system during the culture of different fish and shrimp species such as Nile tilapia, *Oreochromis niloticus* (Mirzakhani *et al.*, 2019); African sharp-tooth catfish, *Clarias gariepinus* (Dauda *et al.*, 2018); Common carp, *Cyprinus carpio* (Najdegerami *et al.*,

2016); crucian carp, *Carassius auratus* (Wang *et al.*, 2015); and Pacific white shrimp, *Litopenaeus vannamei* (Khanjani *et al.*, 2016 and 2017) with hopeful conclusion.

Studies already had proved that an increase in the C:N ratio through feed or direct incorporation of organic carbon were capable of shifting the photoautotrophic or chemoautotrophic communities to heterotrophic-dominated communities (Avnimelech, 1999). This conversion could considerably effects on both the water quality parameters and biofloc biomass production, which could ultimately affect fish feed utilization and fish physiological parameters like immunity and antioxidant grade (Long *et al.*, 2015). In another studies Xu *et al.* (2015) reported that low C:N ratio of 9:1 was more beneficial in compare to high C:N ratio of 18:1 for shrimp growth in high stocking density, zero-water exchange culture systems due to presence of a mixture of both heterotrophic and autotrophic bacteria which was dominated by heterotrophic bacteria in high C:N ratio. Generally in BFT, heterotrophic bacteria were more dominant than nitrifying bacteria because of their higher growth rate and microbial biomass yield per substrate, thus made many fold increase of heterotrophic bacteria could covert the ammonium into bacterial biomass (Hargreaves, 2006).

The carbon nitrogen (C:N) ratio in a system could be manipulated by adding organic carbon to generate different substrate utilization pathways and microbial biomass yields (Hargreaves, 2006). In biofloc systems, a high C:N ratio always led to heterotrophic bacterial dominance (Kamilya *et al.*, 2017), whereas at a low C:N ratio, bacterial communities were composed of mixed heterotrophic and autotrophic bacteria (Xu *et al.*, 2015). The adjustment of C:N ratio (mainly in fish feeds) was necessary for reducing the accumulation of inorganic nitrogen in intensive tilapia ponds. Proper C:N ratio could reduce ammonia to the desired levels within 1-3 days. A ratio of about 15:1 of C:N had been recommended for tilapia (Avnimelech, 1999). Zafar *et al.* (2021) maintained low C:N at 10:1 in the biofloc tanks with the supplementation of sugarcane molasses as a carbon source during culture of Asian stinging catfish (*Heteropneustes fossilis*). Cheap carbon and nitrogen sources could be used as a partial replacer for expensive commercial high-protein feeds. The approach of producing single-cell protein(s) in fish ponds should also be encouraged in developing countries where tilapia culture was widely practiced.

Hargreaves (2013) and Rhode (2014) demonstrated that a carbon to nitrogen ratio of 12:1–15:1 was usually favoured by the heterotrophic bacterial community. Whereas, Ebeling *et al.* (2006) suggested that a carbon to nitrogen ratio of 20:1 was

positive for microbial immobilization of nitrogenous compounds by the heterotrophic bacterial community. Avnimelech (2015) proposed that the carbon to nitrogen ratio should be maintained at 12:1–20:1 during the early stages of the biofloc system to attain best stimulation and stabilization of the heterotrophic bacterial aggregations. The external carbohydrates source which could manipulate the carbon to nitrogen ratio to the desired levels were dextrose, glycerin, sugar, sucrose (Rhode, 2014), starch and cellulose (Avnimelech, 1999). Luo *et al.* (2017) had reported that poly-beta-hydroxybutyrate obtained from microorganisms (Zhang *et al.*, 2016) and polycaprolactone could be considered as stable carbon sources to maintain the optimal C:N ratio in BFT systems. Emerenciano *et al.* (2017) demonstrated that maintaining of the carbon and nitrogen ratio occurred in two phases in BFT system. In the beginning phase it involved a carbon to nitrogen ratio of 12:1–20:1, and the later or maintenance phase involved a carbon to nitrogen ratio of 6:1, based on the TAN levels recorded. The carbon and nitrogen ratio and feed protein content had an inverse relationship in BFT system (Avnimelech, 2015 and Jim´enez-Ojeda *et al.*, 2018). The heterotrophic uptake of nitrogenous waste from the biofloc system was considered more stable and reliable compared with the removal of nitrogenous waste via the nitrification process or by algae (Hargreaves, 2013).

2.12. Fish growth and welfare issues in biofloc

Several studies established that biofloc microbial aggregates were able to reduce the additional feed costs and also put a positive impact on the immunity which resulted reduce mortality rate in both fish and shellfishes (Azim and Little, 2008, Liu *et al.*, 2018a, Ahmad *et al.*, 2016, Kamilya *et al.*, 2017, Kheti *et al.*, 2017, Lee *et al.*, 2017 and Fauji *et al.*, 2018). Bacteria and bacterial products, complex carbohydrates, nutritional factors like Vitamin C, animal extracts, cytokines, lectins, plant extracts and synthetic drugs such as levamisole could be used as an immunostimulants in fish (Wang *et al.*, 2015). As biofloc technology dealt with bacteria and bacterial products it also contained immunostimulatory compounds. The feasible immunostimulatory feature of the bioflocs was a remarkable field of study. The heterotrophic microorganisms cell walls contained microbe-associated molecular patterns (MAMPs) like lipopolysaccharides, glucans or peptidoglycans which could activate the non-specific immune mechanisms and enhanced significant immune response in cultured fishes (Aguilera-Rivera *et al.*, 2019 and Panigrahi *et al.*, 2019a, 2020). Panigrahi *et al.*

(2019b) reported that the biofloc farmed *L. vannamei* exhibited improved immune response and elevated level of total hemocyte count and prophenoloxidase (ProPO) activity from control. As we know that augmentation of the innate immunity of cultured fishes may offer wide range resistance to infections. The biofloc microbial community rich of beneficial bacteria could be used as natural probiotics for its role as an immunostimulants which could improve the immunity and enhance the fish resistance against infectious diseases (Crab, 2010 and Defoirdt *et al.*, 2010). A number of evidences proved that there were noteworthy improvements in the non-specific immunity of the fish cultured in the biofloc system (Decamp *et al.*, 2008 and Tseng *et al.*, 2009).

The heterotrophic microbial biomass in the biofloc mitigated the invasion of pathogenic bacteria (Emerenciano *et al.*, 2013). The working mechanism of the heterotrophic biofloc microbial community against pathogens were by competition of space, substrate and nutrients because essential nutrients like nitrogen were required by both groups (*i.e.*, heterotrophic bacteria and pathogenic bacteria) for their growth. These beneficial microbes contained several nutritional factors and digestive enzymes like amylase and proteases, could definitely altered the gut microbiota and eventually contributed in the normal digestive process, improved the food absorption and efficient utilization and enhanced growth performance, immune response of the host (Liu *et al.*, 2009 and Pérez *et al.*, 2010). It was demonstrated that *Bacillus* sp. supplied vitamins and fatty acids to host and developed the growth and survivability of *Penaeus monodon* postlarvae in zero water exchange provision (Navin Chandran *et al.*, 2014 and Kumar *et al.*, 2016). Zokaeifar *et al.* (2012 and 2014) reported that *Bacillus* sp. appreciably improved the role of digestive enzyme, growth, immune response and resistance of shrimp against bacterial disease. Recently the diversity and abundance of biofloc forming bacteria in the river waters using 16S rDNA sequencing method had been investigated by Kurniawan *et al.* (2020) which exposed that seven bacterial phyla including *Proteobacteria*, *Cyanobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Bacteroidetes*, *Chloroflex*, *Planctomycetes* and 14 bacterial genera *Streptococcus*, *Staphylococcus*, *Bacillus*, *Neisseria* sp., *Bacillus* sp., *Lactococcus*, *Rhodococcus*, *Kocuria*, *Pseudomonas*, *Nitrospira*, *Rhodobacter*, *Sphingomonas*, *Burkholderia* and *Acinetobacter* had potential biofloc forming abilities.

Biofloc bacteria were able to accumulate the compound poly- β -hydroxybutyrate (PHB) that eliminated the invasion of bacterial pathogen in cultured

fish (Defoirdt *et al.*, 2007). A lot of studies also exposed that the PHB accumulating bacteria improve growth performance, food digestibility and also could defend diverse aquaculture species against bacterial infections (De Schryver *et al.*, 2010, Defoirdt *et al.*, 2007, Dinh *et al.*, 2010 and Khanjani and Sharifinia, 2020). Emerenciano *et al.* (2009) found less ectoparasites in gills and ectoderm's mucous in a 60 d of tilapia fingerlings in a limited water exchange as compared to conventional water exchange culture system. Besides that inhibiting compounds excreted by biofloc microorganisms, light strength and type of carbon supply also could diminish the growth of pathogenic microbes. Quality of biofloc was highly influenced by the types of carbon source. Glycerol based bioflocs contained higher protein, vitamin C and n-6 fatty acid than glucose-based bioflocs (Crab *et al.*, 2010), glucose-based bioflocs contained highest protein than starch and glycogen based bioflocs (Wei *et al.*, 2016), glucose and glycogen based bioflocs contained merely similar amount of essential and nonessential amino acid but lesser in compare to starch-based biofocs (Wei *et al.*, 2016).

Light intensity could promote development of green biofloc through photosynthetic process, which contained additional nutrition to benefit biofloc reared species. Several findings were observed where fresh water fishes reared in biofloc water exhibited higher growth performance including improved feed efficiency ratio (FER), specific growth rate (SGR) and feed conversion ratio (FCR) in *Labeo rohita* (Ahmad *et al.*, 2016 and Kamilya *et al.*, 2017), *Oreochromis niloticus* (Mirzakhani *et al.*, 2019, Hwihiy *et al.*, 2021 and Shourbela *et al.*, 2021), *Cyprinus carpio* (Bakhshi *et al.*, 2018 and Aalimahmoudi and Mohammadiazarm, 2019), African sharptooth catfish, *Clarias gariepinus* (Dauda *et al.*, 2018), *Carassius auratus* (Wang *et al.*, 2015) and *Apostichopus japonicus* (Chen *et al.*, 2018). In addition to the growth performance the cultured animals showed better non-specific immune response, supported by notably increased serum protein, serum albumin, total immunoglobulin, lysozyme, respiratory burst and myeloperoxidase activity.

In an experiment *Labeo rohita* fingerlings (4.80±0.12 g) cultured in tapioca based biofloc system exhibited significantly better (p<0.05) growth and feeding performances like feed conversion ratio (FCR), feed efficiency ratio (FER) and specific growth rate (SGR), along with significantly (p<0.05) higher serum protein, serum albumin, total immunoglobulin, respiratory burst activity, myeloperoxidase activity and lower serum glucose and cortisol level when compared to wheat, corn,

sugar bagasse based biofloc system and control (Ahmad *et al.*, 2016). While rearing tilapia (*Oreochromis niloticus*) fingerlings (0.50 ± 0.02 g) in biofloc systems with different C:N ratio of 10:1, 15:1 and 20:1, Liu *et al.* (2018) found that the performance of the digestive enzymes lipase and trypsin, and the activities of lysozyme, alkaline phosphatase, superoxide dismutase, glutathione and malondialdehyde were significantly higher in the C:N-10:1 and C:N-15:1 groups than control group ($p<0.05$). In another experiment Liu *et al.* (2018) reared tilapias *Oreochromis niloticus* fingerlings (0.51 ± 0.05 g) for 120 d with glucose added biofloc system in three different stocking densities of 166 nos./m³ (low density), 333 nos./m³ (middle density) and 600 nos./m³ (high density) to find out the immune and antioxidant abilities like lysozyme, complement-3 and glutathione in the control and experimental group.

Tissue and serum enzymatic antioxidant status like superoxide dismutase (SOD), Catalase (CAT), MDA and glutathione peroxidase (GSH) are directly related to health as these are required to sustain the complex immune systems and also to represent the first line of enzymatic defence against free radicals in fish (Jia *et al.*, 2012). SOD and GSH were associated in resistance against lipid peroxidation in biological systems and in alteration of active oxygen species into non-toxic compounds therefore diminution in SOD and GSH may raise the accumulation of free radicals, injury and impaired cell function (Okamoto and Colepicolo, 1998). In a 56 d culture of golden crucian carp juveniles (5.03 ± 0.22 g) in biofloc system with different C:N ratios (10:1, 15:1, 20:1 and 25:1) Yu *et al.* (2020) examined the activity of superoxide dismutase, total antioxidant capacity, catalase and glutathione peroxidase of crucian carp juveniles. In an 120 d experiment of genetically improved farmed tilapia (GIFT) in light limited and zero-water exchange culture facilities Long *et al.* (2015) observed the serum glutathione peroxidase and lysozyme activities were considerably higher in the BFT treatment than in the control fish ($p<0.05$). Elayaraja *et al.* (2020) showed that the jaggery-based biofloc system had a pronounced effect on hematological, growth performance and immunological parameters. In their 75 d experimental period of biofloc culture of tilapia, *Oreochromis niloticus* in various C:N ratios of 12:1, 15:1 and 20:1 they measured the serum antioxidants, lysozyme, protease, antiprotease and bactericidal capacity of fish after experimental challenge with *Aeromonas hydrophila*. Menaga *et al.* (2019) reared Genetically Improved Farmed Tilapia (GIFT) (5.1 ± 0.05 g) at a density of 15/m³ in lined ponds of 300 m² in

triplicates for 180 d by maintaining the C:N ratio of 10:1 and studied the growth performance, immunological parameters like serum protein, respiratory burst test (RBT) and myeloperoxidase activity and antioxidant indicators (glucose, superoxide dismutase and catalase), immune gene expression and its resistance to *Aeromonas hydrophila* infection. Minabi *et al.* (2020) investigated the body composition of common carp (*Cyprinus carpio* L.) fingerlings (14.17 ± 0.36 g) cultured at stocking densities of 1.06 kg m^{-3} per tank with maintaining different C:N ratio (11:1, 15:1, 19:1 and 23:1) in a 90 d trial.

2.13. Water quality monitoring in biofloc tank

Waste metabolite of the aquatic fauna and unutilized feed often increased the nitrogen metabolites, resulted water quality deterioration and eventually influenced the aquaculture production directly. Accumulation of toxic nitrogenous compounds was the main water quality issue in intensive aqua farming. Keeping that in mind BFT was first introduced to remove $\text{NH}_3\text{-N}$ in aquaculture by applying natural processes which mainly included photoautotrophic removal by algae, autotrophic bacterial alteration of ammonia-nitrogen to nitrate-nitrogen and simultaneously heterotrophic bacterial transformation of ammonia-nitrogen directly to microbial biomass (Ebeling *et al.*, 2006). The said processes were monitored by developing dense heterotrophic microbial community (Azim and Little, 2008) by addition of carbon (Crab *et al.*, 2007 and 2012). Several studies supported that the inclusion of carbon helped the formation of biofloc which not only improved water quality (Avnimelech, 2007, Crab *et al.*, 2007, Hargreaves, 2006, Hari *et al.*, 2004 and MacIntosh, 2000) but also increased dissolved oxygen in the culture system (Lananan *et al.*, 2014 and 2013). Researchers already explained that how the microbial community (periphyton) entrapped organic detritus, removed nutrients by photosynthetic and autotrophic processes to control water quality in aquatic system (Vermatt, 2005). The autotrophic processes of periphytons were capable of assimilating $0.2 \text{ g NH}_3\text{-N/m}^2\text{/d}$ (Azim *et al.*, 2005). The biofloc technology was well-known for its constructive part in maintaining water quality, increasing fish production, giving an alternative source of nutrition and supporting the health and growth of fish (Azim and Little, 2008, Ekasari *et al.*, 2016 and Luo *et al.*, 2014).

Table 2.2. Recent experiments on effects of different carbon to nitrogen (C:N) ratios on freshwater fishes in biofloc culture system

Fish Species	C:N ratio and carbon sources used	Major finding on fish growth performances and welfare issues	References
Rohu, <i>Labeo rohita</i>	C:N = 15:1 (tapioca, wheat, corn and sugar bagasse)	Growth, FCR, FER and SGR were higher in BFT fishes than control. Tapioca based system showed higher serum protein, serum albumin, total Ig, respiratory burst activity, myeloperoxidase activity and lower serum glucose and serum cortisol than other carbon sources.	Ahmad <i>et al.</i> , 2016
Common carp, <i>Cyprinus carpio</i> L.	C:N=11:1,15:1,19:1&23:1 (sugarcane molasses)	Higher weight gain and PER were recorded in C:N=19:1. Increased C:N=23:1, FCR increased but not TFI, FW, WG, PER and SGR.	Minabi <i>et al.</i> , 2020
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 10:1, 20:1 (sugar, molasses and cassava starch)	The growth performance in BFT system managed with different organic carbon sources was found not significantly different between treatments ($p<0.05$).	Silva <i>et al.</i> , 2017
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 14:1, 17:1, 20:1 (molasses)	Growth and FCR were not significantly different among control, C:N=14:1 and 17:1 groups. Low weight gain and high FCR in the C:N=20:1 compared to the control. Proteases and alpha-amylase activities in the liver and digestive tract found similar in all treatments. CAT and SOD activities did not differ significantly among the four groups.	Dilmi <i>et al.</i> , 2022
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 15:1, (molasses, starch, barley flour and corn)	Cultured tilapia using in situ biofloc produced by different carbon sources can promote FCR, survival, body composition, digestive and hepatic enzymes in compare to the control group.	Khanjini <i>et al.</i> , 2021
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 15:1, (broken rice flour) Three different stoking densities (20, 40 and 60 fish/ m ³)	Significantly increased growth and feed utilization were recorded in 40 fish/m ³ groups. RBC, Hb and Ht values were reduced in fish stocked @ 60 fish/ m ³ while ALT and AST increased in fish reared @ 20 fish/ m ³ . Glucose, cortisol, CAT and SOD increased in fish reared at low density and control.	Zaki <i>et al.</i> , 2020
<i>Carassius auratus</i>	C:N= 15:1, 20:1 and C:N=10:1 (Control)	Weight gain, specific growth rate, and protein efficiency ratio were considerably increased when C/N = 20:1 and 25:1. BFT was found effective and highly potential technology in <i>C. auratus</i> aquaculture.	Wang <i>et al.</i> , 2015

Fish Species	C:N ratio and carbon sources used	Major finding on fish growth performances and welfare issues	References
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 10:1, 15:1, 20:1 (glucose)	Better growth in the C:N=10:1 and 15:1 compared to the C:N=20:1 and control. Lysozyme, alkaline phosphatase, SOD, GSH and MDA found higher in the C:N=10:1 and 15:1 groups. The concentrations of glucose and cortisol in the biofloc were significantly lower to control.	Liu <i>et al.</i> , 2018a
Rohu, <i>Labeo rohita</i>	C:N = 15:1, (molasses)	Enhanced growth found in biofloc. Respiratory burst and alkaline phosphatase activity found significantly higher than control, whereas, myeloperoxidase activity and total serum protein content was not significantly different in fish grown in biofloc system.	Kamilya <i>et al.</i> , 2017
Golden crucian carp, <i>Carassius auratus</i>	C:N = 5.48:1 (control), 10:1, 15:1, 20:1 and 25:1. (Anhydrous glucose 99.97 %)	Weight gain, SGR and PER were significantly elevated in C:N= 20:1 and 25:1 than control. Increases in lysozyme, acid phosphatase and ALP activities were found in the C:N=20:1 than control. SOD, total antioxidant capacity, CAT and GSH activity in the C:N=20:1 and 25:1 were higher ($p<0.05$), but MDA was lower than control.	Yu <i>et al.</i> , 2020a
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 15:1, (glucose) Four stocking densities; Low ($166/m^3$), middle ($333/m^3$), high ($600/m^3$) and control ($333/m^3$).	Low and middle density group showed higher final body weight, higher digestive enzymes (lipase, trypsin and amylase) activities, higher lysozyme, complement-3, and glutathione values than control and high density groups. High density groups also showed depressed value of stress indicators and higher ALT, AST and AKP.	Liu <i>et al.</i> , 2018b
Common carp, <i>Cyprinus carpio</i> L.	C:N = 10:1, 15:1, 20:1	C:N=10:1 showed significantly higher serum glucose, ALT but AKP activity was decreased by increasing C:N ratios. No significant influence on albumin and total protein among the treatments. Ig-M level in C:N=15:1 and C:N=20:1 groups were higher than C:N=10:1 group. The humoral immunities, immune-related gene expression and zootechnical responses were found better in BFT at C:N=20:1.	Azimi <i>et al.</i> , 2022
Red Tilapia, <i>Oreochromis niloticus</i>	C:N = 20:1 (molasses) Doses of molasses; 50, 100, 150 & 200 ml/ m^3 .	Significant effect of all BFT groups on absolute weight and specific growth, but did not significantly affect the survival rate. Best growth and feed utilization were found at molasses dose of 200 ml/ m^3 .	Putra <i>et al.</i> , 2020

Fish Species	C:N ratio and carbon sources used	Major finding on fish growth performances and welfare issues	References
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 12:1, 15:1 and 20:1 (Jaggery)	Prominent effects found on innate immunity, serum antioxidant, hematological and growth performance parameters in biofloc fishes compare to control. LYZ, TNF- α , and IL-1 β genes were upregulated in highest fold in C:N=20:1. Lower mortalities and better production in BFT fishes after experimental challenge with <i>A. hydrophila</i> .	Elayaraja <i>et al.</i> , 2020
Dybowski, <i>Opsariichthys kaopingensis</i>	C:N = 10.8:1 (control), 15:1, 20:1 and 25:1. (Anhydrous glucose 99.97 %)	Weight gain, SGR, survival and PER were significantly elevated in C:N=20:1 compared with the control, but not FCR. Significant increases in serum lysozyme, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, AKP, SOD, total antioxidant capacity, CAT and GSH were found in the C:N= 20:1 group.	Yu <i>et al.</i> , 2020b
Asian stinging catfish, <i>Heteropneustes fossilis</i>	C:N = 10:1 (sugarcane molasses)	Weight gain, biomass, SGR and feed utilization were found higher in BFT fishes compared to the control. Protease and amylase activity in the intestine and liver lipase activity and improved hematological profile were found to be significantly higher in the biofloc group.	Zafar <i>et al.</i> , 2021
African catfish, <i>Clarias gariepinus</i>	C:N = 10:1, 15:1 and 20:1 (glycerol)	Survival, growth, and feed utilization were similar among treatments. Muscle cholesterol, lipid peroxidation, serum triglyceride and cholesterol were found lower in biofloc groups, but liver glycogen was higher in the C:N=15:1 group. C:N=15:1 led to the best nutritive value of <i>C. gariepinus</i> as well as their resistance to <i>A. hydrophila</i> .	Dauda <i>et al.</i> , 2017
IMC, e.g., <i>L. rohita</i> , <i>C. catla</i> and <i>C. mrigala</i>	Polyculture at different stocking densities; 4.28, 8.57 and 12.85 fish/ m ³	BFT system improved the growth parameters of fishes as compared to the control system without the application of biofloc. Biofloc system was found efficient to culture IMC in polyculture mode.	Deb <i>et al.</i> , 2020
Red pacu, <i>Piaractus brachyomus</i>	Low TSS: 200–300, Medium TSS: 400–600 mg/l levels and control	High TSS affects the growth, red blood cells and generates changes in gill morphology of fish. Red pacu gained highest biomass, better growth and FCR in control than biofloc treatments.	Angeles- Escobar <i>et al.</i> , 2021
Grass carp, <i>C. idella</i>	C:N = 10:1, 15:1, 20:1 (powdered banana peels)	Average (%) gain in weight and length of fingerlings was obtained noticeably higher in C:N=20:1 than other treatments and control.	Tayyab <i>et al.</i> , 2022

The achievement of biofloc technology depended upon its ability to eliminate, recycle or control harmful nitrogenous substances in the culture unit (Souza *et al.*, 2019). Toxic nitrogenous compound was one of the most significant concerns in aquaculture. But at the same time nitrogen was considered as an essential element for aquatic species for various physiological processes. It also constituted tissues, fluids and molecules including body proteins (Wei *et al.*, 2016), nucleic acids, nucleotide bases, pigments, adenosine phosphates, etc. (Ebeling, 2006). The nitrogen accumulation in aquaculture usually occurred in numerous forms, including ammonia (NH₃), ammonium (NH₄⁺), nitrites (NO₂⁻), nitrates (NO₃⁻), total nitrogen (TN) and total ammonia nitrogen (TAN) (Ebeling *et al.*, 2006). Among all these forms of nitrogen in aquaculture, NH₃ and nitrite (NO₂⁻) accumulation must be controlled immediately. The accumulation of nitrite and NH₃ in the culture units was a vital issue in the BFT culture because even low levels of TAN (0.02 mg/l) and nitrites (2 mg/l) (Bregnballe, 2010) might be harmful to fish (Emerenciano *et al.*, 2017). Nitrates (NO₃⁻) might be considered toxic while accumulate above 100 mg/l in the system (FAO and EUROFISH, 2015). Hence, the nitrification process must be cautiously done for successful operation of a biofloc system (Souza *et al.*, 2019).

Biofloc was the aggregates of heterotrophic microorganism which reduced ammonia and other nitrogenous metabolites from the culture system and thus helped in maintaining water quality. Earlier studies also suggested that 7%–13% nitrogen retention from feed could be assisted by favourable C:N ratio (Hari *et al.*, 2004 and Schneider *et al.*, 2005). Other important water quality parameters which should be continuously monitored in biofloc culture system were temperature, dissolve oxygen (DO), pH, salinity, total suspended solids (TSS), alkalinity, floc volume and orthophosphate (Emerenciano *et al.*, 2017). Proper understanding of these said parameters and their interactions in biofloc culture system played the decisive role for ideal development of floc as well as successful production of crops. Achieving healthy crops without any mortality could not be possible until and unless maintaining safety ranges of dissolve oxygen, pH, total ammonia nitrogen, TSS and alkalinity (Emerenciano *et al.*, 2013). Emerenciano *et al.* (2013) reported that biofloc technology offered a plenty benefit for ensuring zero water exchange through minimal water consumption and pollution.

Biofloc technology decreased the effluent discharge, prevented risks from the disease outbreak, protected the water from pathogen entry and after all developed the

biosecurity at the farm level (Burford *et al.*, 2003). Biofloc system could play a key role in the management of water quality as because of presence of heterotrophic microorganisms (MacIntosh, 2000). In order to attain more growth usually fish were fed with lots of feed. Commonly fish or shrimp feeds were rich in protein which might contained up to 65% of nitrogen content and hence the uneaten feeds were able to deteriorate the pond water and led the aquatic animals to disease susceptibility (Francis-Floyd *et al.*, 2009).

2.14. Physical-chemical characteristics of water under biofloc system

2.14.1. Temperature: Temperature was considered as the most influential parameters in biofloc culture system. Not only the metabolic rate of animal and microorganism and their oxygen consumption rate, but also the pH and concentration of ionized and un-ionized ammonia throughout the culture were highly affected by temperature fluctuations. The most favorable temperature range for biofloc culture was depends on the animal species, bacterial adaptation and also seasonal variations. Biofloc system became more efficient at the temperature between 28-30°C because the efficiency of nitrifying bacteria were reduced by 50% at 16°C and by 80% at 10°C (Emerenciano *et al.*, 2017).

2.14.2. Dissolved oxygen: Generally the ideal dissolve oxygen level in aquatic environment should be greater than 5 ppm, but in biofloc system due to high Biological Oxygen Demand by the algae and bacteria along with cultured fishes, dissolve oxygen must be maintained at 7-8 ppm to ensure suitable operation of the system (Emerenciano *et al.*, 2017).

2.14.3. pH: Alike the pond fish culture system the pH should be maintained in the range 7.5-8.5 in biofloc system because pH reduction generally occurred due to alkalinity consumption during ammonia–nitrate nitrogen conversion processes. According to Furtado *et al.* (2014), pH levels less than 7 for prolonged periods of time can affect the growth performance in biofloc. CaCO₃ could be added @ 20-30 g/1000 l water to maintain pH in biofloc tank.

Table 2.3. Recent experiments on effects of different C:N ratios on monitoring of water quality during freshwater fish culture in biofloc system

Fish Species	C:N ratio and carbon sources used	Major finding on water quality monitoring	References
Common carp, <i>Cyprinus carpio</i> L.	C:N = 11:1, 15:1, 19:1, and 23:1 (sugarcane molasses)	Overall, the biofloc system with C:N=19:1 improved the water quality compare to other treatment groups and control.	Minabi <i>et al.</i> , 2020
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 10:1, 20:1 (sugar, molasses and cassava starch)	The C:N ratio and carbon source affected the alkalinity, settle able solids, turbidity and TSS, which showed significantly higher values at C:N=20:1. Best carbon source for microbial floc formation were the molasses and sugar, at C:N=10:1 and 20:1.	Silva <i>et al.</i> , 2017
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 14:1, 17:1 and 20:1 (molasses)	The dynamics of water quality parameters showed a shift from a predominantly autotrophic to a predominantly heterotrophic biofloc with an increase in the C:N=14:1 to 20:1.	Dilmi <i>et al.</i> , 2022
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 10:1, 12.5, 15:1, 17.5:1, 20:1 (molasses)	Water quality remained similar in all biofloc treatments, but lower value of nitrogen compounds found in C:N=10:1	Pérez-Fuentes <i>et al.</i> , 2016
Red Tilapia, <i>O. niloticus</i>	C:N = 20:1 (molasses, tapioca and sucrose)	Carbon source from molasses was found effective in reducing concentrations of NH ₃ , NO ₂ and NO ₃ in red tilapia culture.	Putra <i>et al.</i> , 2020
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 15:1, (broken rice flour). Three stoking densities (20/40/60 fish/m ³)	pH, BOD, TAN and NO ₂ values were increased significantly (P<0.05) as the density increased and the highest values found in the stocking of 60 fish/ m ³ while dissolved oxygen decreased.	Zaki <i>et al.</i> , 2020
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 10:1, 15:1, 20:1 (glucose)	Inorganic nitrogen dynamics revealed that heterotrophic and autotrophic bacteria existed in low C:N ratio group in contrast domination of heterotrophic bacteria in high C:N ratio group.	Liu <i>et al.</i> , 2018a
Rohu, <i>Labeo rohita</i>	C:N = 15:1, (molasses)	NH ₃ -N immobilization by heterotrophic bacterial population, without nitrification process was a dominant mechanism for removal of inorganic N ₂ from the biofloc system.	Kamilya <i>et al.</i> , 2017
Asian stinging catfish, <i>H. fossilis</i>	C:N = 10:1 (sugarcane molasses)	Ammonia, nitrate, nitrite, and total suspended solids were significantly lower in the BFT than in the control (P < 0.05).	Zafar <i>et al.</i> , 2021

Fish Species	C:N ratio and carbon sources used	Major finding on water quality monitoring	References
Golden crucian carp, <i>Carassius auratus</i>	C:N = 5.48:1 (control), 10:1, 15:1, 20:1 and 25:1. (Anhydrous glucose 99.97 %)	BFT significantly reduced TAN, NO ₂ -N, total phosphorus concentration and water transparency in C:N=15:1 and 25:1 than control (p<0.05), whereas the COD and NO ₃ -N concentration gradually increased over time.	Yu <i>et al.</i> , 2020a
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 12:1, 15:1 and 20:1 (Jaggery)	TAN, NO ₂ -N, and ammonia levels were considerably reduced, while the TSS values elevated significantly in all treated groups compared to the control.	Elayaraja <i>et al.</i> , 2020
Dybowski, <i>Opsariichthys kaopingensis</i>	C:N = 10.8:1 (as control), 15:1, 20:1 and 25:1. Anhydrous glucose 99.97%	BFT significantly reduced TAN, NO ₂ -N, total phosphorus concentrations and water transparency, whereas the COD and NO ₃ -N concentrations gradually increased over time.	Yu <i>et al.</i> , 2020b
Nile Tilapia, <i>Oreochromis niloticus</i>	C:N = 15:1, (glucose) Four stocking densities 166/m ³ , 333/m ³ and 600/m ³ and control (333/m ³)	TAN, NO ₂ -N, and NO ₃ -N in the higher density, i.e., 600/m ³ groups were higher than that in the low (166/m ³) and middle (333/m ³) density group.	Liu <i>et al.</i> , 2018b
African catfish, <i>Clarias gariepinus</i>	C:N = 10:1, 15:1 and 20:1 (glycerol)	Biofloc volume was significantly higher at C:N=20:1, but biofloc biomass was significantly higher at C:N=15:1. Dissolved oxygen was significantly lower at C:N=20:1 while TAN was significantly higher (P<0.05) in control than biofloc groups.	Dauda <i>et al.</i> , 2017
IMC, e.g., <i>L. rohita</i> , <i>C. catla</i> and <i>C. mrigala</i>	Polyculture at different stocking densities; 4.28, 8.57 and 12.85 fish/ m ³	Biofloc system could efficiently remove inorganic nitrogen from culture water. Water quality and floc formation were found higher with a stocking density of 4.28 fish/ m ³ .	Deb <i>et al.</i> , 2020
Common carp, <i>Cyprinus carpio</i> L.	C:N= 15:1, 20:1 and C:N=10:1 (Control) (wheat meal)	Higher C:N ratio increased water quality parameters such as turbidity, TSS, TDS, BOD, alkalinity and NO ₂ -N concentration, but it reduced TAN and phosphate.	Azimi <i>et al.</i> , 2017
Grass carp, <i>Ctenopharyngodon idella</i>	C:N = 10:1, 15:1 and 20:1 (powdered banana peels)	Ammonia and other water parameters were under control in C:N=20:1 compare to other treatments and control groups.	Tayyab <i>et al.</i> , 2021

2.14.4. Alkalinity: It was the ability of water to buffer or resist changes in pH in response to additions of acid or base. Biofloc culture tank should be maintained with sufficient alkalinity because it was frequently used up by the action of nitrifying bacteria present in the system during nitrification. Depletion of alkalinity led to sharp drop of pH and inhibition of heterotrophic bacterial function. In this condition, ammonia accumulated and affected the daily feeding rate and ultimately hampered the production (Emerenciano *et al.*, 2017). Especially in shrimp biofloc culture unit alkalinity should be kept between 100 and 150 ppm as CaCO_3 by regular additions of sodium bicarbonate where every kilogram of feed added to the system should be supplemented with 0.25 kg of sodium bicarbonate.

2.14.5. Total Suspended solids: Maintaining suitable range of suspended solids was required in biofloc culture system because it acted as a substrate for adhesion of bacterial community and also acted as energy source from carbon. In biofloc system, TSS in the range of 250-450 ppm confirmed efficient bacterial activity and an excellent system to deal with ammonia without excess water respiration, but, an surplus of TSS level may led to stress or in intense cases, led to death by clogging gills (Emerenciano *et al.*, 2017).

2.14.6. Total Ammonia Nitrogen: It was produced from the uneaten food components and excretory product of aquatic organisms including phytoplankton and zooplankton. The non-ionized ammonia (gaseous) was considered to be toxic when compared to ionized ammonia or ammonium ion (NH_4^+). The unionized form (NH_3) was directly proportional with low dissolve oxygen, high pH and high temperature. The recommended ammonia concentration for biofloc culture was below 1 ppm at $\text{pH} < 7.0$. Monitoring proper carbon nitrogen ratio by application of external carbon source along with feed was the only way to control TAN in biofloc tank culture (Emerenciano *et al.*, 2017).

2.14.7. Nitrite nitrogen: Nitrite was considered the highly toxic elements in aquatic systems as it affected oxygen transport system, oxidation of vital compounds and tissue injure. The conversion pathway to ammonia nitrogen to nitrite nitrogen and their toxicity type largely depended on the magnitude of chlorides, temperature and oxygen concentration in water. Nitrite-nitrogen concentration should not be exceeds 1 ppm in biofloc culture (Perez-Rostro *et al.*, 2013).

2.14.8. Nitrate nitrogen: The end product of aerobic nitrification was considered as less toxic. To some extent it became toxic for its effects on osmoregulation and oxygen transport in fish body. Nitrate concentration should be less than 10 ppm in biofloc (Emerenciano *et al.*, 2017).

2.14.9. Floc volume (FV): It always should be in the range of 10 to 40 ml/l and this could be monitored using Imhoff cones. When FV concentrations were above 40 ml/l, sludge should be removed, and if below 10 ml/l, carbohydrates should be added (Emerenciano *et al.*, 2017).

2.15. Advantages of biofloc technology

The prime benefits of biofloc farming system over various other advanced fish culture systems was eco-friendliness and zero or minimal water exchange facility. The main benefit of the biofloc system lay on its superior capacity on environmental impact, enhancement of useful exploit of land and water, maintenance of the proper value of water with nominal water usage and replace, and providing supplementary feed to the cultured aquatic species in form of protein-rich biofloc (Reddy, 2019). According to the Arias-Moscoso *et al.* (2018) biofloc was able to minimize the threat of introduction of diseases causing pathogens by reducing wastewater pollution, ensuring clean water and providing better biosecurity. Besides that BFT could be considered as an alternative economical culture procedure as it accelerated the survival rate, growth performance, feed conversion ratio, better health in cultured fish.

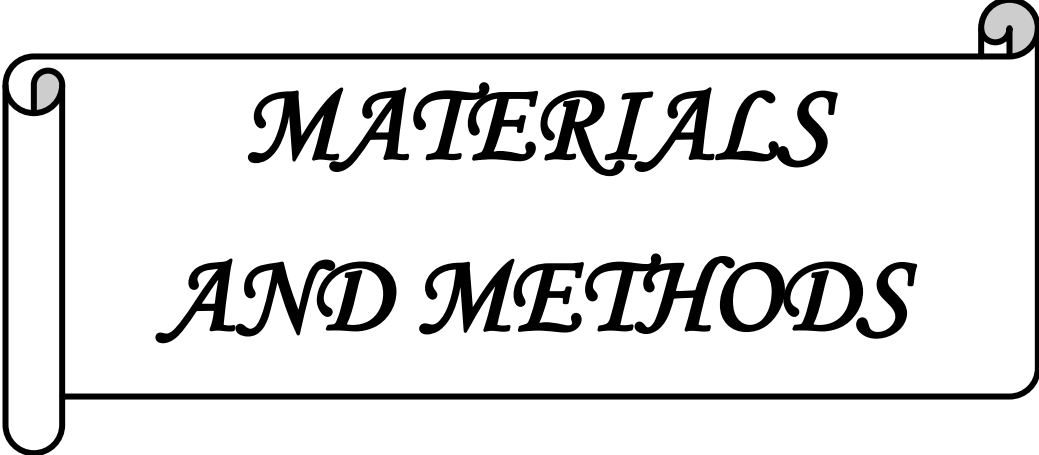
So in nut shell the advantages of this environmental friendly culture system were firstly the water quality in the system could be improved due to the formation of bioflocs (Hari *et al.*, 2006), secondly, zero or low water exchange system could increase feed efficiency with the consumption of flocs, created continuously in the biofloc system (Emerenciano *et al.*, 2012), thirdly, with high density stocking, increased resource productivity could be achieved (Wei *et al.*, 2016), and fourthly the technology could increase bio-security in aquaculture (Pérez-Fuentes *et al.*, 2016).

2.16. Disadvantages of biofloc technology

There were so many drawbacks in the biofloc system, such as the requirement of uninterrupted aeration and continuous water agitation for mixing of suspended solids.

Those aerators and pumps required high energy costs for their regular operation and any occurrence of power failure could cause massive monetary losses. High electricity costs might not make the systems truly practicable to small-scale farmers of our country. Numerous disadvantages were also reported for BFT, such as limited response time due to use of dissolved oxygen in the water and elevated respiration rates, possibility of pollution by nitrate accumulation and the necessity of start-up period and alkalinity supplementation (Reddy, 2019). The slow establishment of nitrifying bacteria within the BFT system was a very common problem and hence it took more than one month to develop the initial bioflocs, which might put negative influence on aquatic life at sensitive stages of their life cycle (Mugwanya *et al.*, 2021). Besides that inconsistent performance, seasonal variation and dependence on the daylight time particularly for sunlight-exposed unit were also reported in BFT (Reddy, 2019). The biofloc system needed regular maintenance which was not inexpensive. Another vital disquiet of the biofloc system was the inferior quality and low market price of BFT-produced fish and shrimps due to development of off-flavors for geosmin and 2-methylisoborneol. The main reasons of these off-flavors were presence of high turbidity, filamentous cyanobacteria and Actinomycetes in the culture system. The problem might be overcome by transferring fishes to clean running water before harvesting which was costly, time taking and unsustainable (Mugwanya *et al.*, 2021).

CHAPTER -3



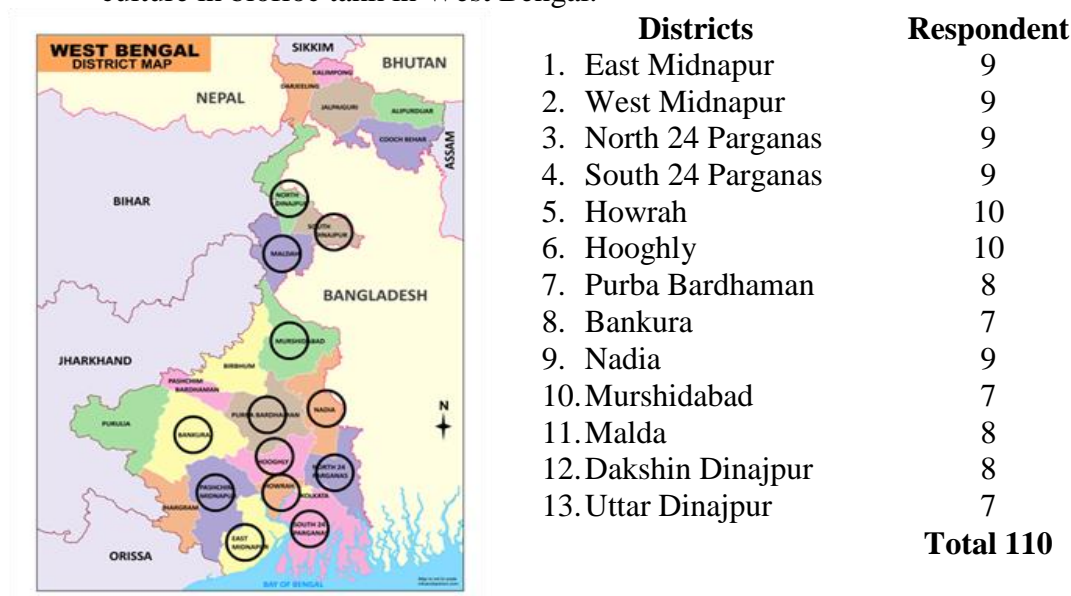
*MATERIALS
AND METHODS*

3. MATERIALS AND METHODS

3.1. Survey on the present scenario of biofloc tank culture practices of *Oreochromis niloticus*, *Heteropneustes fossilis* and others freshwater finfish in West Bengal

Total 13 Districts of West Bengal, i.e., East Midnapur, West Midnapur, North 24 Parganas, South 24 Parganas, Howrah, Hooghly, Purba Bardhaman, Bankura, Nadia, Murshidabad, Malda, Dakshin Dinajpur, Uttar Dinajpur (Fig.3.1) were selected preponderance with biofloc culture of fresh water fish on the basis of information obtained from the progressive farmers, fish seed suppliers, fishery experts and also state fisheries departmental officers. With the help of the afore said sources a preliminary list of 20-30 farmers were prepared from each 13 districts, performing biofloc tank culture till September, 2020 with a criteria that those farmers had already harvested at least one crop earlier.

Fig.3.1. Sources of respondent to study the present scenario of freshwater finfish culture in biofloc tank in West Bengal.



Total 130 biofloc farmers were selected randomly, i.e., 10 from each district, from those prepared farmers list. Majorities of the farmers were asked by telephonic conversations where a few were asked by face to face contact at their culture set up, as per the structured interview schedules regarding every minute aspect on personal details, culture strategies, technical issues, disease management, production, profit gain and prospect (Annexure I). Out of 130 selected farmers, only 110 farmers who were voluntarily shared their views, was considered as population size in this study.

All the collected data were accumulated for percentage calculation and presented in graphical forms by MS Excel 2007. Till then no such information were available on record regarding the biofloc culture practices adopted by the farmers in West Bengal. Perhaps this study was done as a pioneer in that regard.

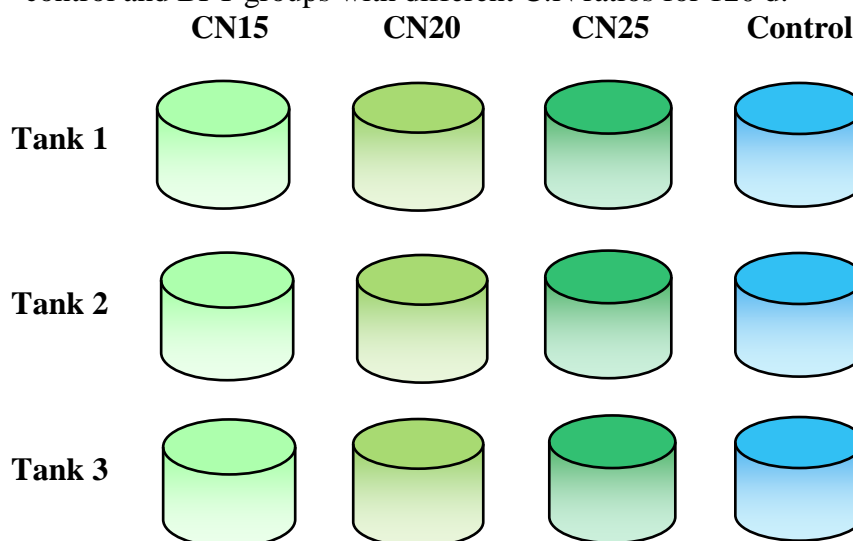
3.2. Place of Experimental Set-up:

The whole experimental set-up for the present studies for rearing of *Oreochromis niloticus* and *Heteropneustes fossilis* in biofloc system with different carbon to nitrogen (C:N) ratios were conducted at the biofloc culture unit of Sasya Shyamala Krishi Vigyan Kendra, Sonarpur, South 24 Parganas, Kolkata-700150, West Bengal (Lat. 22°25'40.22" N; Long. 88°23'17.62" E).

3.2.1. Experimental Set-up and stocking of fish

A completely randomized design of 5000 l tarpaulin tanks with 1.3 m diameter (containing 4000 l water) were used in triplicates, where a control Nile tilapia group without biofloc and three biofloc treated (BFT) Nile tilapia groups were reared in zero water exchange biofloc system with three different C:N ratios for 120 d. For each individual experimental tank including control, a total 2500 numbers of sex reversed Nile Tilapia (*Oreochromis niloticus*) of an average size of 0.5 ± 0.05 g were purchased from an accredited fish farm Anand Krishi Khamar, Kalyani. All the experimental fish were acclimatized for two weeks and were disinfected with 2 ppm potassium permanganate (KMnO_4) for 10 min before releasing in the tarpaulin tanks. At a glance the experimental setup for Nile Tilapia (*Oreochromis niloticus*) involved a conventional system without biofloc as control (C) and experimental BFT system with three different C:N ratios, viz. 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) (Fig.3.2). The control tank was maintained autotrophically without addition of any carbon source and minimal water exchange @ 20% of total volume daily. Feed containing 28-32% crude protein with no additional carbon source was provided @ 5-2% of their body weight to the control fish. In addition to the feed at the same rate, jaggery was added in the BFT groups as carbon source to raise the C:N ratios to 15, 20 and 25 to promote biofloc development. The indoor experimental setup was facilitated of diffuse sunlight though transparent asbestos sheet in day time and well natural air circulation through iron fence windows. This said experiment was started on dated 2th June, 2021 and ended on dated 30th September, 2021.

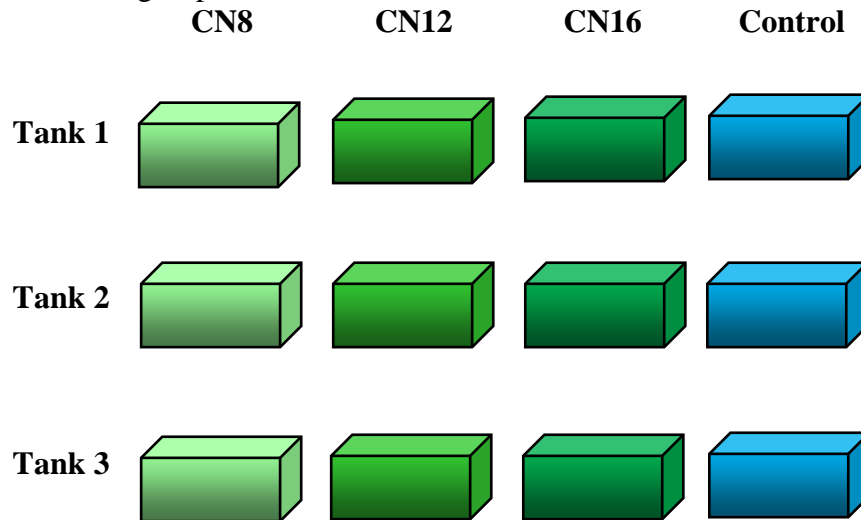
Fig.3.2. Experimental design for rearing of Nile Tilapia (*Oreochromis niloticus*) in control and BFT groups with different C:N ratios for 120 d.



Another randomized design of 2000 l cement rectangular tanks (containing 1500 l water: $2 \times 1 \times 0.75 \text{ m}^3$) were used in triplicate, for rearing of Singhi (*Heteropneustes fossilis*) in clear water as control and three zero water exchange BFT systems with different C:N ratios for 180 d. Prior to use, the tanks were scrubbed, cleaned with chlorinated water (200 ppm), flushed thoroughly with fresh water for three days. For each tank, total 700 numbers Singhi (*Heteropneustes fossilis*) of an average size of $5.0 \pm 0.5 \text{ g}$ procured from the local farmer. Before liberation of fish in experimental tanks they were disinfected by introducing in 5 ppm KMnO_4 solution for 15 min and the weak fish were removed immediately and the selected fish were acclimatized for seven days in the culture tanks. For Singhi (*Heteropneustes fossilis*) the combinations were control (C) without biofloc and, BFT systems with C:N ratios 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16) (Fig.3.3). The control tank was maintained autotrophically without the addition of any carbon source and regular water exchange @ 20% of total volume. Feed containing 28-32% crude protein with no additional carbon source was provided @ 5-2% of their body weight to the control fish. In addition to the feed at the same rate, jaggery was added in the BFT groups as carbon source to raise the desired C:N ratios for promoting biofloc development. The indoor experimental setup were facilitated of diffuse sunlight though transparent asbestos sheet in day time, 100 watt electric light on 4' top of each tank at night and well natural air circulation through iron fence windows. The experiment was conducted in post monsoon, winter and spring season when the temperature became quite low which might not favored biofloc system as the ideal temperature for heterotrophic

bacteria was 25°C above (Azim and Little, 2008). Hence, thermostats (RSElectrical; China made) were used in this study to maintain the water temperature above 26°C throughout the experiment in individual cement tank for rearing of *Heteropneustes fossilis*.

Fig.3.3. Experimental design for rearing of Singhi (*Heteropneustes fossilis*) in control and BFT groups with different C:N ratios for 180 d.



3.2.1. Preparation of bacterial inoculums in tank and culture procedure

All the experimental and control tanks were filled with ground water @ 4000 l for Nile tilapia & 1500 l for Singhi. Raw salt was mixed @ 1 kg/1000 l of water followed by aeration for one day. Commercial Probiotics (Virvac Probiotics V5) and Jaggery (locally procured) were applied @ 25 g/1000 l and 50 g/1000 l, respectively, followed by waiting for 5-7 d for appearance of desired floc. After that stocking of *Oreochromis niloticus* (0.5 ± 0.05 g) @ 312.5 g/m^3 or 2500 nos. in 4000 l, and *Heteropneustes fossilis* (5 ± 0.05 g) @ 2.33 kg/m^3 or 700 nos. in 1500 l were carried out. Supplementary feeding to all including control and carbon addition in BFT tanks to maintain target C:N ratio were conducted throughout the culture period. Apart from that a separate preparation of a ‘FCO’ or ‘Fermented Organic Carbon’ was prepared by mixing 50 g probiotic and 100 g jaggery in 10 l water with aeration for 72 h in a closed container for direct application @ 0.2 ml/l into the BFT tank on daily basis for four weeks and then as per requirements.

3.2.3. Aerator system for the Biofloc tanks

During the experimental period, a central airstream blower system consist of six 1 HP air blowers (China-made; Aqua Blue AP-160 model with a power of 105

Watt and air flow rate of 160 LPM and maximum pressure of 42 Bars) were used to aerate all those tanks. One aerator was connected with water hose pipes and air stones for two circular tarpaulin tanks to ensure continuous aeration and optimum water quality parameters for fish and floc cultivation for Nile tilapia. Whereas, one 1 HP aerator was connected with water hose pipes and air stones for four rectangular cement tanks for rearing of Singhi.

Each tarpaulin tank for Nile tilapia rearing and cement tank for rearing of Singhi were aerated with six and two air stones, respectively with a combine air flow rate of 8-10 LPM, so that the dissolved oxygen content was maintained above 6 mg/l throughout the experiment.

3.2.4. Estimation of organic carbon (%) in jaggery

The organic carbon (%) in jaggery was estimated by Walkley and Black (1934) rapid titration method. First 2 g of jaggery was taken in a 500 ml conical flask with addition of 10 ml $K_2Cr_2O_7$ (1 N) (49.04 g reagent grade in 1 l distilled water), 20 ml concentrated H_2SO_4 (Specific gravity 1.84) and pinch of silver nitrate and mixed thoroughly. The mixture was digested by slight heating, shaking for 30 min in dark room. When the mixtures became green, known volume of $K_2Cr_2O_7$ (1 N) solution was added to convert the mixtures in yellow in colour and then the mixture was diluted with 200 ml distilled water. After that 5 ml of Phosphoric acid (85%) was added to it and then the mixtures was titrated for excess dichromate with standard $FeSO_4$ solution (278 g $FeSO_4 \cdot 7H_2O$ in water mixed with 15 ml concentrated H_2SO_4 and volume made up to 1 l by distilled water) by using 1 ml diphenylamine (0.5 g diphenylamine mixed with 20 ml concentrated H_2SO_4 and 20 ml distilled water) as indicator, upto an end point blue to brilliant green. Similarly a blank was also run without jaggery in the same way. The Organic Carbon (%) was estimated by the formula given below.

$$\text{Organic Carbon (\%)} = \frac{(B-U) \times D \times N \times A}{B \times W} \times 100$$

Where,

B = Volume of $FeSO_4$ required for blank; U = Volume of $FeSO_4$ required for sample;
D = ml of $K_2Cr_2O_7$ used; N = Normality of $K_2Cr_2O_7$ (1 N); W = Weight of sample used; A = mEq (Milliequivalent) of Carbon (0.003).

3.2.5. Carbon Nitrogen Ratio Maintenance

Maintenance of different C:N ratios (CN15, CN20 & CN25 for Tilapia) and (CN8, CN12 & CN16 for Singhi) was performed as per Elayaraja *et al.* (2020) according to the following formula;

Q_{Feed} (quantity of feed, g) = Fish initial weight (g/biomass) \times feeding rate (%).

N_{Feed} (amount of nitrogen produced, g) = $Q_{\text{Feed}} \times \text{Crude protein\%} \times 6.25^{-1}$, as each g of feed having 16% of nitrogen.

$N_{\text{Excretion}}$ (amount of nitrogen excreted, g) = $N_{\text{Feed}} \times 70\%$, as only 70% of produced nitrogen was excreted, while the rest was digested and transformed into muscle.

$C_{\text{Requirement}}$ (amount of carbohydrate requirement, g) = $N_{\text{Excretion}} \times [\text{desired (C:N)}] \times [\%C \text{ in the utilized carbohydrate}]$

Organic %C; in jaggery was 46.7% as measured by the procedure for estimation of organic carbon in soil and sediment sample by Walkley and Black's rapid titration method.

3.2.6. Feeding of fish and addition of carbon source

Initial stocking density for Nile Tilapia and Singhi were 312.5 g/m³ and 2.33 kg/m³, respectively. All the control and experimental fishes were fed daily with floating commercial feed containing 28-32% crude protein. Nile Tilapia were fed @ 5-2% of their body weight (BW) daily for 120 d, while Singhi were fed @ 5-2% BW daily for 180 d (Table 3.1). Feed were spelt into two equal daily amounts (8:00 am & 4:00 pm) and required amount of jaggery was added daily after 2 h of morning meal to maintain the desired C:N ratios. Fish biomasses of individual tank were checked weekly by random sampling of 50 nos. fish. Accordingly, the amount of daily feed and jaggery addition were revised in every week. The C:N ratios were maintained strictly up to 12th weeks of the culture of Nile tilapia or *Oreochromis niloticus* by incorporation of proper calculated amount of jaggery. After that, a continuance dosage of jaggery was applied to each BFT tanks for rest of the culture duration for proper maintenance of floc volume and heterotrophic bacterial load without uncontrolled elevation of the total suspended solids. Whereas, the C:N ratios were maintained strictly during culture of Singhi or *Heteropneustes fossilis* for 180 d.

Table 3.1. Regular Feeding rate of *Oreochromis niloticus* and *Heteropneustes fossilis*

Fish size		Feeding rate (% BW)	% of crude protein in feed
<i>Oreochromis niloticus</i>	<i>Heteropneustes fossilis</i>		
0.5-20 g	5-20 g	5	32
20-50 g	20-30 g	3	28
> 50 g	> 30 g	2	28

Table 3.2. Total amount of feed and jaggery applied for rearing of *O. niloticus* and *Heteropneustes fossilis* in biofloc system for 120 d and 180 d, respectively.

<i>Oreochromis niloticus</i>	C:N=15:1	C:N=20:1	C:N=25:1	Control
Jaggery applied (kg)	409.79 ± 5.64	523.37 ± 8.25	583.25 ± 1.50	Nil
Supplementary feeds (kg)	407.93 ± 8.62	395.98 ± 9.06	378.44 ± 5.14	371.25 ± 14.55
<i>Heteropneustes fossilis</i>	C:N=8:1	C:N=12:1	C:N=16:1	Control
Jaggery applied (kg)	49.40 ± 1.90	71.56 ± 1.41	91.14 ± 0.74	Nil
Supplementary feeds (kg)	87.67 ± 1.74	91.31 ± 3.00	83.11 ± 0.59	79.19 ± 1.78

3.3. Estimation of growth and feeding performances of *Oreochromis niloticus* and *Heteropneustes fossilis*)

Weekly growth was calculated by weighing 50 fishes taking randomly from each tank. For this purpose they were first anesthetized with MS222 at 50 µl in 10 l of clear water and after taking total length (TL) and weight (W) they were again liberated in their respective tank. At the end of the 120 d culture of Nile tilapia and 180 d culture of Singhi, growth performance and feeding parameters were calculated using the following equations (Luo *et al.*, 2014).

$$a) \text{ Survival rate (\%)} = \frac{N_t}{N_o} \times 100$$

Where,

N_t = Final fish count

N_o = Initial fish count

$$b) \text{ Weight gain (\%)} = \frac{\text{FBW} - \text{IBW}}{\text{IBW}} \times 100$$

Where,

FBW = Final body weight (g)

IBW = Initial body weight (g)

$$c) \text{ Specific growth rate (SGR) (\% d)} = \frac{L_n(\text{FBW}) - L_n(\text{IBW})}{N} \times 100$$

Where,

FBW = Final body weight (g)

IBW = Initial body weight (g)

L_n = Natural Log

N = Duration of culture (d)

d) Total feed intake (g) = Total sum of feed consumed by all the survived fish for
120 d

$$\text{e) Feed conversion ratio (FCR)} = \frac{\text{Dry weight of feed consumed by fish}}{(W_t - W_0)}$$

Where, W_t : Total weight of fish at the end of the study (g)
 W_0 : Total weight of fish at the beginning of the study (g)

$$\text{f) Feed Efficiency Ratio (FER)} = \frac{1}{\text{FCR}}$$

$$\text{g) Protein efficiency ratio (PER)} = \frac{(W_t - W_0)}{P_i} \times 100$$

Where, W_t : Total weight of fish at the end of the study (g)
 W_0 : Total weight of fish at the beginning of the study (g)
 P_i : Protein content of the feed ingested during experiment (g)
(NB: Total protein ingested was determined from the daily feed intake multiplied by diets protein content.)

$$\text{h) Hepatosomatic index (\%)} = \frac{\text{Weight of liver (g)}}{\text{Total weight of fish (g)}} \times 100$$

$$\text{i) Gastrosomatic index (\%)} = \frac{\text{Weight of intestine (g)}}{\text{Total weight of fish (g)}} \times 100$$

$$\text{j) Condition factor (CF)} = \frac{\text{Weight of fish (g)}}{\text{Length of fish (cm)}^3} \times 100$$

Plate 3.1. Experimental Set-up for rearing of Nile Tilapia (*O. niloticus*) and Singhi (*Heteropneustes fossilis*) in biofloc system for 120 d and 180 d, respectively.



Plate 3.1A. Biofloc unit of Sasya Shyamala KVK, Sonarpur, South 24 Pgs., Kol - 150

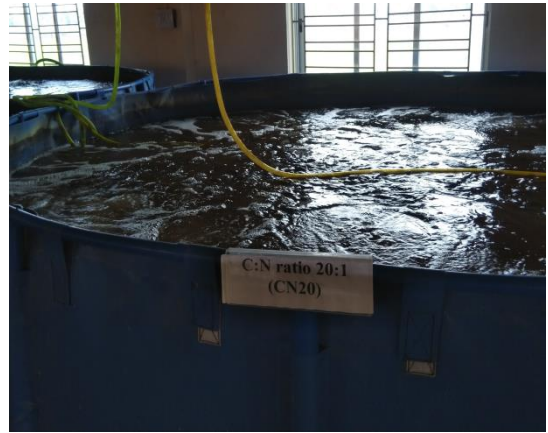


Plate 3.1B. Biofloc culture of Nile tilapia



Plate 3.1C. Circular tanks for *O. niloticus*



Plate 3.1D. Circular tanks for *O. niloticus*



Plate 3.1E. Rectangular cement tanks for *Heteropneustes fossilis*



Plate 3.1F. Rectangular cement tanks for *Heteropneustes fossilis*

Plate 3.2. Aerators, thermostat, pH and dissolve oxygen meters, supplementary feeds and other ingredients for biofloc culture.



Plate 3.2A. Aerators



Plate 3.2B. pH and D.O. meters



Plate 3.2C. Thermostat



Plate 3.2D.
Supplementary feeds (0.8 mm)



Plate 3.2E. Supplementary feeds (1.5 mm)



Plate 3.2F. Raw salts



Plate 3.2G. Probiotics



Plate 3.2H. Jaggery

3.4. Proximate composition analysis of Biofloc and *Oreochromis niloticus* and *Heteropneustes fossilis*)

Proximate composition of whole fish body of both *Oreochromis niloticus* and *Heteropneustes fossilis* and floc collected from each experimental tank maintaining different C:N ratios were measured by standard method of AOAC, 2012. *Oreochromis niloticus* and *Heteropneustes fossilis* were collected on the day of harvesting i.e., 120 d and 180 d, respectively. Concentrated biofloc sample was collected from each tank by passing tank water through a 10- μ m nylon mesh after 60 d of culture. The samples was dried in an oven at 105°C until they reached a constant weight followed by preservation in a refrigerator (-20°C) (Wei *et al.*, 2016) until proximate composition analysis.

3.4.1. Determination of moisture content

The estimation of moisture content of fish only was done by following the method given by AOAC (2012). After measuring appropriate weight of 5 g of sample in a pre weighed petri dish, it was placed in a hot air oven without lid. The samples were kept overnight (16 h) for drying in hot air oven at fixed temperature of 100 \pm 5°C. Then the dishes were cooled in desiccators at room temperature. Estimation of total moisture content was done by the formula given bellow:

Moisture (%) = {(Wt. of dish with sample before drying – wt. of dish with sample after drying)/ (wt. of dish with sample before drying – wt. of empty dish)} \times 100

3.4.2. Estimation of protein content

Crude protein content of the fish and biofloc sample was estimated by using Kjeldahl method (AOAC, 2012). One gram sample were weighed in a dry kjeldahl flask followed by addition of 5 g of digestion mixture and 20 ml of concentrated H₂SO₄ and few glass beads to avoid bumping. Initially the mixtures were digested slowly by heating followed by vigorously for 4-6 h until the sample became clear and colourless to ensure absolute conversion of nitrogen in the sample to ammonium sulphate. The flask was then cooled and volume was made up to 250 ml by distilled water. The kjeldahl steam distillation unit was rinsed well by steaming before. Addition of 10 ml of 2% boric acid with 2-3 drops of Toshiro's indicator was made in a 100 ml conical flask and it was placed in such a way that tip of the outlet of the condenser of distillation unit remain dipped into the boric acid solution. Then 5 ml of the made up digested sample was added to the previously cleaned distillation chamber and about 10 ml of 40% NaOH was added before rinsing with distilled water. The

steam distillation was continued up to 7-8 min until collection of 30 ml distillate in the receiving flask. At the end the receiving flask was lowered and hold for about 1 min at about 1 cm below the tip of the condenser. The condenser outlet was washed into the receiving flask with distilled water. The boric acid turned green when ammonia was absorbed. A blank was also done through all the steps by taking distilled water in place of sample. The collected distillate was titrated with 0.02 (N) H₂SO₄ until the original red colour was obtained. Total protein content was estimated with the formula given bellow:

1 ml of 0.02 (N) H₂SO₄ = 0.00028 g of nitrogen

$$\% \text{ Total nitrogen} = \frac{0.00028 \times \text{titre value} \times 250 \times 100}{5 \times \text{weight of sample}}$$

$$1 \text{ g nitrogen} = \frac{100}{16} = 6.25 \text{ (conversion factor)}$$

Hence; % Protein = % nitrogen × 6.25 (conversion factor)

3.4.3. Determination of fat content

The fat content was estimated by Soxhlet method (AOAC, 2012). Five gram dry sample were weighed in a thimble and plugged with cotton. The thimble was then fixed in to Soxtech apparatus (Socsplus, Pelican India Ltd.). The fat was extracted with anhydrous ether (Boiling point: 40-60°C) for about 16 h. After completion of the extraction, the thimble was removed and the solvent from the receiving flask was collected out by distilling it off, before it returned to the flask by siphoning. Thus, maximum possible amount of solvent was restored. Finally, the traces of solvent were removed from the flask by overnight drying it in oven at 100°C. After cooling the flask in the desiccators, its weighed was recorded. Final fat content was estimated by using the formula given bellow:

$$\text{Fat content (g per 100 g of sample)} = \frac{\text{Weight of fat in the sample}}{\text{Original weight of the sample}} \times 100$$

3.4.4. Determination of ash content

The estimation of ash content was done by following the method given by AOAC (2012). Five gram of sample was weighed accurately in a porcelain crucible and dried in a hot air oven overnight at temperature of 100±5°C. Sample were char drier completely by heating over a burner and incinerated in a muffle furnace at a temperature of 550±50°C with adequate air supply until it became completely white.

After that the crucible was taken out and cooled in a desiccator at room temperature.

Total ash content was estimated using the formula given below:

$$\text{Ash (\%)} = \frac{(\text{Weight of Crucible with the material after incinerating} - \text{weight of empty crucible})}{(\text{Weight of crucible with sample before incinerating} - \text{weight of empty crucible})} \times 100$$

3.5. Collection of serum from *Oreochromis niloticus* and *Heteropneustes fossilis*

3.5.1. Sample collection

For the fish welfare study, blood samples were collected from all the biofloc groups and control. Blood samples for Nile tilapia (*Oreochromis niloticus*) were collected on 60th and 120th d from all the experimental groups and control. Similarly, blood samples for Singhi (*Heteropneustes fossilis*) were collected on 90th and 180th d from all the experimental groups and control.

3.5.2. Blood collection

Ten fish from each experimental tank including control were sampled with minimum handling stress and transferred to the plastic buckets containing water of the same tank and instantly anaesthetized with clove oil (Merck, Germany) @ 20 µl/l. The blood from the experimental fish was drawn using 2 ml sterile syringes through caudal puncture (Roberts, 2012).

3.5.3. Serum collection

The non-heparinized blood was allowed to clot ≈30°C by keeping the syringe in slanting position and kept at 4°C overnight. The serum samples were collected by centrifugation at 4500 rpm for 15 min (Eppendorf Centrifuge 5418 R), transferred to Eppendorf tubes and stored at –20°C for further analysis.

3.6. Serum biochemical analysis of *O. niloticus* and *H. fossilis*

3.6.1. Serum biochemical analysis of stress parameters

3.6.1.1. Serum glucose

The serum glucose were determined by using a glucose test kit (DiaSys Diagnostics Systems, GmbH, Germany) following the GOD-POD method. To 1000 µl of the reagent solution taken in a clean and dry test tube, 10 µl of serum was added. Simultaneously a standard, using standard glucose (100 mg/100 ml), and a blank (10 µl distilled water and 1000 µl reagent) were prepared (1010 µl each). All the tubes were mixed thoroughly and incubated at 37°C for 5 min. Then the absorbance was taken at 546 nm using a Photometer (Model: 5010 v5+, Robert Riele KG, Berlin,

Germany). The final glucose concentration were calculated by putting the absorbance values (optical density) into the standard formula and expressed as mg/dl (Trinder, 1969).

3.6.1.2. Total protein

The serum total protein were determined by using Total protein FS test kit (DiaSys Diagnostics Systems, GmbH, Germany) following photometric test according biuret method. In alkaline solution proteins formed a violet colour complex with copper ions, which absorbance was directly proportional to the protein concentration.

To 5 µl of the serum sample taken in a clean and dry test tube, 200 µl of reagent 1 and 50 µl of reagent 2 were added. Simultaneously a standard, using 5 µl bovine serum albumin (<5%), 200 µl of reagent 1 and 50 µl of reagent 2 were prepared. All the tubes were mixed thoroughly and incubated at 37°C for 5 min. Then the absorbance was taken at 545 nm using Photometer (Model: 5010 v5+, Robert Riele KG, Berlin, Germany). The final protein concentrations were calculated by putting the absorbance values (optical density) into the standard formula given below and expressed as g/dl.

$$\text{Total protein (g/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times \text{Protein conc. of standard (g/dl)}$$

3.6.2. Serum biochemical analysis of liver functions

3.6.2.1. Serum Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activity

The serum ALT and AST levels were determined by using SGPT (Serum glutamic pyruvic transaminase) and SGOT (Serum glutamic oxaloacetic transaminase) test kits (ERBA, Transasia Bio-Medicals Ltd., India) following the modified UV (IFCC) and kinetic assay methods, respectively. The serum ALT and AST were measured from 100 µl serum samples at room temperature by following the kit procedure through Photometer (Model: 5010 v5+, Robert Riele KG, Berlin). The ALT and AST concentrations were calculated by putting the absorbance values as 340 nm (optical density) into the standard formula and expressed as IU/l (Thomas, 1998).

3.6.2.2. Serum alkaline phosphatase (ALP) activity

The serum ALP activity were determined by using an ALP test kit (DiaSys Diagnostic Systems, Holzheim, Germany) following the kinetic photometric method

(Tietz, 1994) recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). By ensuring the kit protocol, the serum ALP levels were measured at 37°C using a Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) at 405 nm subsequently over 3 min. The mean absorbance per minute was used to calculate the ALP level.

3.6.3. Serum biochemical analysis of ionic balance activity

3.6.3.1. Serum calcium (Ca²⁺)

The serum calcium ion levels were determined by using a calcium test kit (DiaSys Diagnostic Systems, Holzheim, Germany) following a photometric method using arsenazo III (Michaylova and Iikova, 1971). By ensuring the kit protocol, the serum calcium ionic levels were measured at 37°C using a Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) at 650 nm subsequently over 3 min. The mean absorbance per minute was used to calculate the calcium ions concentration.

3.6.3.2. Serum chloride (Cl⁻)

The serum chloride ion levels were determined by using a chloride test kit (DiaSys Diagnostic Systems, Holzheim, Germany) following a photometric test using the ferric (III) perchlorate method (Schoenfeld and Lewellen, 1964). By ensuring the kit protocol, the serum chloride ionic levels were measured at 37°C using a Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) at 340 nm. The mean absorbance per minute was used to calculate the chloride ions concentration.

3.7. Analyses of hematological parameters

3.7.1. Collection of blood samples

Ten fish from each tank including experiment and control were sampled with minimum handling stress and transferred to the plastic buckets containing water of the same tank and instantly anaesthetized with clove oil (Merck, Germany) @ 20 µl/l. The blood from the experimental fish were drawn using 2 ml sterile syringes filled with 3% ethylenediaminetetraacetic acid (EDTA) through caudal vein puncture (Roberts, 2012). The anticoagulant mixed blood was stored in Eppendorf tubes for further analysis.

3.7.2. Enumeration of total erythrocyte count (TEC)

The enumeration of TEC of fish blood were made in Neubauer's counting chamber using Hayem's fluid (Math *et al.*, 2016). An aliquot (0.20 µl) of the collected blood sample from the Eppendorf tubes after thorough shaking was sucked into a

micropipette and immediately diluted (1:200) using Hayem's fluid in a test tube. The diluted blood was mixed gently by tilting the test tubes to avoid cell destruction. Some of the diluted blood were drawn into the micropipette and expelled out touching the tip of the pipette to the edge of the cover slip on the counting chamber. Capillary action will draw the blood onto the chamber followed by enumeration under trinocular microscope. The cells occurring in five small squares at the center of the grid (Total area: 0.02 mm^3 ; $1/50$ of 1 mm^3) was counted. The number of erythrocytes per mm^3 was calculated as follows:

$$\text{TEC} / \text{mm}^3 = \text{Nos. of cells counted in } 0.02 \text{ mm}^3 \times 50 \text{ (area counted)} \times 200 \text{ (dilution)}$$

3.7.3. Estimation of total leucocyte count (TLC)

Total leucocyte count (TLC) of fish blood were made in Neubauer's chamber using Shaw's WBC diluting fluid (Shaw, 1930). An aliquot ($0.20 \mu\text{l}$) of the collected blood sample from the Eppendorf tubes after thorough shaking was sucked into a micropipette and immediately diluted (1:20) using Shaw's fluid in a test tube. The diluted blood was mixed gently by tilting the test tubes to avoid cell destruction. Some of the diluted blood were drawn into the micropipette and expelled out touching the tip of the pipette to the edge of the cover slip on the counting chamber. Capillary action will draw the blood onto the chamber followed by enumeration under trinocular microscope. During enumeration, cells in the large four squares were considered. The TLC per mm^3 was calculated as follows,

$$\text{TLC} / \text{mm}^3 = \text{Nos. of cells counted in } 0.1 \text{ mm}^3 \times 10 \text{ (area counted)} \times 20 \text{ (dilution)}$$

3.7.4. Estimation of hemoglobin concentration (Hb)

The estimation of Hb levels were estimated by the acid-haematin method using Sahil's hemocytometer (Ashford, 1943). The blood was drawn into the Sahil's pipette upto the 0.02 mark. The blood was expelled into a hemometer tube containing 0.1 N HCL, upto the lowest mark (10). The pipette was then removed and the content was thoroughly mixed using a stirrer. The mixture were diluted with distilled water by adding a few drops at a time with thorough mixing until the colour of the solution matches with the glass plate of the comparator. The level of the fluid was noted at the lower meniscus. The amount of hemoglobin (g/d) was directly read from the comparator.

3.7.5. Estimation of Hematocrit value (Ht)

The hematocrit value (Ht) was determined by micro-hematocrit centrifugation (Giron-Perez *et al.*, 2008). The hematocrit tube (HiMedia, India: $7 \text{ cm} \times 11 \text{ mm}$) was

filled with anticoagulant mixed blood (2/3rd) by capillary action and surface tension, while the other end of the tube was sealed with plasticine. These tubes were placed onto the grooves of the centrifuge with the sealed ends facing away from the centre. The centrifugation was done at 10,000 rpm for 5 min (Giron-Perez *et al.*, 2008). The blood column in the tube upon centrifugation showed a 3 layer profile, viz., a tall bottom layer of red cells, a thin middle layer of WBC and the top layer of clear plasma. Hematocrit values were calculated by the following formula:

$$\text{Ht (\%)} = \frac{L_1}{L_2} \times 100 \quad \text{Where, } L_1 = \text{Height of the RBC column (in mm) and,} \\ L_2 = \text{Total length of the column (in mm).}$$

3.8. Measurements of non-specific immune parameters

3.8.1. Respiratory burst activity (RBA)

Nitroblue tetrazolium (NBT) assay were performed to estimate RBA using the method described by Anderson and Siwiki (1995). Briefly, one-hundred microliters of blood were mounted in the wells of a flat microtitre plate and incubated at 37°C for 1 h to promote cell adhesion. The supernatant were removed from wells by washing at least three times with phosphate buffered saline (PBS, pH 7.4), followed by an addition of 100 µl of 0.2% NBT. Then, the plate was incubated for another 1 h and washed once with 100% methanol and thrice with 70% methanol, followed by air dry. Potassium hydroxide (2N; 120 µl) and dimethyl sulphoxide (DMSO) (140 µl) was added into each well to form blue formazone precipitate. The absorbances of the wells were measured in an ELISA reader (ECIL, India) at 620 nm.

3.8.2. Myeloperoxidase content

Total myeloperoxidase content present in serum were measured according to Quade and Roth (1997) with some modifications. About 15 µl of the serum was diluted with 135 µl of hank's balanced salt solution (HBSS) without Ca²⁺ or Mg²⁺ in 96 well 'U' bottom microtitre plate. The wells were added with 25 µl of 20 mM 3, 3'-5,5'- tetramethyl benzidine hydrochloride (TMB) (Hi-media) and 25 µl of 5 mM H₂O₂ (Both substrates of MPO) followed by 2 min incubation at room temperature. The reaction was stopped by addition of 50 µl of 4 M sulphuric acid (H₂SO₄).The plate was centrifuged (400×g) for 10 min, and 150 µl of the supernatant from each well were transferred into a new 96 well plates. The absorbances were measured at 450 nm in a micro plate reader (ECIL, India) against 150 µl of HBSS as blank. The % release of MPO was expressed as OD₄₅₀.

3.8.3. Serum lysozyme activity

The serum lysozyme activities were measured by using turbidimetric assay described by Parry *et al.* (1965) with the microplate adaptation of Hutchinson and Manning (1996). In a 96 well microtitre plate 250 µl of *Micrococcus lysodeiticus* suspension (0.3 mg/ml) in phosphate buffer was taken, to which 10 µl of serum sample was added. The reductions in absorbances at 490 nm were determined after 0.5 and 4.5 min of incubation at 28°C in a micro plate reader (ECIL, India). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min expressed as U/min. Difference in OD was for 10 µl of serum for 4 min. Hence, calculation for 1 ml of serum for 1 min was expressed by the following formula.

$$\text{Lysozyme activity (U/ml)} = \frac{\text{Difference in OD} \times 1000 \times 1}{10 \times 4}$$

3.9. Detection of enzymes of oxidative stress

3.9.1. Superoxide dismutase (SOD)

The Superoxide dismutase assay of fish serum was performed by the spectrophotometric method developed by Mishra and Fridovich (1972) with some modifications. During sample preparation initially a stock solution (10%) of test serum was prepared with 10 µl serum and 90 µl 100 mM Phosphate buffer, pH 7.4 and, then 25 µl from that stock solution were mixed with 0.5 ml of 0.1 mM EDTA (4 mg EDTA in 100 ml distilled water), 1 ml of 0.05 M Na₂CO₃ buffer (530 mg Na₂CO₃ in 100 ml distilled water, pH 10.2) and 0.5 ml of freshly prepared 9 mM Epinephrine (30 mg Epinephrine bitartrate in 10 ml double distilled water). Absorbance was measured at 480 nm with time kinetics for 120 seconds (60 s lag time). A blank was also prepared without serum stock solution by mixing 0.5 ml of 0.1 mM EDTA, 1.025 ml of 0.05 M Na₂CO₃ buffer and 0.5 ml of freshly prepared 9 mM Epinephrine and, absorbance was measured at 480 nm with time kinetics for 120 s (60 s lag time).

The calculation of % of inhibition and SOD activity (Unit/ml serum) was measured by the following equations;

$$\% \text{ of inhibition} = \frac{\text{Absorbance rate of blank} - \text{Absorbance rate of test}}{\text{Absorbance rate of blank}} \times 100$$

$$\text{SOD activity (U/ml serum)} = \frac{\% \text{ of inhibition} \times 1000 \times 0.1}{50}$$

3.9.2. Catalase (CAT)

The Catalase activity of fish serum was performed by the spectrophotometric method developed by Beers and Sizer (1952) with some modifications. First the 10 µl of test serum was diluted at 1:10 by mixing with 90 µl distilled water. Then 10 µl of serum diluents mixed with 2920 µl of 100 mM Phosphate buffer, pH 7.4 (8.9 g Na₂SO₄ in 500 ml distilled water, pH 7.4 adjusted with KH₂PO₄ solution of 34 g KH₂PO₄ in 250 ml distilled water) and 70 µl of diluted H₂O₂ (120 µl of 30% H₂O₂ in 1880 µl distilled water). The absorbances were measured at 240 nm with time kinetics for 120 seconds (60 s lag time). The calculation of catalase activity (Unit/ml) in serum was measured by the following equations.

$$\text{Cat (U/ml)} = \frac{\text{Rate of absorption} \times \text{Total volume of assay} \times \text{dilution factor} \times 10^3 \times 0.01}{43.6 \times \text{Sample volume}}$$

3.10. Histology

The digestive organs like liver and intestine of 120 d Nile tilapia and 180 d Singhi were collected from each experimental BFT and control tank followed by fixation in Bouin's fixative for 48-72 h. After fixation, the tissues were transferred to 70% ethyl alcohol and kept overnight for histological analyses as described by Roberts (2012).

3.10.1. Tissue cutting and processing

The different tissues were taken out of the 70% alcohol and cut into small pieces. Then they were dehydrated by a series of ethyl alcohol with a gradually increasing concentration (85%, 90% and 100%). In each alcoholic concentration, tissues were kept for 90 min with two changes. The tissues after dehydration were transferred to xylene to make them transparent. Then the tissues were kept in liquid paraffin for at least 2 h and 30 min. The paraffin was allowed to penetrate into the tissues.

3.10.2. Tissue embedding

Triple filtered matured paraffin (melting point 58-60°C) was used for this purpose. The organs were kept in molten paraffin contained in L-mould and the paraffin was allowed to solidify.

3.10.3. Preparation of blocks for sectioning

The solid paraffin blocks containing tissues were trimmed into small square blocks and the tissues were exposed for proper sectioning.

3.10.4. Sectioning

The trimmed blocks were then fixed to the block holder of the microtome (Medimeas Model: MRM-RM 1191) and cut into sections or ribbons of 5 μm thickness. Good sections were carefully transferred to clean grease free glass slide. The grease free slides were layered with Mayer's albumin for better attachment of the tissues to the slides.

3.10.5. Stretching of tissues

The slides with ribbons of required lengths were transferred on a hot plate containing warm water maintained at 50-55°C to render the wrinkled tissues stretched and flat.

3.10.6. Preparation of slides and staining

The dried slides were stained by haematoxylin and eosin double staining (H&E) method described by Roberts (2012). Slides were permanently mounted using DPX (Dibutyl Phthalate Xylene) mountant.

3.10.7. Microscopy and photomicrography

The sections were screened with the help of a monitor attached microscope. The digital colour microphotographs were taken from the selected slides at different magnification with advanced Trinocular Research Microscope (Olympus, Japan, Model: BX51) using SCO-LUX camera 16 MP attached to the microscope. The images were taken with the help of ToupTek ToupView software (Version x64, 4.11).

3.11. Water Quality Parameter test

Water temperature ($^{\circ}\text{C}$), pH and dissolved oxygen (DO, mg/l) were monitored daily throughout the experimental period. Total ammonia nitrogen (TAN, mg/l), nitrite ($\text{NO}_2\text{-N}$, mg/l), nitrate ($\text{NO}_3\text{-N}$, mg/l) and total suspended solids (TSS) was measured weekly by the APHA (2005) standard methods for the examination of water and wastewater. Biofloc or floc volume was determined by Imhoff cones at every week. Measurements of total alkalinity (mg/l) and the total heterotrophic bacterial count of the biofloc tank were determined fortnightly by APHA (2005) standard methods throughout the rearing of both *Oreochromis niloticus* and *Heteropneustes fossilis*.

3.11.1. Dissolve Oxygen

Dissolved oxygen (DO, mg/l) was monitored daily throughout the experimental period using Digital DO meter (EUTECH DO600).

3.11.2. pH

Observation of pH of biofloc tank water was conducted daily throughout the experimental period using Digital pH meter (EUTECH pH700).

3.11.3. Temperature

Water temperatures (°C) were monitored daily throughout the experimental period using thermometer. As Singhi culture was conducted during winter season the temperature was adjusted at 26°C by thermostat throughout the experiment.

3.11.4. Total ammonia nitrogen (TAN)

Ammonia in the pretreated sample produced a yellow coloured compound when reacted with alkaline Nessler's reagent. The pretreatment was done by addition of zinc sulphate (ZnSO₄) and sodium hydroxide (NaOH) which precipitate calcium (Ca), Iron (Fe), Magnesium (Mg), and Sulfide (S) and removed turbidity and apparent colour. Addition of EDTA prevented precipitation of residual Ca and Mg in the presence of Alkaline Nessler's reagent. Initially, 25 ml of sample were taken in a conical flask. Then 1 ml of phenol solution (11.1 ml liquefied 89% phenol mixed with 95% ethyl alcohol to a final volume of 100 ml) was added and mixed thoroughly. After that 1 ml of solution nitroprusside solution (0.5 g sodium nitroprusside in 100 ml deionized water) was added followed by mixing of 2.5 ml oxidizing solution [100 ml alkaline citrate solution (200 g tri-sodium citrate and 10 g sodium hydroxide diluted up to 1000 ml by deionized water) mixed with 25 ml sodium hypochloride (commercial 5%)]. The flask was covered with paraffin wrapper film and was kept at room temperature (22-27°C) at subdued light for 1 h to develop yellow colour. Absorbance was measured at 649 nm by spectrophotometer. The same procedure was followed for a blank.

Calculation: Standard curve was prepared by plotting absorbance readings of standards against ammonia concentrations of standards. Sample concentrations were computed by comparing sample absorbance with the standard curve.

NB: Standard ammonia solution was prepared by dissolving 53.5 mg of ammonium chloride in 1 l of distilled water and that solution contained nitrogen at 14 mg/ml. Different working standard solution (i.e., 0.5, 2, 4, 5, 10 and 20 mg) was prepared from the above stock solution.

$$\text{TAN (mg/l)} = \frac{\text{Concentration of the standard solution}}{\text{OD value of the corresponding standard solution}} \times \text{OD of the sample}$$

3.11.5. Nitrite (NO₂-N)

Sulphanilamide was used as the amino compound which was coupled with N-(1-naphthyl) ethylene diamine dihydrochloride. This reaction led to the formation of azo dye at pH 2.0 to 2.5. The amount of azo dye formed was proportional to the initial concentration of nitrite. The extinction of the dye solutions were measured at 540 nm in a spectrophotometer. Initially 50 ml of water sample was taken in a conical flask. Then 1 ml of sulphanilamide solution (10 g of sulphanilamine, 100 ml of concentrated hydrochloric acid and 300 ml of distilled water were mixed to make up to 500 ml) was added and mixed thoroughly for 5 min. After that 1 ml of N-(1-naphthyl) ethylene diamine dihydrochloride (NEDD) solution (0.5 g of NEDD in 500 ml of distilled water) was added. The absorbance of the solution was measured within 2 h at 540 nm by spectrophotometer when water samples show visible brownish coloration. Similarly 50 ml of different standard solutions and 50 ml of blank solution were taken in conical flasks and the above procedure was followed. The nitrite value was found from the following calculation.

NB: Nitrite standard solution was prepared by dissolving 0.4926 g of anhydrous sodium nitrite in 1000 ml of distilled water. The solution contained 100 mg NO₂-N/ml was stored in a brown glass bottle with a few drops of chloroform as preservative (to retard growth of bacteria).

$$F = \frac{\text{Standard concentration } (\mu\text{g NO}_2\text{-N/l)}}{E_1 (\text{Standard}) - EB_1}$$

The concentration of NO₂-N in samples = $F \times E_1 \text{ Sample} - (E_0 + EB_1)$

Where, $EB_1 = \text{Absorbance of distilled water + reagents}$

$E_0 = \text{Absorbance of sample without NEDD reagents}$

$E_1 = \text{Absorbance of standards or samples with reagents.}$

3.11.6. Nitrate (NO₃-N)

Nitrate reacted with phenol disulfonic acid and produced nitro-derivative which in alkaline solution developed yellow colour. The colour that produced was proportional to the concentration of NO₃ present in the sample. Fifty microlitre of sample was taken in volumetric flask. Then 2 ml of buffer solution (phenol + sodium hydroxide) was added to it. Then after 2 min 1 ml of reducing reagent (CuSO₄ + N₂H₄ H₂SO₄) was added to the sample. The solution was kept for 20 h in dark. After adding

2 ml of acetone and 1 ml of sulphenilamide, the sample was kept for 2-8 min. Then 1 ml of N1-naphthyl ethylene diamine dihydrochloride (NNED) was added to it. Finally the absorbance of the sample was taken after 10 min but before 2 h of adding NNED at 543 nm through spectrophotometer. The NO₃-N was calculated from the standard curve prepared by plotting the absorbance with their respective known concentration of NO₃-N obtained through the same procedure which was expressed as mg/l.

3.11.7. Total Alkalinity

Phenolphthalein alkalinity was determined by titration of sample aliquot with a standard solution of strong acid to a pH 8.3. Total alkalinity (methyl organe) was determined by titration of sample aliquot with a standard solution of strong acid using methyl orange indicator which determined the equivalence point pH of the titration. First 20 ml sample was taken in a conical flask. Then 2-3 drops of phenolphthalein indicator was added. In case of development of pink colour the sample was titrated with 0.02N H₂SO₄ (0.56 ml in 1000 ml) till it disappeared (pH 8.3). The volume of H₂SO₄ used for titration was noted down (A). In case non appearance of pink colour after addition of phenolphthalein indicator, 2-3 drops of methyl orange were added to the same sample and it was titrated with standard H₂SO₄ till orange colour changes to pink (pH comes down to 4.5 or 4.4). The volume of H₂SO₄ added for titration was noted down (B).

$$\text{Total Alkalinity as CaCO}_3 \text{ in mg/l} = \frac{\text{Total volume of titrant (A+B) (ml)}}{\text{Volume of sample (ml)}} \times 1000$$

3.11.8. Total Suspended Solids

The TSS determination were accomplished by filtration using glass fiber Whatman GF/C within 1 µm pore-size followed by evaporation at 105°C for 2 h. The weigh difference between filter and the filter after evaporation corresponds to the concentration of suspended solids were determined by the following formula.

$$\text{TSS (mg/l)} = \frac{(M_1 - M_0) \times 10^6}{V_s}$$

Where,

M₀: the initial mass of the clean filter (g), M₁: mass of the filter with suspended solids after evaporation at 105°C for 2 h (g), and V_s: volume of sample that is filtered (ml).

3.11.9. Floc volume (ml)

Floc volume was determined by glass made Imhoff cones of one litre capacity at every week. One l water from biofloc tank was taken in the Imhoff cone and the volume of the floc plugs accumulated at the bottom of the cone was measured after 15 min of sampling.

3.11.10. Total Heterotrophic Bacterial count

The total heterotrophic bacteria were determined fortnightly by counting the colonies on Trypticase soy agar (TSA, Himedia) plates. Before plating biofloc tank water sample onto agar medium, serial dilutions was made in a physiological saline solution composed of 0.9% NaCl. Levels of bacteria were expressed in colony forming units per ml of water (CFU/ml).

3.12. Statistical analysis:

Statistical analysis was performed using Statistical Package for the Social Sciences (IBM-SPSS) Version 22. The differences were considered statistically significant at $p \leq 0.05$. One-way analysis of variance (ANOVA) with Duncan post-hoc was used for the comparison of means of the different experimental parameters within the control and different biofloc groups in connection with growth, feeding performances, proximate compositions, water quality parameters, antioxidant properties, immunity condition, hematological status and other welfare indicators.

Student's t-test ($p \leq 0.05$) was conducted to evaluate the effects of the two sampling date on welfare parameters in different experimental groups and control, i.e., 60th and 120th d for Nile tilapia and 90th and 180th d for Singhi.

Pearson correlation coefficient analysis ($p \leq 0.01$) was done for analysis of correlation between different water quality parameters measured in control and BFT experimental tanks for both Nile tilapia and Singhi.

Histological alterations of fish liver and intestines were analyzed by Kruskal–Wallis test ($p \leq 0.05$) with histological changing scores, starting from 1 to 5 (Score 1 indicated 0-5% changes, score 2: 5-15% changes; score 3: 15-25% changes; score 4: 25-50% changes and score 5 indicated 50-100% changes).

Plate 3.3: Floc managements during culture of Nile Tilapia (*O. niloticus*) and Singhi (*Heteropneustes fossilis*) in biofloc system for 120 d and 180 d, respectively.



Plate 3.3A. Floc measurements



Plate 3.3B. Imhoff cone

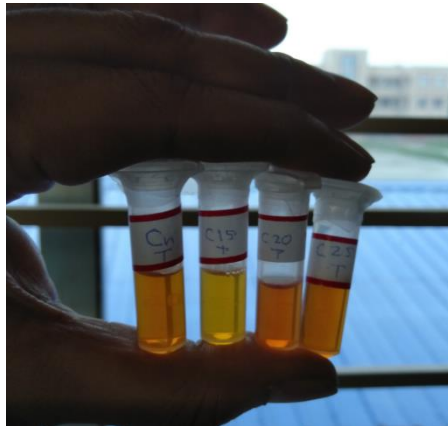


Plate 3.3C. Fish serum



Plate 3.3D. Total Heterotrophic Count



Plate 3.3E. Floc collection for proximate



Plate 3.3F. Water quality testing

CHAPTER -4



*RESULTS AND
DISCUSSION*

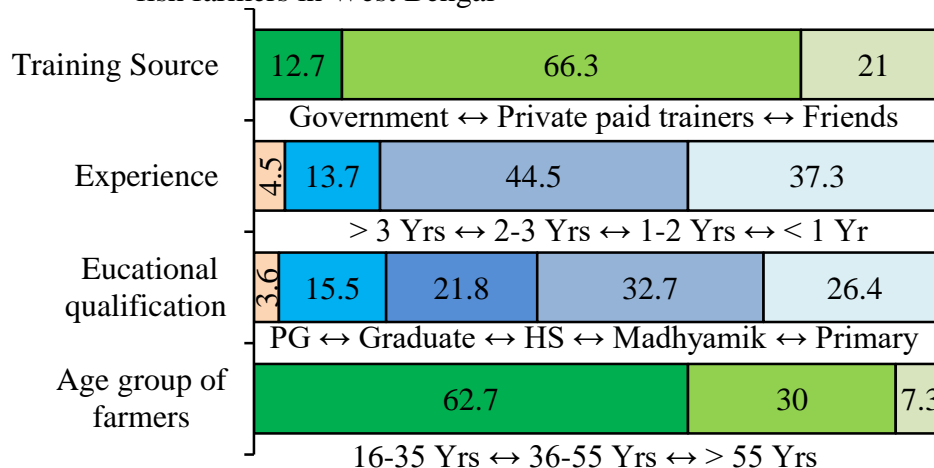
4. RESULTS AND DISCUSSION

4.1. Objective 1: To study the present scenario of biofloc tank culture practices of *Oreochromis niloticus*, *Heteropneustes fossilis* and others freshwater finfish in West Bengal.

4.1.1. Age, education and experience of biofloc farmers

The survey was undertaken during October-December, 2020 to assess the farming practices of biofloc technology in tanks by the randomly selected 110 biofloc farmers from thirteen districts of West Bengal (Fig.3.5). Till then no such information were available on record regarding the biofloc culture practices adopted by the farmers in West Bengal. Perhaps this study was done as a pioneer in that regard. Majority of the biofloc farmers were found younger with good educational background (Fig.4.1), however most of them had very little experience in biofloc culture (Fig.4.1). Unemployment during the pandemic COVID-19 situation since March, 2020, was the driving force to adopt this technology by the young enthusiastic generations of West Bengal. Most were trained by different private paid trainers or friends who already started biofloc farming but trained by Government organizations was found negligible (Fig.4.1).

Fig.4.1. Age, education, experience and training of fresh water biofloc fish farmers in West Bengal

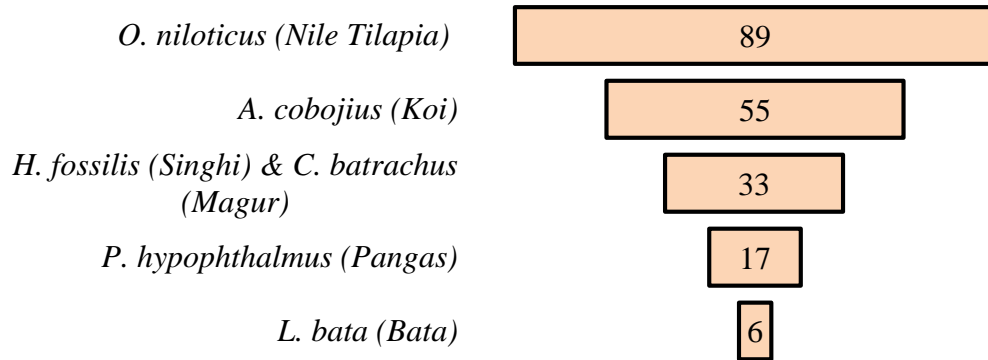


4.1.2. Culture practices of biofloc farmers

Majority of the farmers were found culturing more than one species but not multispecies in one tank. Nile tilapia or *Oreochromis niloticus* was found the most common species cultured by 89 farmers, followed by *Anabas cobojius* (Koi) (55 farmers), Catfishes like *Heteropneustes fossilis* (Singhi) and *Clarias batrachus*

(Magur) (33 farmers), and *Pangasianodon hypophthalmus* or commonly known as Pangas by 17 farmers. Unexpectedly, 6 respondents were found to be culturing *Labeo bata* which was not considered as a suitable species for biofloc (Fig.4.2).

Fig.4.2. Fish species cultured by 110 respondents (Values are in number)



Few farmers took initiation for culturing *Ompok bimaculatus* (Butter catfish or Pabda) and *Mystus* sps. (Tengra) but were unable to harvest due to sudden mortality, while few were found still struggling on it. As complete data were not obtained on Pabda and Tengra from the selected respondents, these two fish species were not considered in this survey. Among the cultured species, the most preferred stoking sizes were found 0.5-1 g/fish (Fig.4.3a). Low stoking densities were maintained for bata, pangas, singhi and magur, whereas tilapia and koi farmers were found to maintain higher stocking densities (Fig.4.3b). Higher stocking densities usually impact water quality and impart main stress to the fish (de Oliveira *et al.* 2012).

Fig.4.3a. Stocking size (g/fish) of different species in biofloc

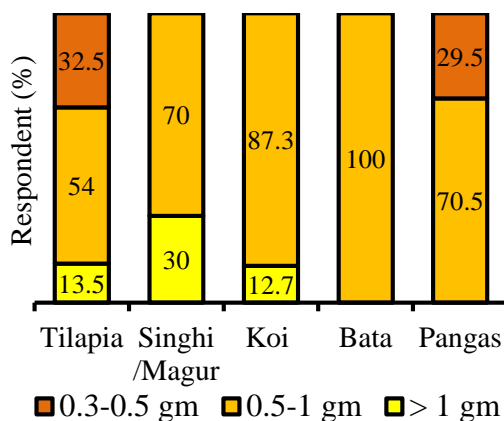
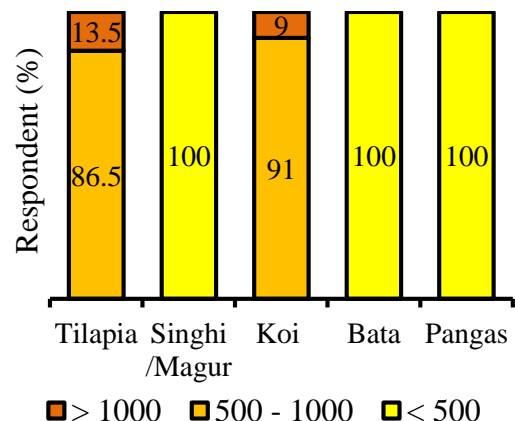


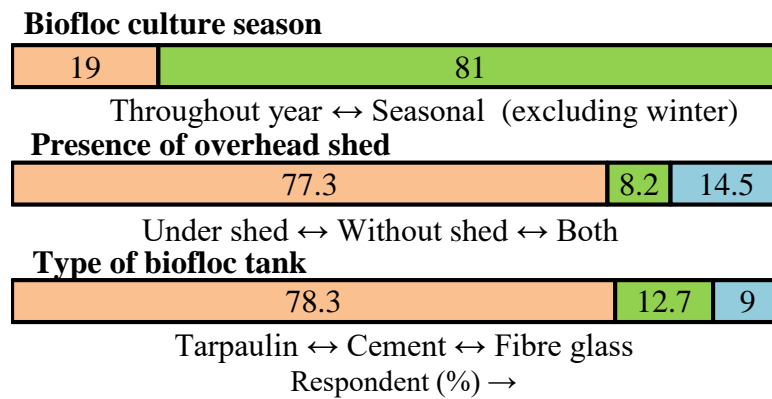
Fig.4.3b. Stocking density (Nos./1000 l water) of different species



Tarpaulin tank supported by iron mesh was found most popular over cement and fibre glass or plastic tub (Fig.4.4). Due to easy installation and comparatively lower cost the tarpaulin tanks were preferred by most of the farmers. Maximum

culture setup was found in indoor provision with well constructed or temporary covered (Fig.4.4). Due to disease predominance and difficulties in maintaining heterotrophic bacteria at low temperature, seasonal culture (excluding December-February) was found most common by 81% of respondent (Fig.4.4). According to the Azim and Little (2008) the optimal temperature to maintain floc stability should be maintained from 26°C to 32°C. Mainly the catfishes were reported to be reared throughout the year by the biofloc farmers of the present study.

Fig.4.4. Tank materials, overhead and seasonality of biofloc culture



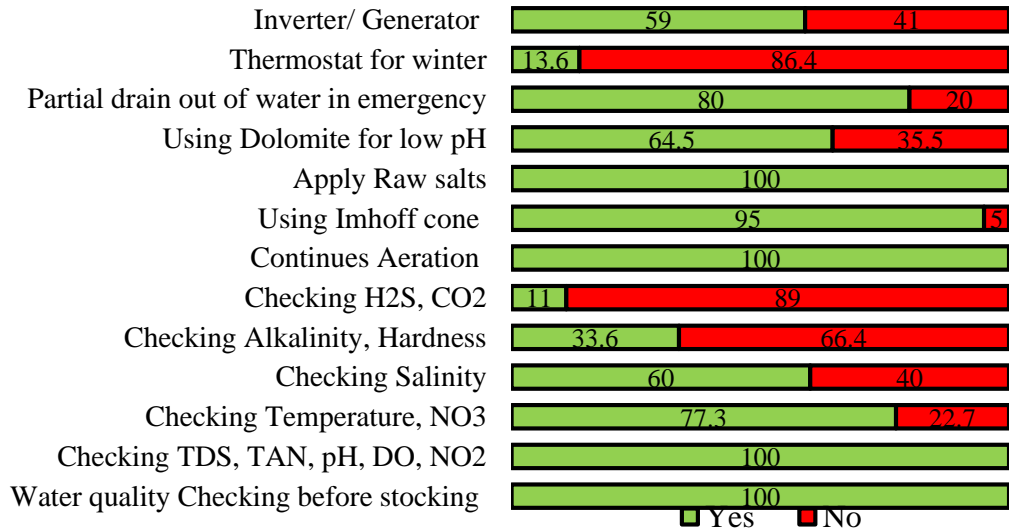
4.1.3. Water quality monitoring practices by biofloc farmers

Water quality monitoring was an important aspect of biofloc management and almost all of the farmers were found aware about the ideal physical and chemical water quality parameters required to run the biofloc system. They knew that deviation from the optimum range might cause adverse affects on crop. Monitoring of major water quality parameters like pH, DO, temperature, NO₂-N, TAN, TDS and floc volume measurement by Imhoff Cone was found common practices (Fig.4.5).

But monitoring of others parameters like NO₃-N, salinity, alkalinity, hardness, H₂S and free CO₂ were not found common. In general aquaculture system water exchange was important to maintain water quality from deterioration. In minimum or zero-water exchange culture techniques there is always a possibility of elevated nitrogen levels, which could be immobilized by increase heterotrophic bacteria with addition of external carbon (Avnimelech, 1999). Most respondents used to perform partial water exchange during emergency or when it was apprehended the TAN level could cause mass mortalities to cultured fishes. That practice could not be appreciated as it might slow down the dominance of heterotrophic bacterial community over chemoautotrophic bacteria. Provisions for emergency power supply and thermostats were found limited (Fig.4.5). Farmers not having inverter or generator facilities often

faced culture failure during long power cut-off, mainly in rural areas. The worst experience in this regard was faced by many farmers after super cyclonic storm Amphan on 16th May, 2020 which affected electricity supply badly in West Bengal.

Fig.4.5. Water quality monitoring practices adopted by the biofloc farmers (%)



4.1.4. Heterotrophic bacteria management practices by biofloc farmers

Preparation of heterotrophic bacterial floc was the most vital in biofloc culture. Majority (71%) of the farmers were found preparing bacterial inoculums outside the rearing tank. They usually mixed the carbon source and probiotics in small bucket with continuous aeration to prepare slurry, which was called as FCO or “Fermented Carbon Organic”. While rest of others (29%) prepared it directly into the culture tank with constant aeration prior to the fish stocking and applied the required carbon amounts directly to the culture tank on regular basis.

Maintaining optimum floc levels was also considered as a critical issue because high fluctuation of pH and alkalinity, high concentrations of inorganic nitrogen might have chronic effects on fish health (Azim and Little, 2008). In biofloc system the microbial communities formed with phytoplankton, bacteria, and aggregates of living and dead particulate organic matter (Ahmad *et al.*, 2017). Most commonly maintained floc volume by farmers for culturing tilapia and koi was found 10-15 ml and, for singhi, magur and bata it was 5-10 ml and, for pangas it was highest 15-20 ml (Fig.4.6a). It was due to the feeding habits of the cultured fish species. Biofloc formation in the water column of tank was correlated to the dissolved ammonia nitrogen from feed as well as fish excretions by heterotrophic bacteria (Wang *et al.*, 2016), so the optimum range of TAN must be controlled within suggested ranges. Hence all the research papers suggested maintaining higher floc volume 20-30 ml or

more to immobilize the inorganic nitrogen. Most of the farmers believed that higher floc volume created much problem as it became beyond control over time. Limited farmers were found having clear idea about the estimation of carbon nitrogen ratio which they tried to maintain. In spite of lack of scientific knowledge, all the farmers were found familiar with the term ‘Carbon and Nitrogen (C:N) ratio’ and expressed their views on preferred C:N ratio (Fig.4.6b) and, majority of the farmers were found to have conception that higher C:N ratio was always directly proportional to the floc volume which was true as heterotrophic count might increase with higher C:N ratios (Kamilya *et al.*, 2017).

Fig.4.6a. Ideal Imhoff cone/ floc volume measurements

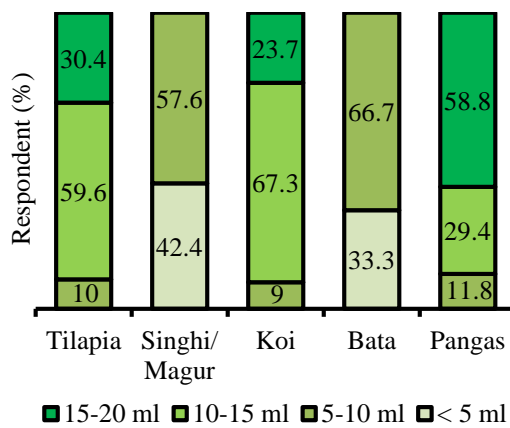
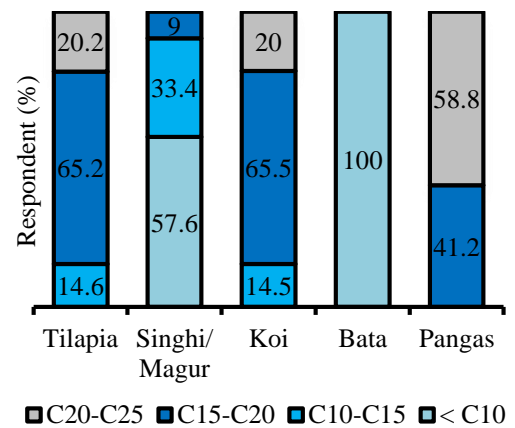
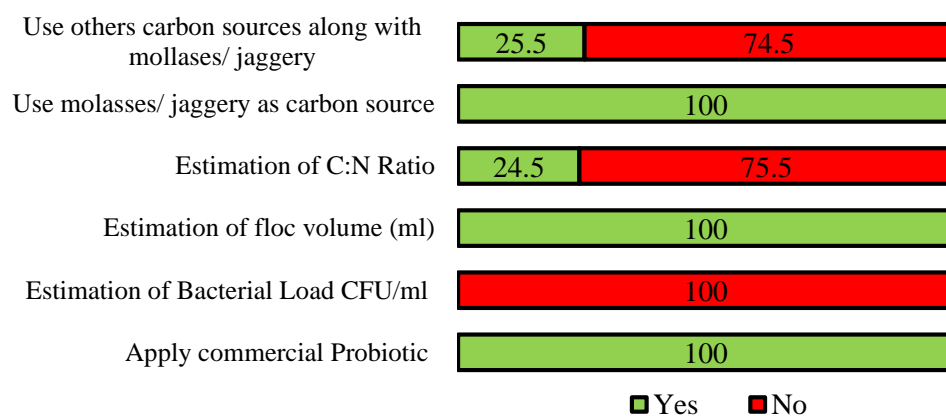


Fig.4.6b. Carbon to nitrogen ratios to be achieved for different fish



Controlled CN ratio by adding organic carbon was the prime management measures for biofloc tank, but availability of inexpensive and easily available carbon source was also important to run this system (Zaki *et al.*, 2020).

Fig. 4.7. Heterotrophic bacterial floc management practices by biofloc farmers (%)



All farmers were using mollasses and/or jaggery in case of non availability of mollasses as carbon sources, but their assumptions on organic carbon (%) in their given carbon sources were found different throughout the state (Fig.4.7). One fourth

of the respondents were found to be utilizing rice bran and corn starch along with molasses. Commercial probiotics were used by all of them for floc preparation but neither had they provision to estimate bacterial load (cfu/ml) in their tank, nor did they even try to estimate it through outsourcing (Fig.4.7). Though it was practically impossible for them, but unfortunately, most farmers blindly believed that observation of water colour and floc volume was enough to realize the existence of sufficient heterotrophic bacteria in tank water.

4.1.5. Feed management practices by biofloc farmers

All had used supplementary feed mainly the commercial floating feeds of different sizes according to the age groups of cultured fishes. Major lacuna in feeding practice was associated with limited practices of assessing left out feed, checking stored feed for possible rancidity as well as quality and, calculating feed performances like FCR during harvesting (Fig.4.8). Consideration of carbon content in uneaten supplementary feed was completely ignored by the majority.

Fig.4.8. Fish feed management practices by the biofloc farmers (%)

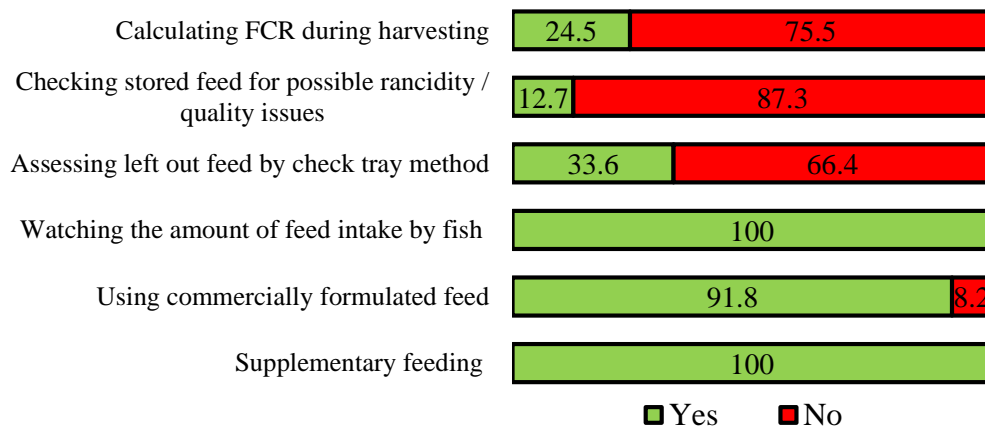
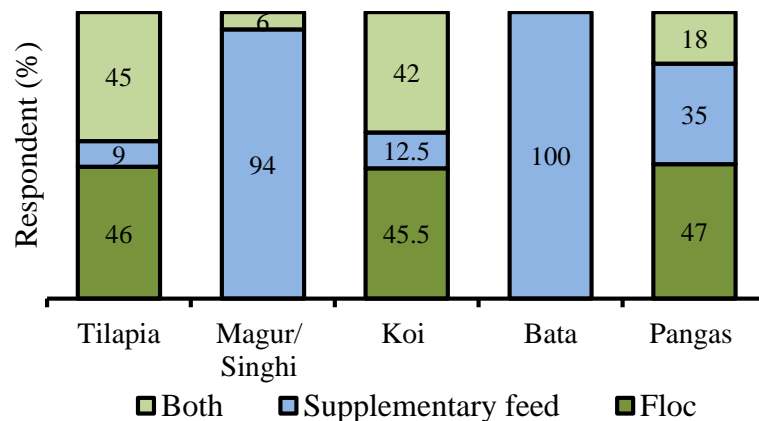


Fig.4.9. Feed preferences for different species in biofloc culture

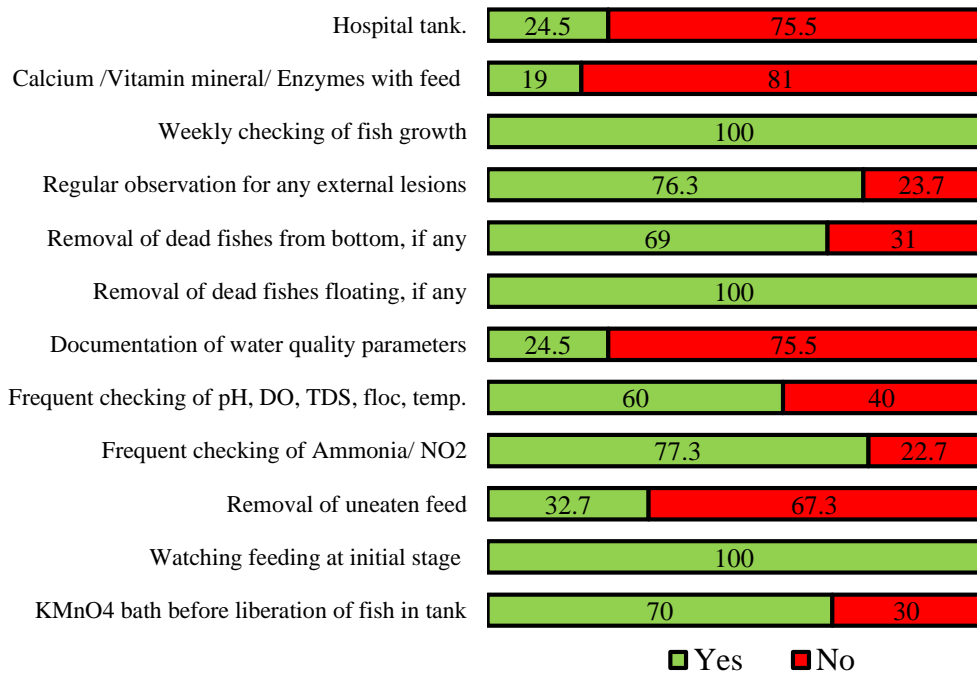


Regarding the farmers opinion about feed preferences between floc and supplementary feeds by their culturing fish species, merely all singhi, magur and bata growers selected supplementary feeds unanimously, whereas, tilapia, koi and pangas farmers answered floc as well as supplementary feeds both (Fig.4.9).

4.1.6. Preventive health management practices by the biofloc farmers

The health status of aquatic organisms largely depends on the farming system (M’balaka *et al.*, 2012). Lots of demerit was found in respect of taking preventive measures for disease concerned. Non-removal of uneaten feed led to subsequent water quality deterioration was found very common (Fig.4.10).

Fig.4.10. Preventive measures taken by the biofloc farmers (%) in connection with fish health management practices.



In spite of checking important water quality parameters frequently by majorities, few of them documented their findings or any observation in well manner which might be an important tool for taking preparatory measures to combat any diseases and for case studies by fishery experts. Farmers used to check fish growth rate weekly found very common. Incorporation of calcium, vitamin-mineral mixtures or any digestive enzyme along with feed to enhance the fish health status was practised rare by. Rehab tank for quarantine and observation of any suspected fishes was performed by only one fourth of respondents which should a compulsory approach (Fig.4.10).

4.1.7. Diseases and mortalities of fish in biofloc tank culture system

Occurrences of disease and fish mortality were the major problems in all type of culture practices including biofloc tank culture system. Only, 42.7% tilapia farmers never faced any disease, followed by 27.3% singhi/magur farmers (Fig.4.11). But, disease occurrences were very abundant in pangas and koi farming. Presence of ulcer and haemorrhages, fin rots and tail rot, might be of bacterial aetiology, was found very common. Occurrences of dropsy were confirmed by bata farmers only. Parasitic infections were negligible as clinical signs described by them. Scale loss, possibly due to poor water quality, and fin erosion either due to infection or might be the attack by co-species during shortage of feed, were reported as one of the vital problems for tilapia and koi. The major disease occurrence in pangas, koi, singhi, magur was appearance of fungal like mat. For koi, first a hazy film appeared on skin or fin which rose into white to pink bumps and was reported untreatable. This symptom was quite similar to *Lymphocystis* viral infection and needs to be investigated. Slow growth as well as non-uniform size was also reported for all cultured fishes (Fig.4.11).

Advanced fingerling stage was reported as the most disease affected group for tilapia, singhi, magur, koi and bata, whereas, fingerling was found the mostly affected group for pangas. Fry size was reported as the least disease affected group (Fig.4.12a). That clearly indicated that most of the time diseases occurred in biofloc system in later stages instead of initial stages might be due to appearance of unexpected issues in biofloc tank environment.

Fig.4.12a. Fish age group mostly affected by disease outbreaks in biofloc system as stated by respondent (%)

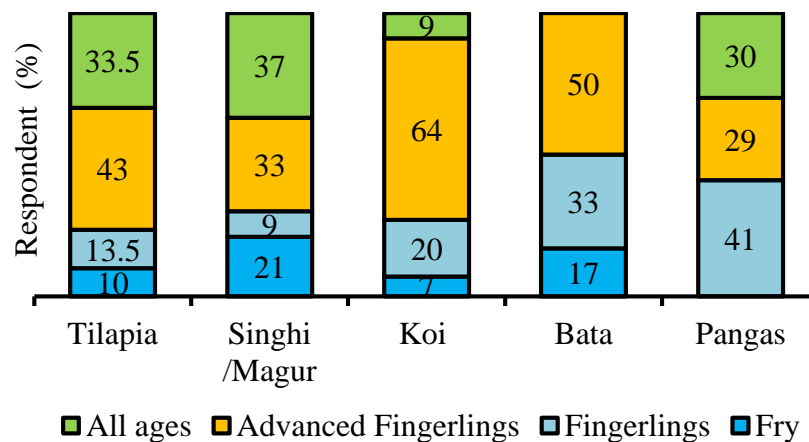
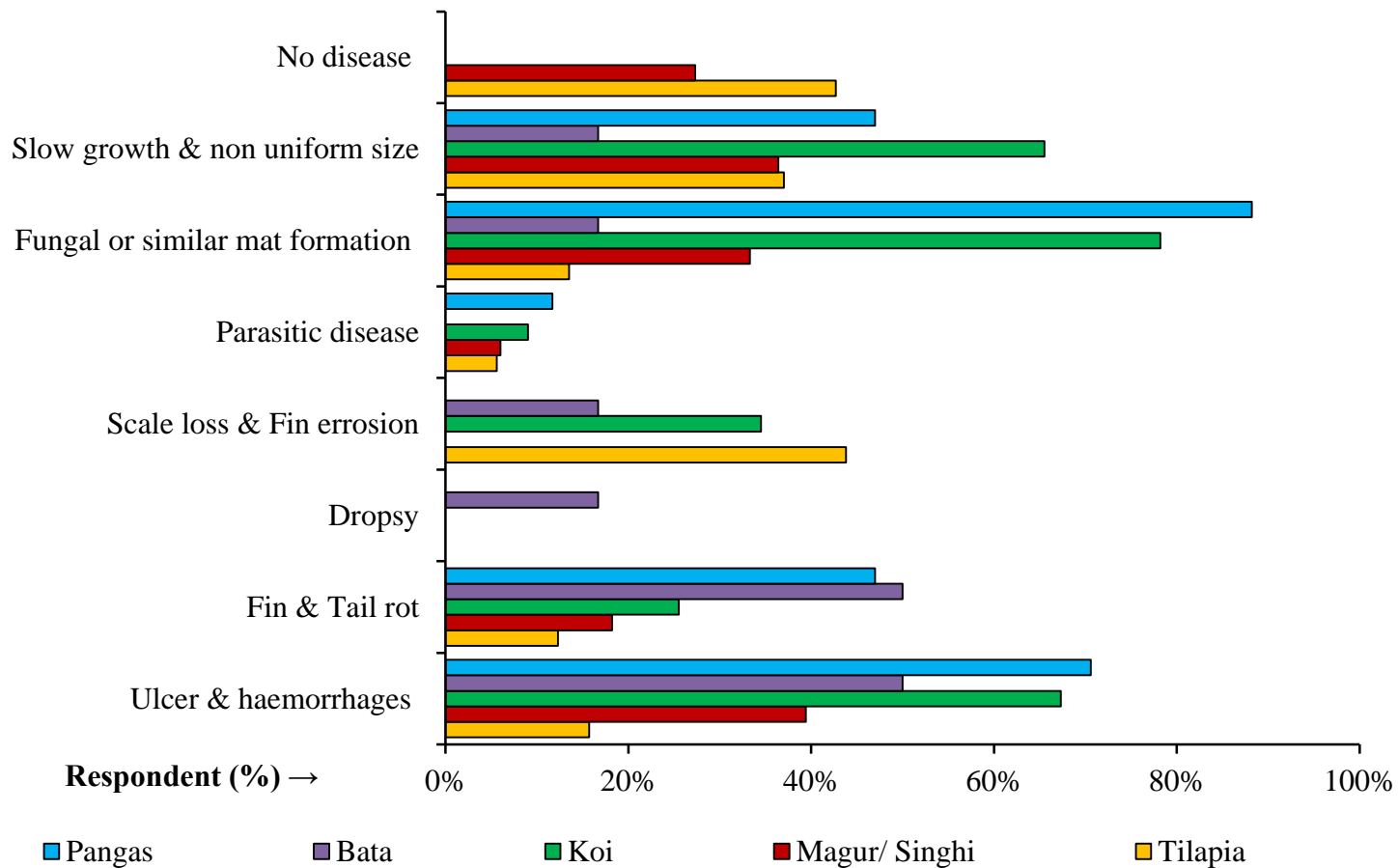
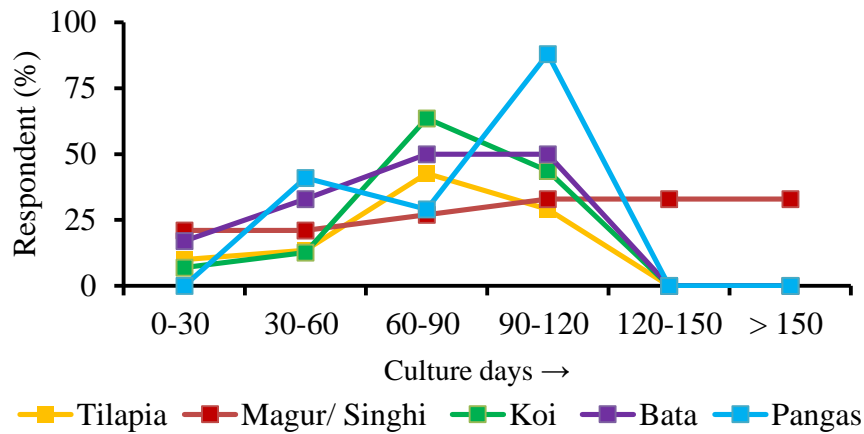


Fig.4.11. Assumption on occurrences of freshwater fish diseases in biofloc tank based on the observation by biofloc farmers (%)



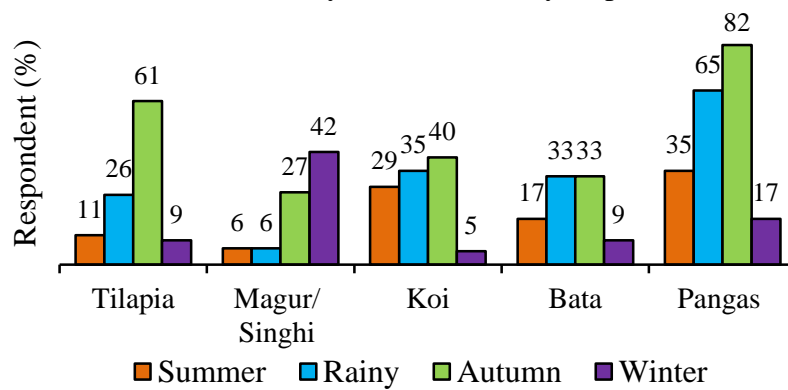
While concerning the disease occurrences from commencement of culture to the end of 120th d, it was found that maximum diseases occurred during 3rd and 4th month for tilapia, koi and bata whereas, for pangas it was 4th month. Disease outbreaks for singhi/ magur occurred in every month uniformly during 6 month culture (Fig.4.12b).

Fig.4.12b. Most disease occurrence period from commencement of culture of fish in biofloc system as stated by respondent (%)



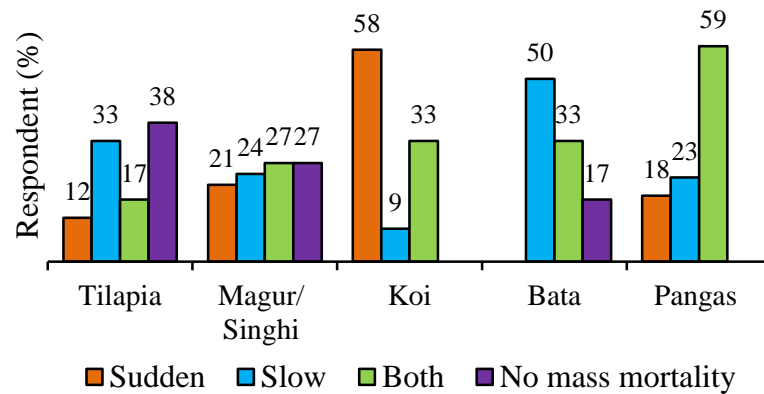
Most farmers skipped culture during the winter season except singhi/magur farmers. So, highest disease prevalence occurred during autumn, followed by rainy and summer season for all fishes, whereas, for singhi/magur it was found to be the winter (Fig.4.12c).

Fig.4.12c. Seasonal occurrence of diseases in different fishes in biofloc culture system as stated by respondent (%)



Fluctuation of temperature and stress were the major triggering factors. Mass mortalities of cultured fishes were also reported. Sudden mass mortalities were found most common in koi and slow mass mortalities in bata. Both sudden and slow mortalities were found highest in pangas. Farmers who never faced mass mortalities were mostly tilapia growers (Fig.4.12d). It was needless to say that mass mortality of cultured fish was found the major panic among farmers.

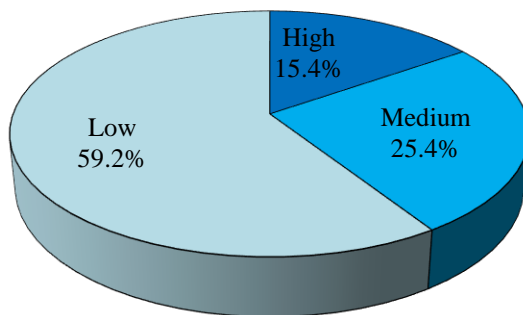
Fig.4.12d. Type of mass mortalities for different fish species in biofloc culture system as stated by respondent (%)



4.1.8. Therapeutic measures taken by the biofloc farmers

Tropical intensive aquaculture was highly affected by heavy mortalities as well as huge economic losses due to occurrence of diseases in West Bengal, India (Sen and Mondal, 2018). Disease management and therapeutic measures adopted by the respondents were found very poor as only few farmers were found confident to treat their fishes in case of emergency (Fig.4.13a).

Fig.4.13a. Self confidence of the biofloc farmers on disease therapy



High: Able to treat fish without taking help from others.

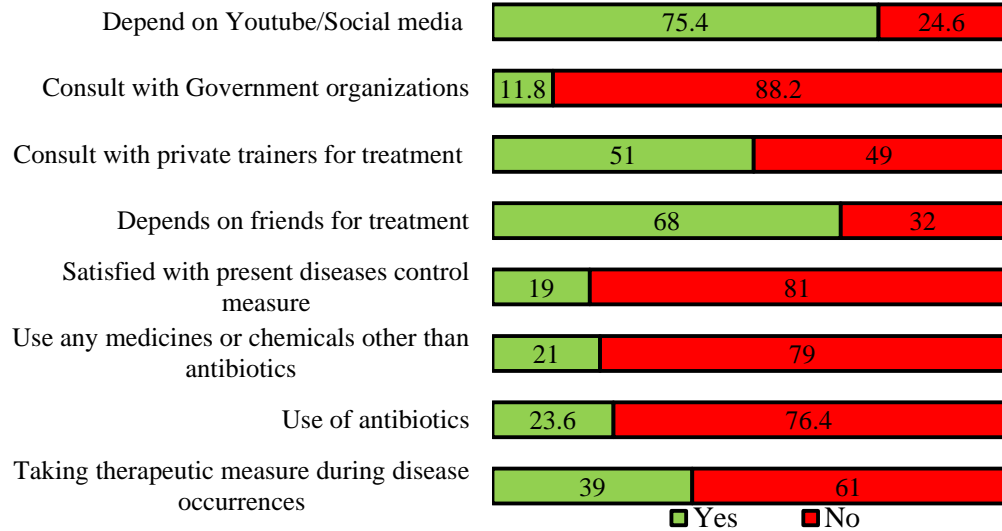
Medium: Can treat fish by taking advice from others.

Low: Unable to treat fish even after getting advice from others.

Therapeutic measures for disease treatments with antibiotics and medicines or chemicals were found limited, as most of the farmers failed earlier or had no idea (Fig.4.13b). Therapeutic treatment got effective results for catfishes only. Most of the farmers were found influenced by Youtube videos, social media groups in Facebook, Whatsapp and also depended on their friends, private trainers for disease control (Fig.4.13b). From practical point of view treatment of fish in biofloc system was merely possible because of incorporation of any medicine could directly hamper the heterotrophic bacterial population which turned the culture system failure to immobilize the toxic inorganic nitrogenous substances. Hence the only way to avoid fish disease in such intensive culture system was following good management

practices in every step like selection of healthy species, floc preparation and maintenance, maintaining ideal water quality parameters, correct feeding and frequent sampling for thorough observation throughout the rearing period.

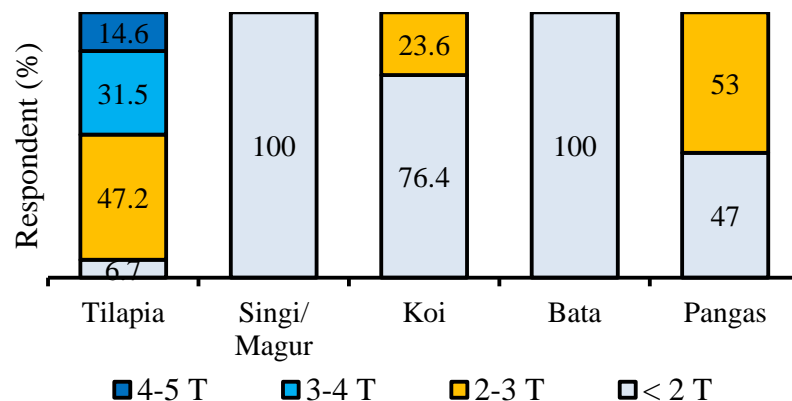
Fig.4.13b. Therapeutic measures practiced by biofloc farmers against fish diseases



4.1.9. Fish production in biofloc tank culture system

Biofloc technology had gained importance especially among unemployed youths because they believed that highly profitable intensive culture system could be manipulated easily, but in reality most of the fish seeds did not survive until the end of the culture, majorly due to adverse water quality, cannibalism and diseases. An overall scenario of fish production clearly indicated that harvesting 3-4 ton and/or above 4 ton per 10,000 l water volume was achieved by only 31.5% and 14.6% tilapia farmers, respectively (Fig.4.14). The third highest production rate of 2-3 ton/ 10000 l were also achieved by the 47.2% tilapia farmers followed by 53.6% pangas and 23.6% koi farmers. Low production rate were reported by the all singhi, magur, bata and 76.4% koi growers (Fig.4.14).

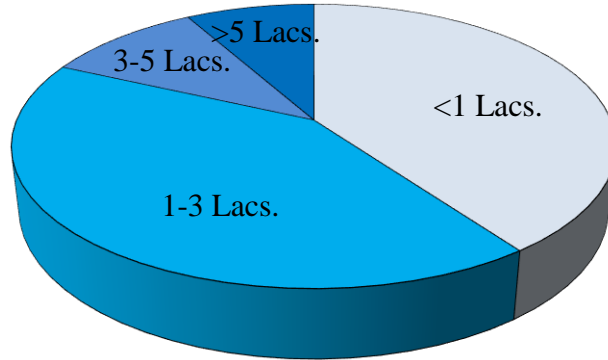
Fig.4.14. Production of different cultured species in biofloc tank (Ton/10000 l)



4.1.10. Profit and loss gained by the biofloc farmers

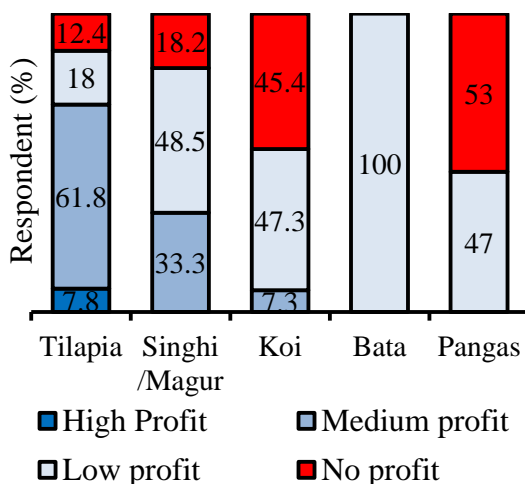
Majorities of respondent were found small scale investors which were accounted less than Rs. 1 lacs and Rs. 1-3 lacs by 40% and 41.8% of the respondents, respectively. Only 10% and 8.2% of the farmers were found who invested Rs. 3-5 lacs and more than 5 lacs, respectively (Fig.4.15a).

Fig.4.15a. Total investment in biofloc project by the farmers in Rs. (Lacs.)



The profit gain was categorised in four groups as presented graphically in Fig.4.15b. High profit was reported by 7.8% tilapia farmers only, while majority belonged to medium profit groups. In spite of high production, the profit from tilapia was not satisfactory because of low market price. Singhi/magur farmers were also unable to reach their target due to slow growth, non uniform size and disease occurrences. All the bata farmers belonged to the low profit group because of low production in contrast to investment cost. Profits earned by koi and pangas farmers in 4 months were found very unsatisfactory, as majority faced financial loss due to excessive mortalities (Fig.4.15b). Apart from disease and mortality, the problem of koi was basically associated with low acceptability by consumer for unpleasant colour, whereas, for pangas it was low market price.

Fig.4.15b. Profit gain by the biofloc farmers (%) for different cultured species



High Profit: (>10% of total project cost including capital cost in first crop and >50 % of culture cost in subsequent crop).

Medium Profit: (5-10% of total project cost including capital cost in first crop and 25-50% of culture cost in subsequent crop).

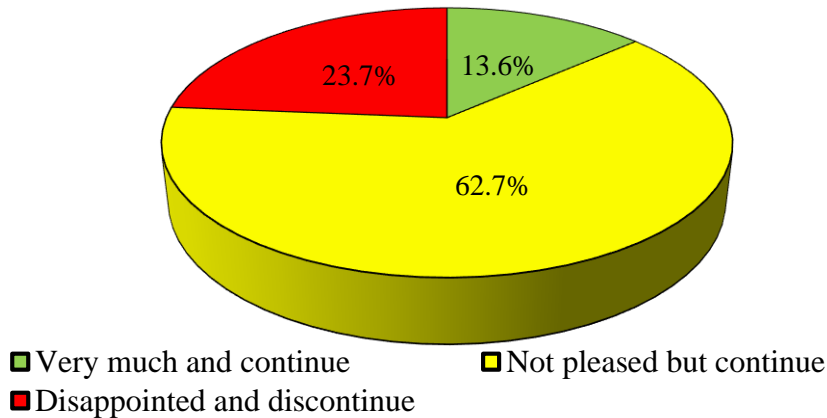
Low Profit: (<5% of total project cost including capital cost in first crop and <25% of culture cost in subsequent crop).

No Profit: (Less than project cost in first and subsequent crop).

4.1.11. Farmers satisfaction and prospect of biofloc culture

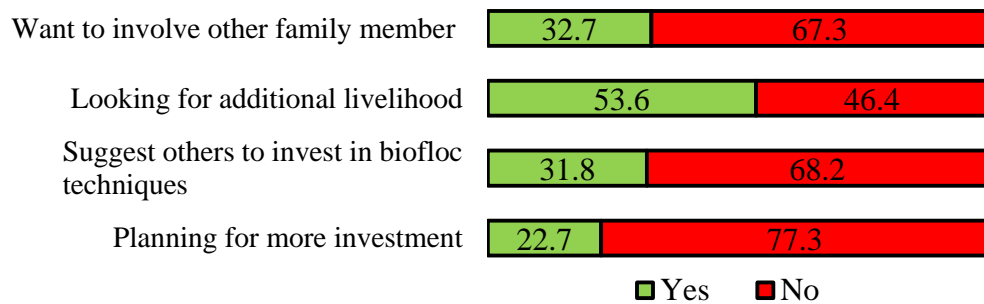
Only 13.6% respondents were found satisfied with the tank biofloc culture techniques and decided to go forward without vacillate. Majority of the respondent were not found happy with this technique yet they did not give up. Whereas, 23.7% was found disappointed and decided to stop the culture thenceforth (Fig.4.16a).

Fig.4.16a. Respondent satisfaction with biofloc technology



Possibility for more investment, suggesting others to adopt biofloc culture and involving family members were also found limited among the respondents as they apprehended the techniques might not be sustained in coming days (Fig.4.16b).

Fig.4.16b. Prospect of biofloc culture by the respondent (%)



4.2. Objective 2: To evaluate the effects of Biofloc Technology (BFT) with different C/N ratios on growth and feeding performances of *Oreochromis niloticus* and *Heteropneustes fossilis*.

4.2.1. Growth performances of Nile tilapia (*Oreochromis niloticus*)

Nile tilapia or *Oreochromis niloticus* were competent species for absorbing suspended biofloc and also could adapt high stocking densities (Avnimelech, 2007). Tilapias could efficiently utilize heterotrophic bacteria and were considered as suitable for cultivation in biofloc systems (Choo and Caipang, 2015). The findings of the present study clearly confirmed that the Nile Tilapia in all of the biofloc treatments with different carbon to nitrogen ratio of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) had superior growth performance than the control (Table 4.1). The initial average size of the tilapia juveniles were 0.53 ± 0.04 g. The initial biomasses of 2500 tilapia fry were 1.33 kg for each individual culture tank (4000 l) for control and biofloc treatment (BFT) with different carbon to nitrogen ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25). After 120 d of culture significant ($p < 0.05$) higher survival rate (%) were found among biofloc treatments groups with carbon to nitrogen ratio of 15:1 and 20:1 in compare to the CN25 and control Nile tilapia groups (Table 4.1). The survival rate of 91.39 ± 0.86 % and 90.43 ± 0.21 % for BFT treated CN15 and CN20 groups indicated that in spite of high stocking densities, biofloc culture system could provide excellent survivability to the Nile tilapia. Survivability (%) rate of CN25 Nile tilapia groups were 88.61 ± 1.49 % which was significantly higher ($p < 0.05$) to control groups (86.72 ± 0.69 %).

The maximum mortalities were observed upto the 7th weeks of rearing from commencement of culture for all biofloc treatment groups which were became almost nil during rest of the culture period (Fig.4.17). That was due to improvement of water quality parameters in terms of fewer amounts of total ammonia and nitrite content in biofloc system which we had discussed in section 4.6.5. Whereas, in control fish group the rate of mortality was found constantly downward throughout the 120 d culture period (Fig.4.17). Elayraja *et al.* (2020) also found considerable higher survivability among Nile tilapia reared in biofloc with C:N of 15:1 and 20:1 compare to control in their 75 d experiment. Our results were also agreed with previous studies where significant higher survival rate were noticed in tilapia reared in biofloc system compare to control (Khanjani *et al.*, 2021 and Menaga *et al.*, 2019). The growth

parameters and feed utilization were found enhanced in biofloc units than the control groups. Azim and Little (2008) reported that the biofloc system could augment the fish productivity by around 45% over the usual aquaculture productions. High percentage of survival rate with no significant difference ($p>0.05$) in the mortality rate between two different carbon to nitrogen ratio (15:1 and 20:1) biofloc treatment fish groups in this study was also supported by the previous studies of Ekasari *et al.* (2015), Wang *et al.* (2015) and Minabi *et al.* (2020).

Table 4.1. Growth performances of Nile Tilapia (*Oreochromis niloticus*) reared in control and biofloc system with different carbon to nitrogen (C:N) ratios of 15:1, 20:1, 25:1 for 120 d in circular tarpaulin tanks with stocking densities of 2500 nos./ 4000 l.

	CN15	CN20	CN25	Control
IBW (g)	0.53±0.04	0.53±0.04	0.53±0.04	0.53±0.04
FBW (g)	160.35 ^b ±2.16	158.68 ^b ±2.38	155.51 ^b ±3.73	122.17 ^a ±2.65
IBL (cm)	2.1±0.2	2.1±0.2	2.1±0.2	2.1±0.2
FBL (cm)	20.7 ^b ±0.1	20.4 ^b ±0.1	20.6 ^b ±0.4	19.0 ^a ±0.2
IBM (kg)	1.33	1.33	1.33	1.33
FBM (kg)	366.31 ^c ±3.54	358.73 ^{bc} ±5.48	344.58 ^b ±13.16	264.87 ^a ±6.42
SR (%)	91.39 ^c ±0.86	90.43 ^c ±0.21	88.61 ^b ±1.49	86.72 ^a ±0.69
WG (g)	159.82 ^b ±2.16	158.15 ^b ±2.39	154.98 ^b ±3.74	121.64 ^a ±2.66
SGR (% d)	4.76 ^b ±0.01	4.75 ^b ±0.02	4.74 ^b ±0.02	4.54 ^a ±0.02
DWG (g)	1.33 ^b ±0.02	1.32 ^b ±0.02	1.29 ^b ±0.03	1.01 ^a ±0.02

Values presented as Mean (\pm SD, n=3) in the same row with different superscripts differ significantly for each parameter between experimental group (ANOVA, $p<0.05$). [CN15, CN20 and CN25: Biofloc groups with different carbon to nitrogen ratios of 15:1, 20:1 and 25:1; IBW: Initial average body weight; FBW: Final body weight; IBL: Initial body length; FBL: Final body length; IBM: Initial biomass; FBM: Final biomass; SR: Survival rate; FWG: Final weight gain; DWG: Daily weight gain; SGR: Specific growth rate]

The final body weight (FBW), weight gain (WG), daily weight gain (DWG) and specific growth rate (SGR) (% d) of all biofloc treatment groups were found higher ($p<0.05$) than the control but no significant differences were found among the different biofloc treatment groups with different C:N ratios (Table 4.1). Though the non significant ($p>0.05$) highest value of all those said parameters were recorded for the BFT system with C:N of 15:1 (CN15) followed by 20:1 (CN20) and 25:1 (CN25)

(Table 4.1). The highest FBW, WG, SGR and DWG were recorded in CN15 groups i.e., 160.35 ± 2.16 g, 159.82 ± 2.16 g, 4.76 ± 0.01 % d and 1.33 ± 0.02 g, respectively which was followed by CN20 and CN25 Nile tilapia groups (Table 4.1).

In a semi large scale project those minute differences among growth performances might effects the final fish production which was observed in our studies. The final biomasses (FBM) of the all biofloc treatment groups were found significantly higher than the FBM of control Nile tilapia groups i.e., 264.87 ± 6.42 kg (Table 4.1). In this study the highest FBM were obtained 366.31 ± 3.54 kg from the CN15 biofloc groups which was found significantly ($p < 0.05$) higher than the FBM obtained from CN25 groups i.e., 344.58 ± 13.16 kg but not significantly higher ($p > 0.05$) than the production of CN20 groups i.e., 358.73 ± 5.48 kg (Table 4.1). The higher FBW obtained in all biofloc based treatment groups with different carbon to nitrogen ratios compare to control in the present study indicated the potentiality of Nile tilapia fingerlings to utilize the microbial protein efficiently (Khanjani *et al.*, 2021, Menaga *et al.*, 2019 and Elayraja *et al.*, 2020).

The average growth curve of triplicate tanks of each BFT treatment and control Nile tilapia also found best fitted in polynomial curve with order three by showing highest possible regression coefficient or R^2 values of 0.999 (Fig.4.18). The highest final average body weight (FBW) was recorded in biofloc system of CN15 (160.35 ± 2.16 g) followed by CN20 (158.68 ± 2.38 g), CN25 (155.51 ± 3.73 g) and control (122.17 ± 2.65 g) Nile tilapia group (Fig.4.18). Overall uniform growths of Nile tilapia were achieved on the 120th d with moderate size variation (Fig.4.19).

The experimental setup for rearing Nile Tilapia in devoid of biofloc as control and with biofloc by maintaining three different C:N ratios of 15:1, 20:1 and 25:1 for 120 d were carried out in triplicate. The weekly growth rate, final growth and feeding performances in each triplicate culture tank including biofloc and control Nile tilapia groups had been illustrated in the Fig. 4.20a for CN15, Fig. 4.20b for CN20, Fig.4.20c for CN25 and Fig.4.20d for control. Growth curves showed a typical sigmoid pattern with highest possible regression coefficient or R^2 values of 0.999 or 0.998 for all BFT treated and control Nile tilapia groups, and were found best fitted in polynomial curve with order three in Excel 2007 (Fig.4.20a to Fig.4.20b).

Fig.4.17. Cumulative survival (numbers) in every week during 120 d culture of Nile Tilapia (*Oreochromis niloticus*) in biofloc system with different carbon to nitrogen (C:N) ratios of 15:1 (CN15), 20:1 (CN20), 25:1 (CN25) and control tanks.

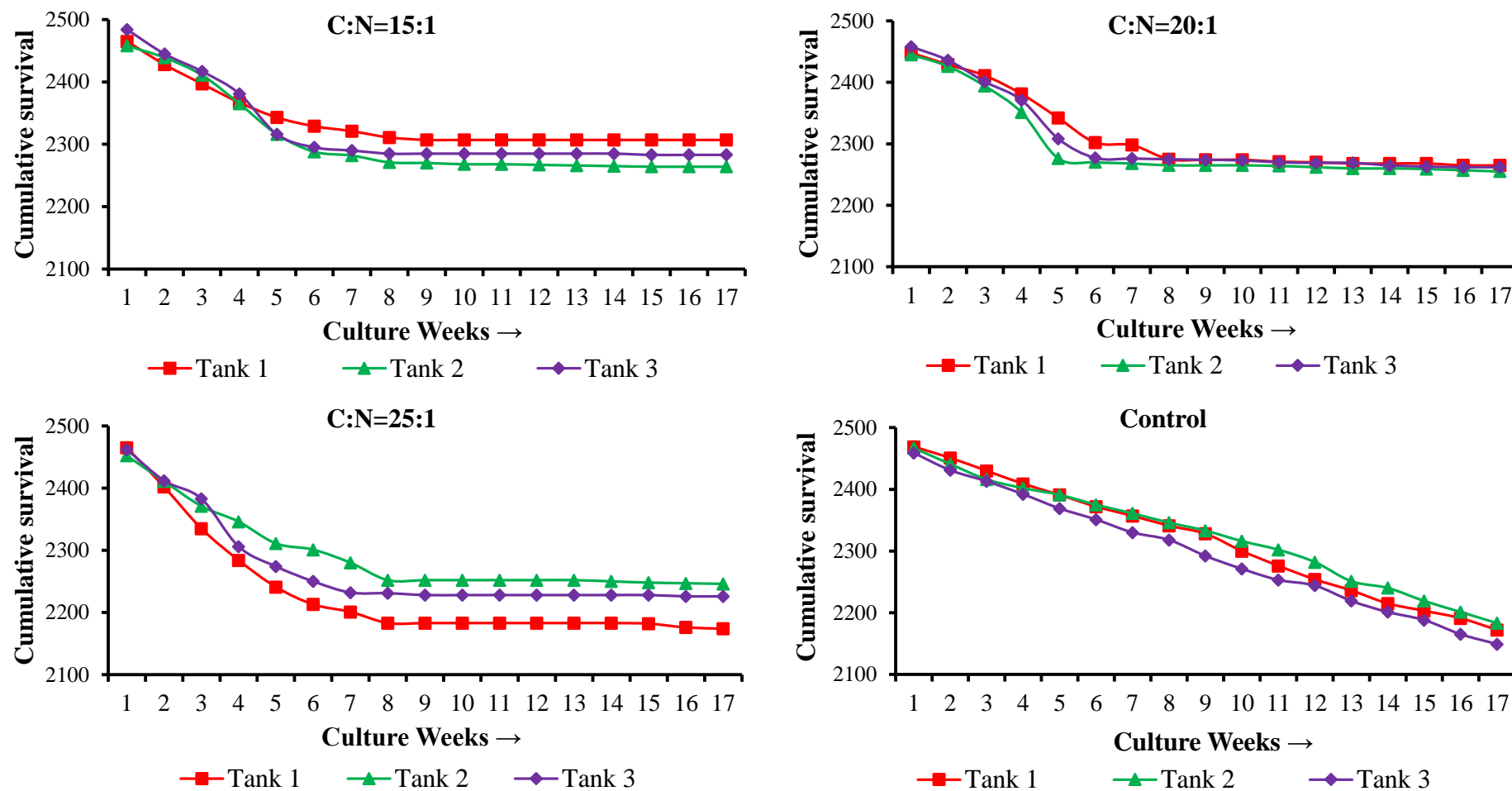


Fig.4.18. Average growth rate of Nile Tilapia (*O. niloticus*) reared in control and biofloc with C:N of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) for 120 d

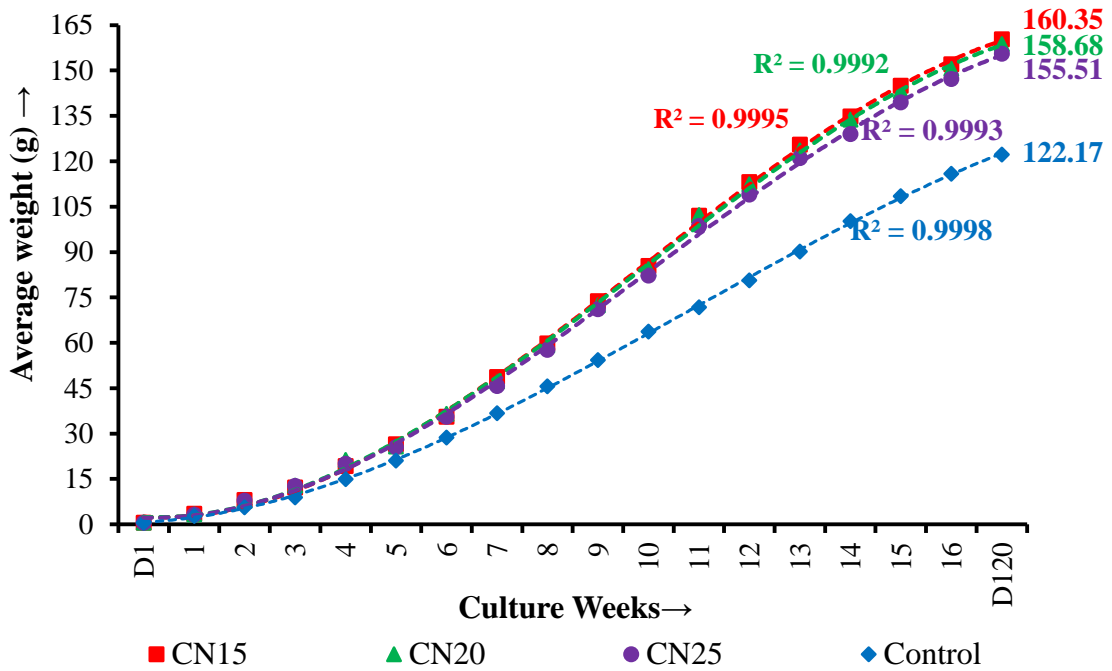


Fig.4.19. Final weight (n=150) of Nile Tilapia (*O. niloticus*) reared in control and BFT system with different C:N of 15:1, 20:1 and 25:1 on 120th d

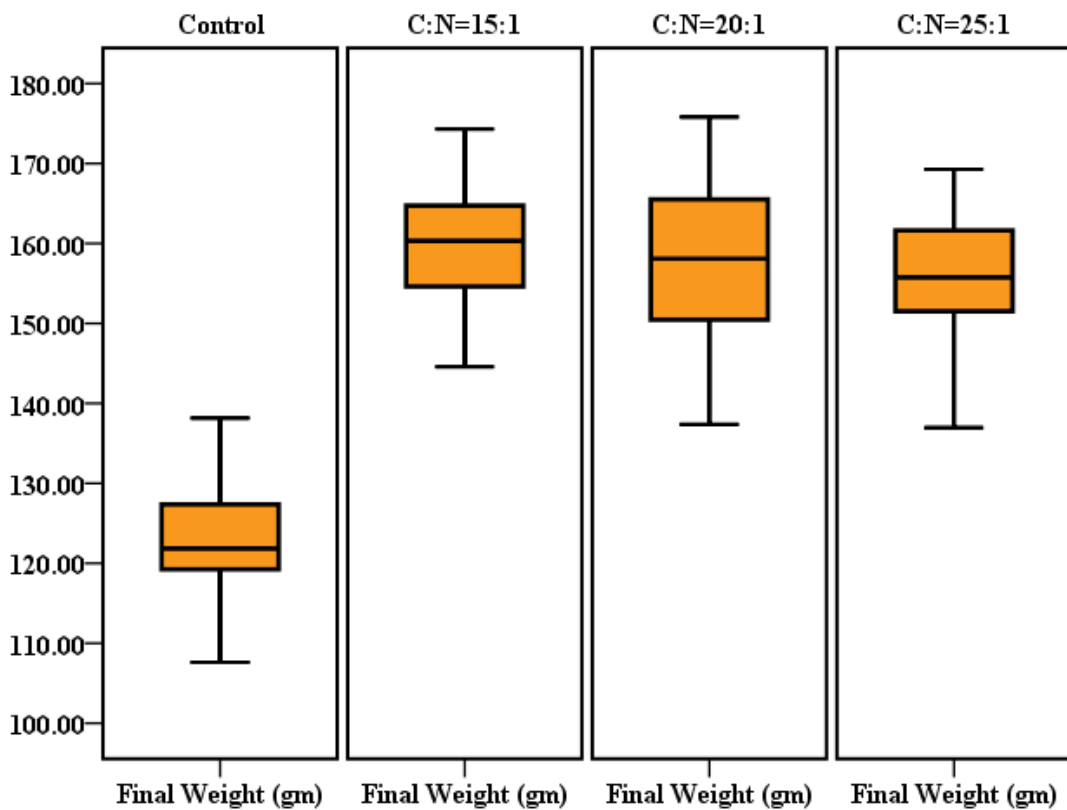


Fig.4.20a. Growth and feed performances of Nile Tilapia (*O. niloticus*) cultured in biofloc system with carbon to nitrogen ratio of 15:1 (CN15) for 120 d.

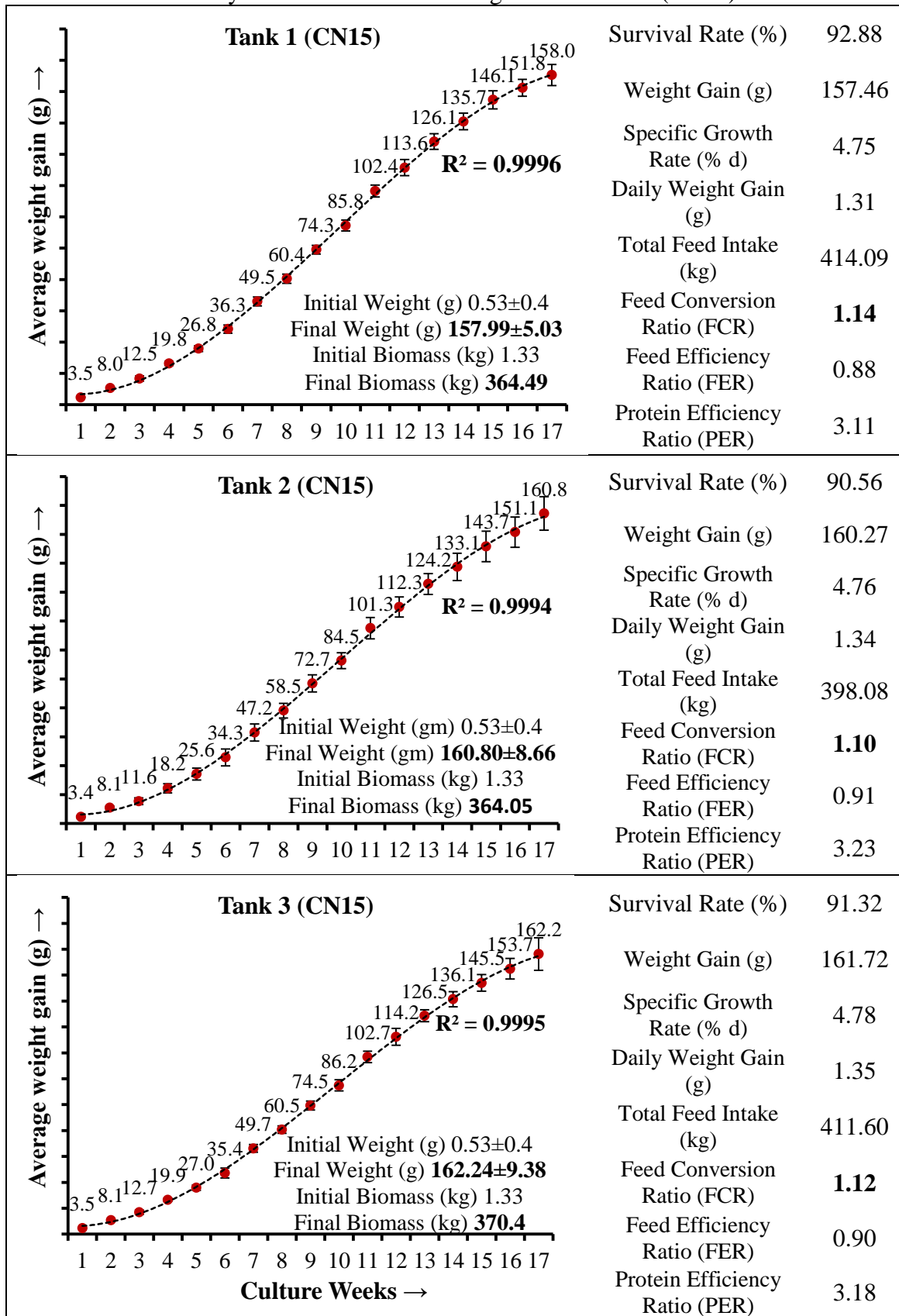


Fig.4.20b. Growth and feed performances of Nile Tilapia (*O. niloticus*) cultured in biofloc system with carbon to nitrogen ratio of 20:1 (CN20) for 120 d

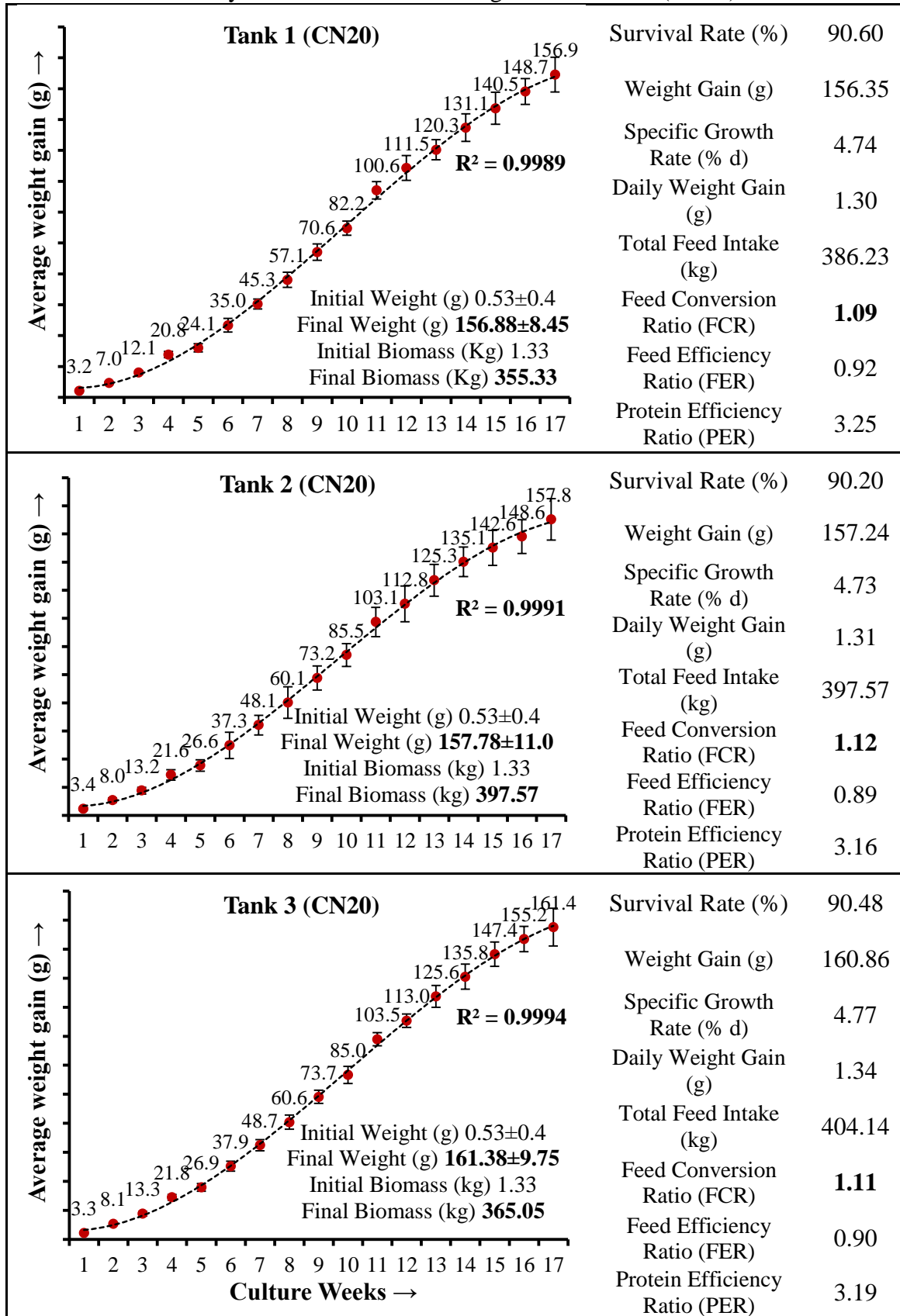


Fig.4.20c. Growth and feed performances of Nile Tilapia (*O. niloticus*) cultured in biofloc system with carbon to nitrogen ratio of 25:1 (CN25) for 120 d

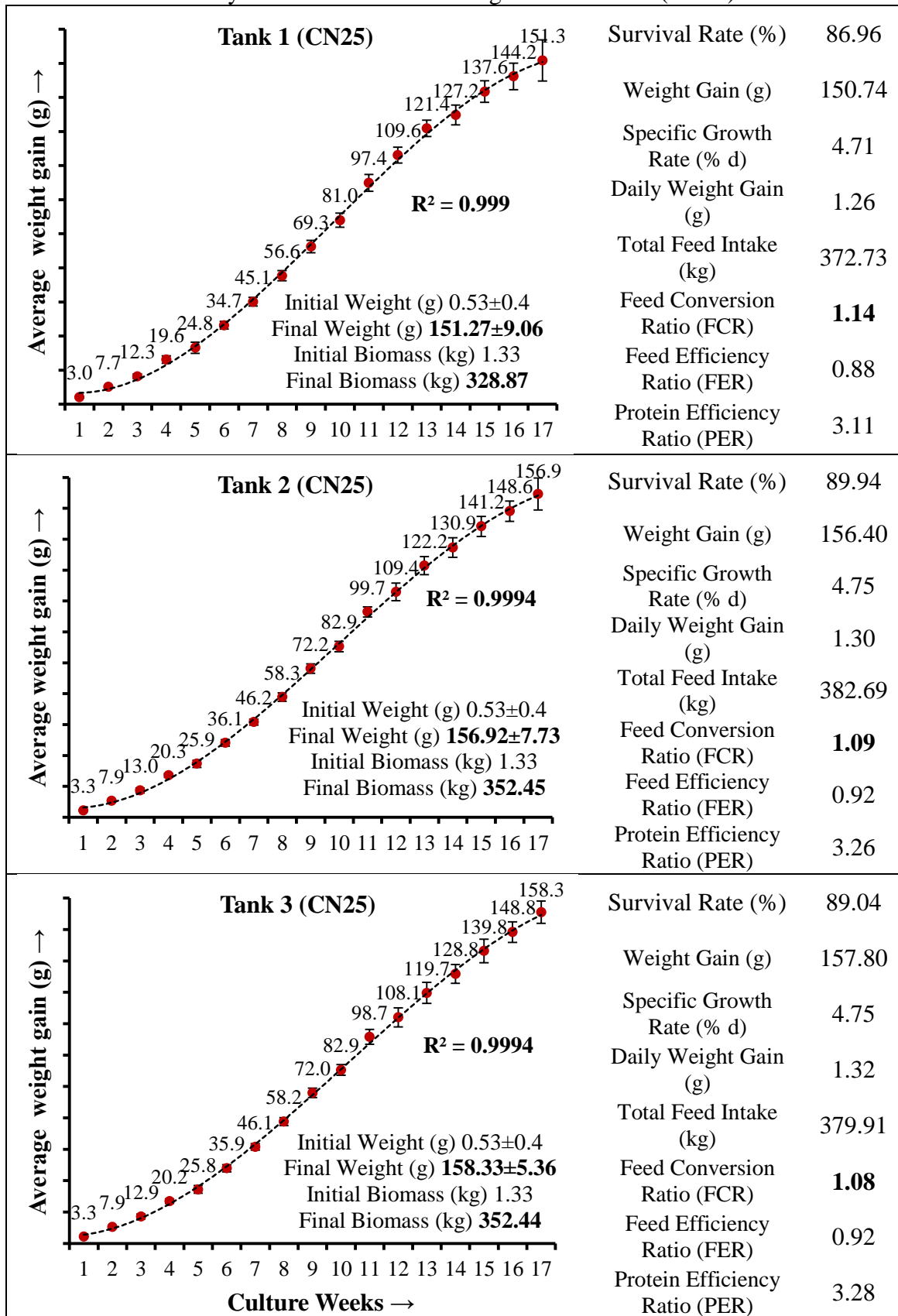


Fig.4.20d. Growth and feed performances of Nile Tilapia (*O. niloticus*) reared in control tanks without addition of jaggery as carbon source for 120 d

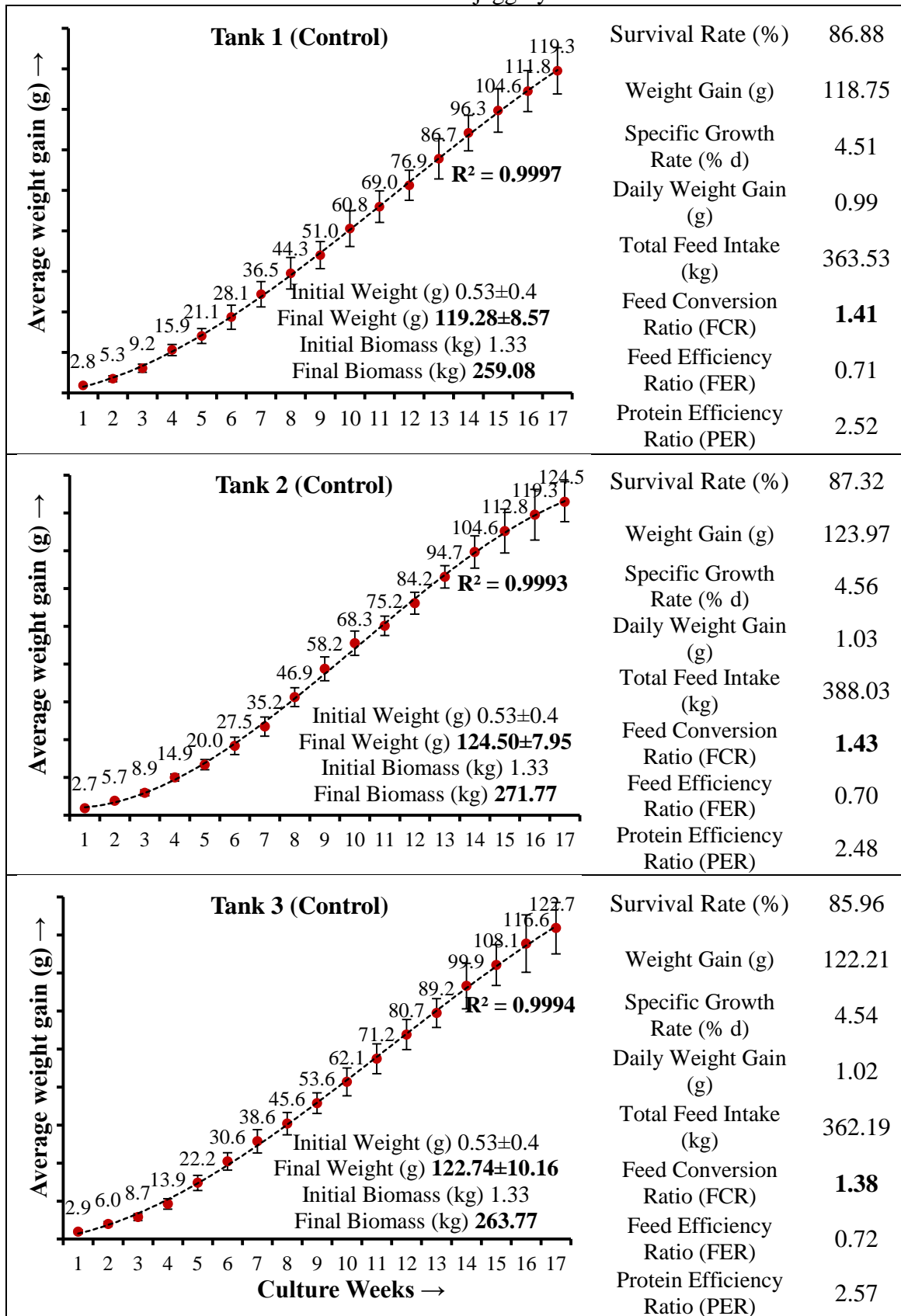


Plate 4.1. Length and weight measurements of Nile Tilapia (*O. niloticus*) and final harvesting after 120 d culture in biofloc system.

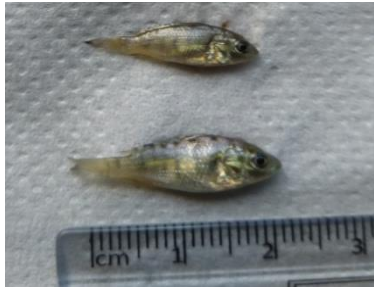


Plate 4.1A



Plate 4.1B



Plate 4.1C



Plate 4.1D



Plate 4.1E



Plate 4.1F



Plate 4.1G

4.2.2. Feeding performances of Nile tilapia (*Oreochromis niloticus*)

The average total feed intake (kg) of triplicate tanks in all biofloc treatments with different carbon to nitrogen ratios and control Nile tilapia groups were found significantly indifferent ($p>0.05$) during the 120 d culture period, except the CN15 groups where significantly higher ($p<0.05$) feed intake (407.93 ± 8.62 kg) were noticed than control (371.25 ± 14.55 kg) but not from other two BFT treated Nile tilapia groups i.e., CN20 (395.98 ± 9.06 kg) and CN25 (378.44 ± 5.14 kg) (Table 4.2). That might be because of highest survival rate of 91.39% in CN15 group out of all other BFT treated and control Nile tilapia groups. That definitely proved that compare to the other conventional aquaculture techniques, biofloc system could reduce the utilization of supplementary feed requirement and could be considered as an economically benefitted low-cost sustainable techniques in aquaculture as suggested by De Schryver *et al.* (2008). Particulate organic substances, colloidal particles and various suspended matters form biological flocculation feed could be eaten by omnivorous fish like Nile tilapia which led promotion of the growth of fish (Ahmad *et al.*, 2017).

The feed conversion ratio (FCR), feed efficiency ratio (FER) and protein efficiency ratio (PER) for all the biofloc treatment groups with different C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) were found superior than the FCR, FER and PER of control Nile tilapia group i.e., 1.41 ± 0.03 , 0.71 ± 0.01 and 2.52 ± 0.05 , respectively. Among the all biofloc treatment groups the CN25 group showed lowest value of FCR (1.10 ± 0.03) and highest values of FER and PER i.e., 0.91 ± 0.02 and 3.22 ± 0.08 , respectively. But those were not significantly superior ($p>0.05$) than the other two biofloc treatment groups CN15 and CN20 (Table 4.2). So in this study the lowest FCR and highest FER and PER were recorded for the CN25 groups followed by the CN20 and CN15 groups. No significant differences ($p>0.05$) of feeding performances were observed among the three different biofloc treatment groups CN15, CN20 and CN25 (Table 4.2). So, our studies confirmed that both C:N ratios of 15:1 and 20:1 could be considered as an optimal range for tilapia culture in BFT system for superior feed performances along with good survival rates.

So the survival rate, final body weight, weight gain, daily weight gain, SGR (% of d) and final biomass were found at the uppermost levels in Nile tilapia among the biofloc treatment groups with C:N ratios of 15:1 (CN15) followed by 20:1 (CN20) and 25:1 (CN25). Whereas, the FER and PER were at the top levels and FCR at the least level in Nile tilapia were found in the fish reared in BFT system with C:N ratios

of 25:1 (CN25) and 20:1 (CN20) followed by 15:1 (CN15) Nile tilapia groups. The positive effects of the application of biofloc system on growth and feeding performance had already been reported in previous studies for different species, e.g., in *O. niloticus* (Azim and Little 2008 and Mirzakhani *et al.*, 2019), *L. vannamei* (Khanjani *et al.*, 2017 and Panigrahi *et al.*, 2019), *L. rohita* (Mahanand *et al.*, 2013b; Ahmad *et al.*, 2016 and Kamilya *et al.*, 2017), *C. auratus* (Wang *et al.*, 2015 and Yu *et al.*, 2020a), Dybowski, *Opsariichthys kaopingensis* (Yu *et al.*, 2020b) and *C. carpio* (Najdegerami *et al.*, 2016 and Minabi *et al.*, 2020).

Table 4.2. Feeding performances and somatic indices of Nile Tilapia (*Oreochromis niloticus*) reared in control and biofloc system with different carbon to nitrogen (C:N) ratios of 15:1, 20:1, 25:1 for 120 d in circular tarpaulin tanks with stocking densities of 2500 nos./ 4000 l.

	CN15	CN20	CN25	Control
<i>Feed Performances</i>				
TFI (kg)	407.93 ^b ±8.62	395.98 ^{ab} ±9.06	378.44 ^{ab} ±5.14	371.25 ^a ±14.55
FCR	1.12 ^b ±0.02	1.11 ^b ±0.02	1.10 ^b ±0.03	1.41 ^a ±0.03
FER	0.89 ^b ±0.02	0.90 ^b ±0.01	0.91 ^b ±0.02	0.71 ^a ±0.01
PER	3.17 ^b ±0.06	3.20 ^b ±0.05	3.22 ^b ±0.08	2.52 ^a ±0.05
<i>Somatic Indices</i>				
GSI (%)	4.12 ^a ±0.05	4.11 ^a ±0.04	4.27 ^b ±0.07	4.34 ^b ±0.06
HSI (%)	1.70 ^a ±0.04	1.70 ^a ±0.07	1.78 ^b ±0.01	1.81 ^b ±0.01
CF	1.83±0.04	1.88±0.03	1.81±0.13	1.80±0.02

Values presented as Mean (± SD, n=3) in the same row with different superscripts differ significantly for each parameter between experimental group (ANOVA, p<0.05). [CN15, CN20 and CN25: Biofloc groups with different carbon to nitrogen ratios of 15:1, 20:1 and 25:1; TFI: Total feed intake; FCR: Feed conversion ratio; FER: Feed efficiency ratio; PER: Protein efficiency ratio; GSI: Gastrosomatic index; HSI: Hepatosomatic index; CF: Condition factor]

On the other hand, Bakhshi *et al.* (2018) observed a small increase of final weight, final productivity, weight gain, and SGR (% day) of common carp fingerlings, experimentally cultured in BFT treatments with different carbon sources but did not noticed any significant difference between the control and BFT treatments. Angeles-Escobar *et al.* (2021) showed Red pacu, *Piaractus brachypomus* gained highest biomass, better growth and FCR in control than biofloc treatments. During the culture of *C. gariepinus* in biofloc system Dauda *et al.* (2018) did not found any significant

different for biomass gain, SGR, and FCR among the control and BFT treatments with different C:N ratios perhaps that species was an incompetent filter feeder of bioflocs (Ekasari *et al.*, 2016). In our semi large scale studies the better growth and feeding performances of Nile tilapia in biofloc systems were strongly supported by the previous experiment done on *O. niloticus* by Menaga *et al.* (2019), Elayaraja *et al.* (2020) and Khanjini *et al.* (2021).

Our results were found contradictory with Dilmi *et al.* (2022) who did not observed any significant different growth and FCR among control and biofloc system with C:N of 14:1, 17:1 and 20:1 groups, and lower performances in all the said aspect was noticed in C:N of 20:1. Parallel results were noticed by Hernández-Vergara *et al.* (2018) and Widanarni *et al.* (2012) while reared tilapia in BFT system. Our finding suggested that too high carbon to nitrogen ratio in biofloc system could weaken the growth of Nile tilapia in BFT system. In those cases the microorganisms might not be benefited for growth of cultured fishes because of the negative effect of the microorganisms on controlling water quality. The heterotrophic bacterial community might increase the biological oxygen demand in a short time led declining the growth of fish in biofloc system. Avnimelech *et al.* (1994) estimated that the requirement of feed ratio was 20% lower than conventional culture whereas feed utilization was higher for tilapia in biofloc ponds. Similar results were obtained in our studies where, the FCR in the biofloc treatment groups were significantly lower ($p < 0.05$) than in the control group, whereas the FER, PER, FBW, DWG, and SGR were significantly higher in the CN15, CN20 and CN25 groups compare to the control. Our findings were also matched with Liu *et al.* (2018a) who observed better growth of *O. niloticus* in the lower C:N of 10:1 and 15:1 compared to the higher C:N of 20:1 and control.

However, the highest feeding performances with increasing carbon to nitrogen ratio in our studies indicated that biofloc system could reduce supplementary fish utilization due to the development of microbial flocs, recycling of the remaining feeds and wastes and the feed reutilization of the nutrients by fish which resulted in the enhancement of growth and feeding performance (Avnimelech, 2006). According to Avnimelech (1999) heterotrophic bacteria needed about 20 units of carbon per unit of nitrogen assimilated in the culture water whereas majorities of artificial feeds used in semi-intensive aquaculture had C:N ratio of 10:1. The majority of the previous studies considered the microbial flocs were available for all time might be considered as a supplementary source of food. Those flocs contained additional protein (essential

amino acids), polyunsaturated fatty acids and different vitamins and minerals which were the key factors for the beneficial effects of biofloc system on growth and feeding performance (Azim and Little, 2008, De Schryver *et al.*, 2008, Luo *et al.*, 2014 and Wang *et al.*, 2015). De Schryver *et al.* (2010) also reported that the bioflocs contained a biodegradable polymer poly- β -hydroxybutyrate (PHB) created by bacteria. The advantages of those PHB included improve intestine digestibility, raised the amount of unsaturated fatty acid and improve the growth of cultured species (Crab *et al.*, 2007 and Emerenciano *et al.*, 2013). Another possible explanation for the growth promotion and better nutrient digestibility performances in BFT was the microbial flocs could be helpful prospective exogenous and endogenous enzymes sources that stimulated the cultured species (Najdegerami *et al.*, 2016, Zhang *et al.*, 2016 and Bakhshi *et al.*, 2018).

4.2.3. Somatic Indices of Nile tilapia (*Oreochromis niloticus*)

Conditioning factor (CF) was a somatic index to provide vital information regarding the physiological parameters of fish (Lima-junior *et al.*, 2002). In this study the conditioning factor (CF) of Nile tilapia at the end of 120 d of culture was not significantly differs between the control and biofloc treatments with different C:N ratio of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25), whereas the hepatosomatic index (HSI) and gastro-somatic index (GSI) of control and CN25 Nile tilapia were found significantly higher ($p < 0.05$) than CN15 and CN20 groups (Table 4.2). The higher values of GSI in control and CN25 Nile tilapia might be due to the adverse health status compare to the CN15 and CN20 Nile tilapia groups which was supported by Khanjani *et al.* (2021) where significantly higher GSI was noticed in control Nile tilapia compare to Nile tilapia reared in BFT system with different carbon sources. Overall better CF, HSI and GSI values in BFT treated CN15 and CN20 groups indicated the elevated carbon to nitrogen ratio in the biofloc farming provoked no negative effects on the Nile tilapia somatic indices up to carbon to nitrogen ratio of 20:1. In this study the CF and HSI values of the experimental Nile tilapia in both control and BFT treated groups were recorded in the range of 1.80 to 1.88 and 1.70 to 1.81, respectively. Different values of CF and HSI had been reported by others, viz., CF 1.6-1.8 and HSI 3.03-3.5 for *C. carpio* (Bakhshi *et al.*, 2018); CF 1.56–1.92 and HSI 1.71–2.51 for *C. carpio* (Adineh *et al.*, 2019); CF 1.21-1.28 and HSI 1.54-1.59 for *C. carpio* (Minabi *et al.*, 2020); CF 1.71–1.80 and HSI 1.15-2.51 for *O. niloticus*

(Durigon *et al.*, 2020) and CF 1.89–2.07 for *O. niloticus* (Khanjani *et al.*, 2021) which might be associated to the specific experimental conditions. Bakhshi *et al.* (2018) found no significant alterations in HSI and CF of *C. carpio* fingerlings between the control and biofloc treatments with different carbon sources. An increasing trend but not significant of condition factor in the biofloc system was recorded from C:N ratio of 15:1 to 20:1 but dropped in the higher C:N ratio of 25:1 (Table 4.2). Declining CF in the C:N ratio of 25:1 from C:N ratio of 20:1 and 15:1 was probably due to the increases in the TSS level and deteriorating water quality (Minabi *et al.*, 2020). Any adverse aquatic environmental conditions might affect the physiological status of the fish and increase stress which eventually led to reduce in body weight gain (Khanjani *et al.*, 2021). HSI of cultured Nile tilapia could be influenced by the nutritional factors (Durigon *et al.*, 2020). In this study the HSI was influenced by the jaggery as carbon source added to the biofloc system which promoted the growth of biofloc for the cultured Nile tilapia. Another possible reason for elevated HSI of Nile tilapia of CN25 and control Nile tilapia in this study might be associated with hypertrophy of hepatocytes and cytoplasmic vacuolation, whereas for higher GSI it could have been due to swollen lamina propria and epithelial cells due to poor nutrient absorption in those particular environment for a prolonged period over 120 d. All those had been discussed in section 4.5.1 and 4.5.2.

4.2.4. Growth performances of Singhi (*Heteropneustes fossilis*)

Singhi or *Heteropneustes fossilis* reared in the biofloc treatments with different carbon to nitrogen ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16), showed better growth than control after 180 d culture. The initial average size of the *H. fossilis* juveniles were 5.53 ± 0.47 g and the initial biomass of those 700 numbers of *H. fossilis* juveniles for each rearing tank were 3.87 kg which were randomly distributed in each individual triplicate tank (1500 l) including BFT with different C:N of 8:1 (CN8), 12:1 (CN12), 16:1 (CN16) and control. At the end of 180 d of culture the survival rate of BFT systems of CN8 and CN12 were found significantly higher ($p < 0.05$) than CN16 and control. Though the survival rates were found very satisfactory for BFT treatment and control tanks which were recorded highest $96.81 \pm 0.58\%$ and lowest $93.86 \pm 0.87\%$ for CN8 and CN16 BFT systems, respectively (Table 4.3). No sudden mortalities of *H. fossilis* during the 180 d rearing were observed in any triplicate rearing tank among control and three biofloc systems

(Fig.4.21). Zafar *et al.* (2021) found above 96% survivability in biofloc culture but not significantly different from control, during 120 d of culture of *H. fossilis* with stocking densities of 450 numbers juveniles with average body weight (ABW) of 4.40 g in 500 l tanks. Das *et al.* (2021) found higher survival rate in BFT system than control during 90 d culture of *H. fossilis* in 1000 l tanks with a stocking density of 500 numbers of juveniles with ABW of 4.3 g. The findings of the present study revealed that the *H. fossilis* in the biofloc culture systems had better significant ($p < 0.05$) growth and feeding performance than in the control (Zafar *et al.*, 2021 and Das *et al.*, 2021) but the carbon to nitrogen ratio played a deceive roles for growth and feed performances among different BFT systems. An increasing trend of growth parameters from C:N ratio 8:1 to 12:1 and then decreasing trend from C:N of 12:1 to 16:1 were observed, while measuring final average body weight (FBW), final body length (FBL), final biomass (FBM), final weight gain (FWG), daily weight gain (DWG) and specific growth rate (SGR-% d) at the end of 180 d of culture (Table 4.3).

The maximum final body length (FBL) was recorded 19.4 ± 0.2 cm in BFT groups of CN12 which was significantly higher from others biofloc treatment groups CN8 (18.9 ± 0.2 cm), CN16 (18.8 ± 0.2 cm) and control (17.3 ± 0.2 cm). The highest growth was observed in the *H. fossilis* cultured in CN12 biofloc system where the FBW (57.65 ± 1.53 g), FBM (38.92 ± 1.21 kg), FWG (52.12 ± 1.53 g) and DWG (0.29 ± 0.01 g) were found significantly higher ($p < 0.05$) than the fishes reared in CN16 BFT system and control but not significantly higher ($p > 0.05$) compare to the fishes reared in CN8 biofloc group in respect of FWG and DWG (Table 4.3). The FBW (54.50 ± 2.19 g), FWG (48.97 ± 2.19 g) and DWG (0.27 ± 0.01 g) of fishes in CN8 group were not significantly higher than the results obtained in fishes in CN16 group i.e., FBW (50.96 ± 1.01 g), FWG (45.43 ± 1.01 g) and DWG (0.25 ± 0.01 g) but both were significantly higher ($p < 0.05$) than control *H. fossilis* groups (Table 4.3). No significant differences of specific growth rates or SGR (% d) were found among BFT treated Singhi groups while they were found significantly higher than control fish groups. Highest SGR (% d) was recorded as 1.30 ± 0.01 in CN12 group. The overall growths of *H. fossilis* in 180 d culture were not found satisfactory in this study in respect of Nile tilapia production in our previous experiment.

Table 4.3. Growth performances of Singhi (*Heteropneustes fossilis*) reared in control and biofloc system with different carbon to nitrogen (C:N) ratios of 8:1, 12:1, 16:1 during the culture period of 180 d in rectangular cement tanks with a stocking densities of 700 nos./ 1500 l.

	CN8	CN12	CN16	Control
IBW (g)	5.53±0.47	5.53±0.47	5.53±0.47	5.53±0.47
FBW (g)	54.50 ^{bc} ±2.19	57.65 ^c ±1.53	50.96 ^b ±1.01	43.34 ^a ±1.66
IBL (cm)	5.5±1.1	5.5±1.1	5.5±1.1	5.5±1.1
FBL (cm)	18.9 ^b ±0.2	19.4 ^c ±0.2	18.8 ^b ±0.2	17.3 ^a ±0.2
IBM (kg)	3.87	3.87	3.87	3.87
FBM (kg)	36.93 ^c ±1.40	38.92 ^c ±1.21	33.48 ^b ±0.40	28.70 ^a ±1.27
SR (%)	96.81 ^b ±0.58	96.43 ^b ±0.43	93.86 ^a ±0.87	94.57 ^a ±0.57
FWG (g)	48.97 ^b ±2.19	52.12 ^c ±1.53	45.43 ^b ±1.01	37.81 ^a ±1.66
DWG (g)	0.27 ^b ±0.01	0.29 ^c ±0.01	0.25 ^b ±0.01	0.21 ^a ±0.01
SGR (% d)	1.27 ^b ±0.02	1.30 ^b ±0.01	1.23 ^b ±0.01	1.10 ^a ±0.09

Values presented as Mean (± SD, n=3) in the same row with different superscripts differ significantly for each parameter between experimental group (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different carbon to nitrogen ratios of 8:1, 12:1 and 16:1; IBW: Initial average body weight; FBW: Final body weight; IBL: Initial body length; FBL: Final body length; IBM: Initial biomass; FBM: Final biomass; SR: Survival rate; FWG: Final weight gain; DWG: Daily weight gain; SGR: Specific growth rate]

The FBW and FBL of control tank 43.34±1.66 g and 17.3±0.2 cm, respectively, were recorded significantly lower (p<0.05) than all biofloc treatment groups (Table 4.3). Prominent variation in weekly fish growth rates were observed in every BFT and control groups after one month of culture (Fig.4.22). The individual size differences were found highest at the time of harvesting on 180th d (Fig.4.23). Females were found much larger than males. No research data on non uniform growth of *H. fossilis* in biofloc system was reported till date. However the size differences between male and female *H. fossilis* was reported by Talwar and Jhingran (1991).

The weekly growth rate, final growth performances and feeding performances of each triplicate culture tank of both biofloc and control had been illustrated in the Fig.4.24a for CN8, Fig.4.24b for CN12, Fig.4.24c for CN16 and Fig.4.24d for control Singhi (*H. fossilis*) groups. Growth curves of all BFT treated and control Singhi groups showed a typical sigmoid pattern with highest possible regression coefficient or R² values of 0.995 to 0.998, when best fitted in polynomial curve with order three in Excel 2007 (Fig.4.24a to Fig.4.24d).

Fig.4.21. Cumulative survival (numbers) in every week during 180 d culture of Singhi (*Heteropneustes fossilis*) in control and biofloc system with different carbon to nitrogen (C:N) ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16).

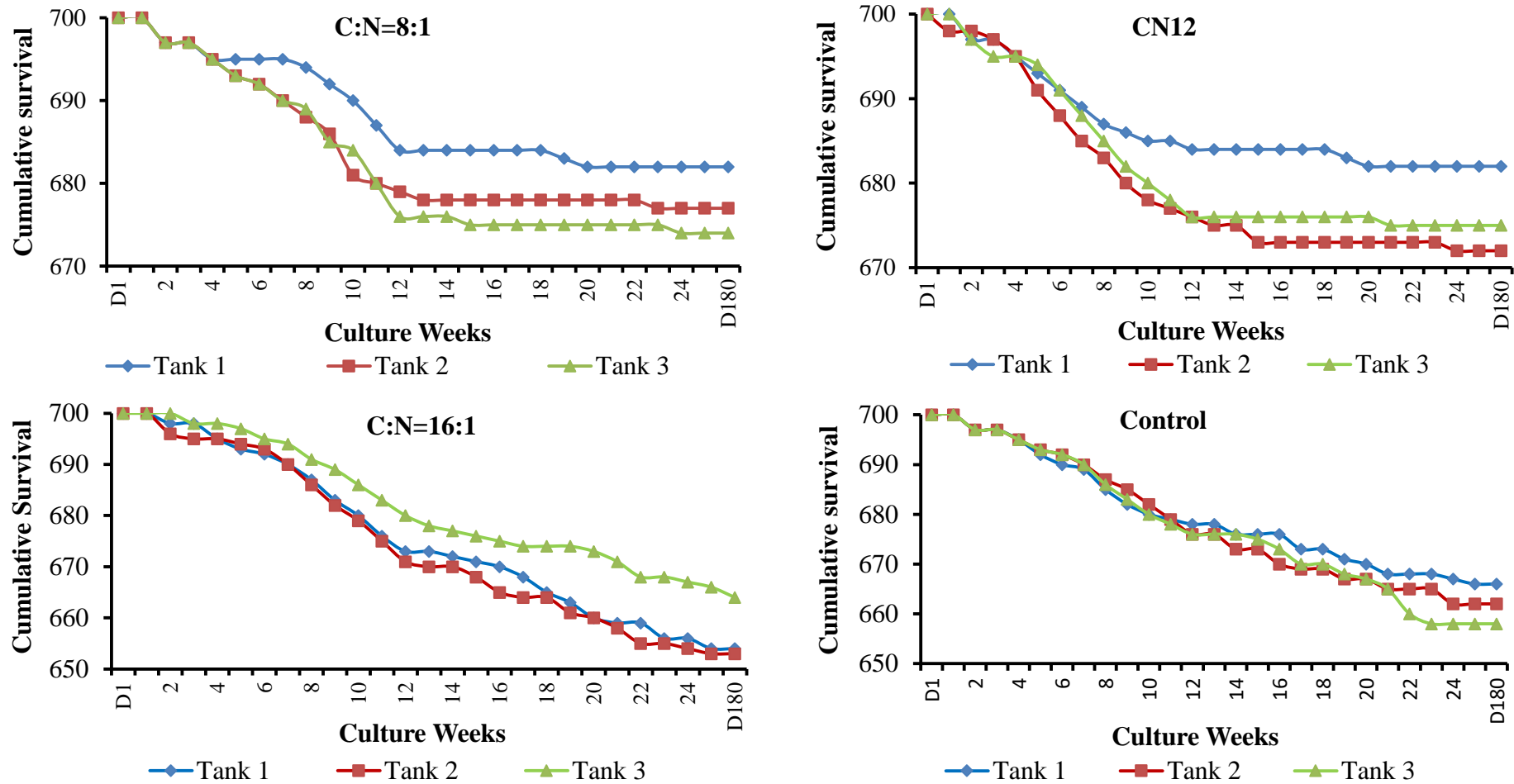


Fig.4.22. Average growth rate of Singhi (*Heteropneustes fossilis*) cultured in control and biofloc system with C:N of 8:1, 12:1 and 16:1 for 180 d

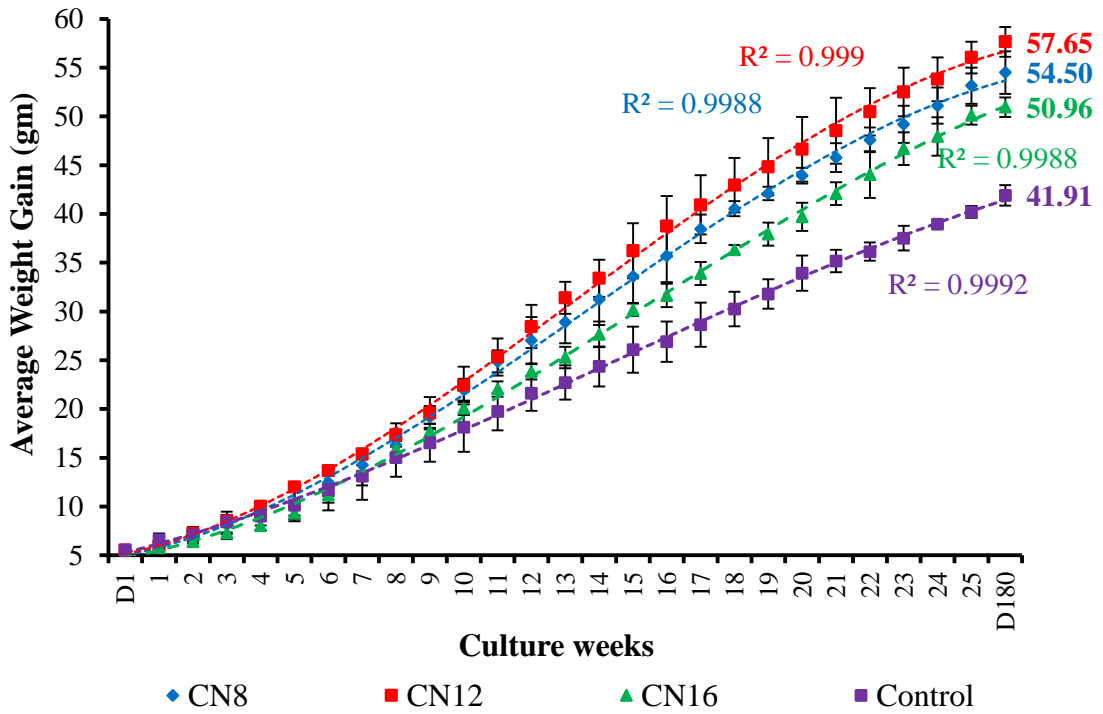


Fig.4.23. Final weight range (g) (n=90) of Singhi (*Heteropneustes fossilis*) cultured in BFT system with C:N of 8:1, 12:1, 16:1 and control tanks on 180th d

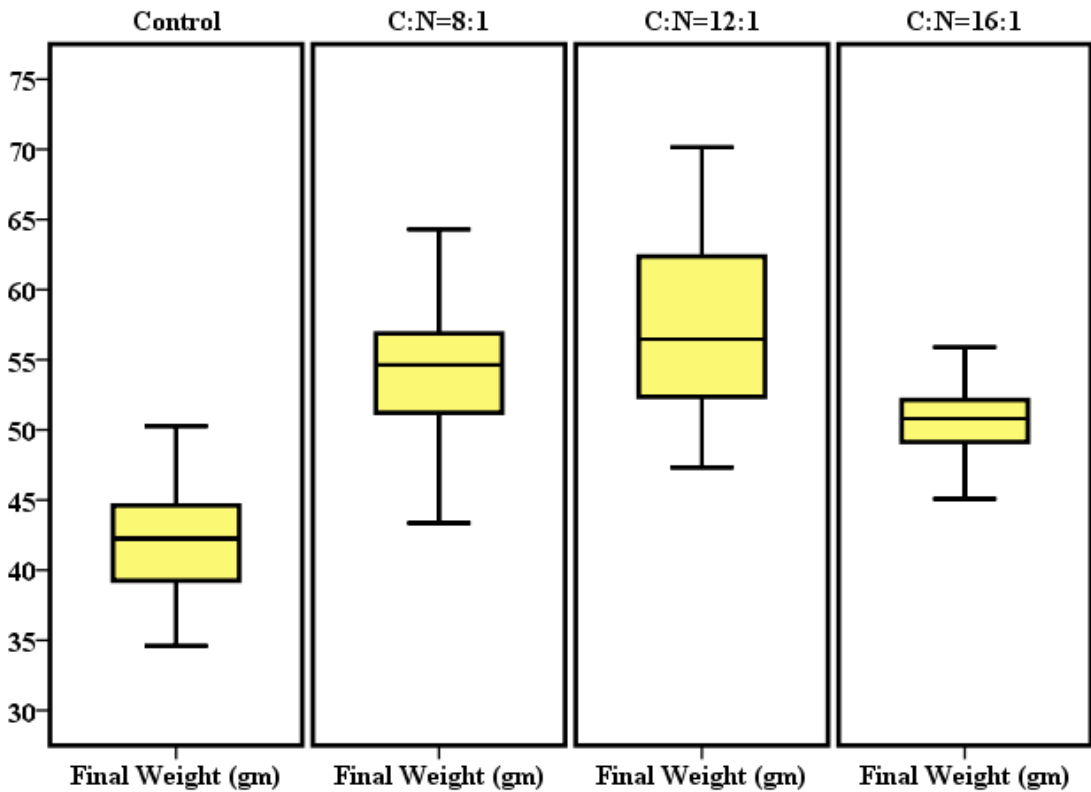


Fig.4.24a. Growth and feed performances of *Heteropneustes fossilis* reared in biofloc with C:N of 8:1 (CN8) for 180 d (Stocking density 700 nos./ 1500 l)

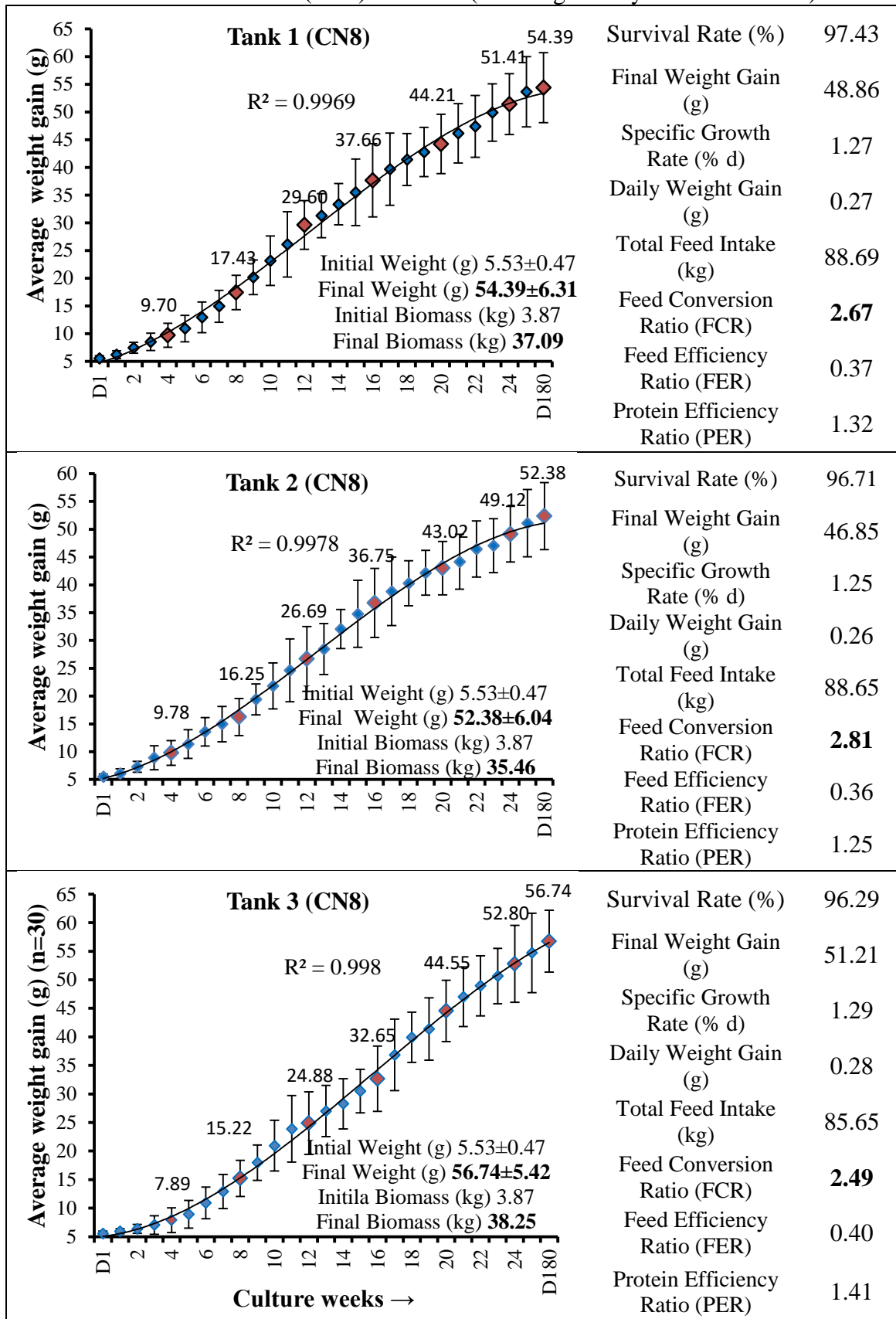


Fig.4.24b. Growth and feed performances of *Heteropneustes fossilis* reared in biofloc with C:N of 12:1 (CN12) for 180 d (Stocking density 700 nos./ 1500 l)

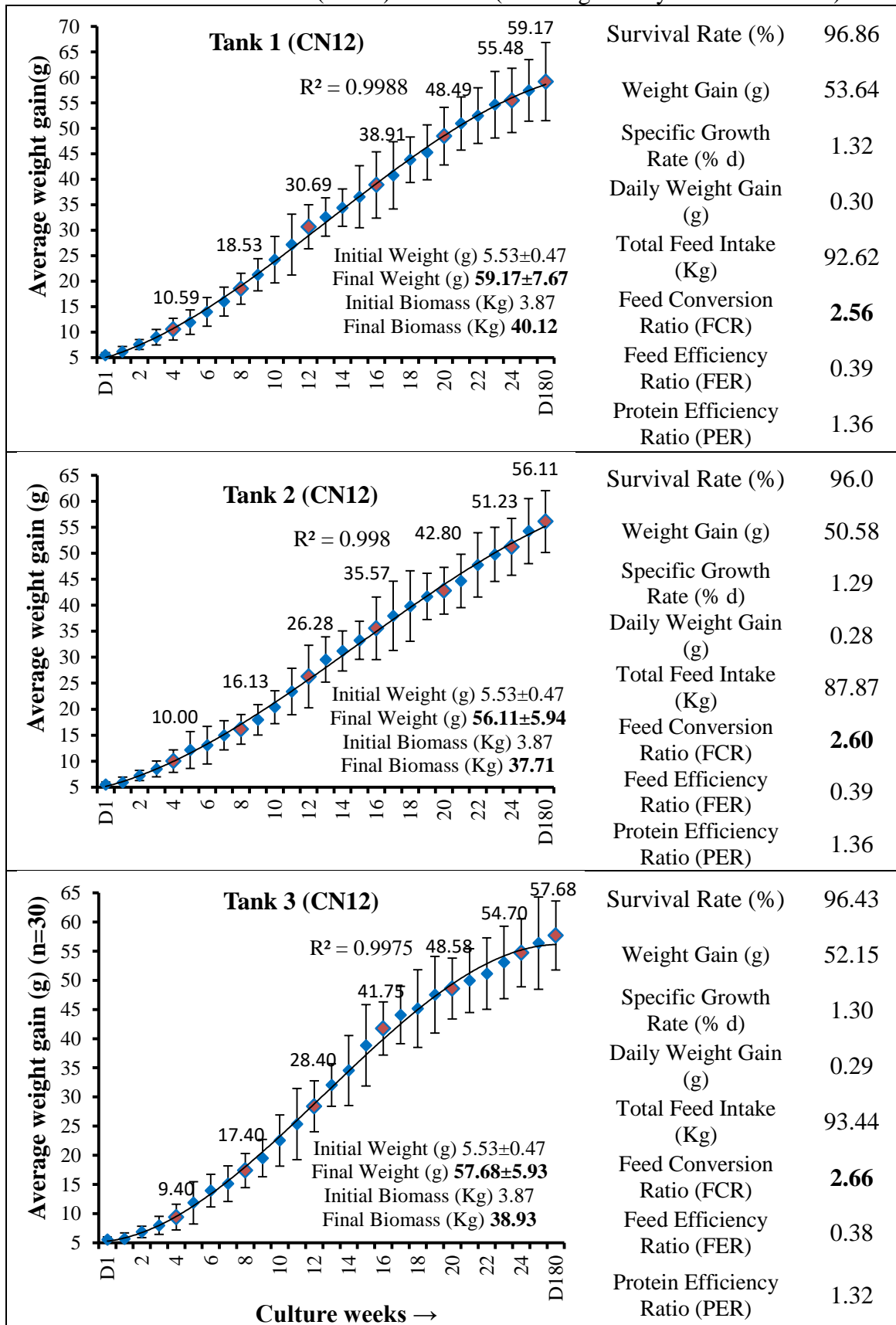


Fig.4.24c. Growth and feed performances of *Heteropneustes fossilis* reared in biofloc with C:N of 16:1 (CN16) for 180 d (Stocking density 700 nos./ 1500 l)

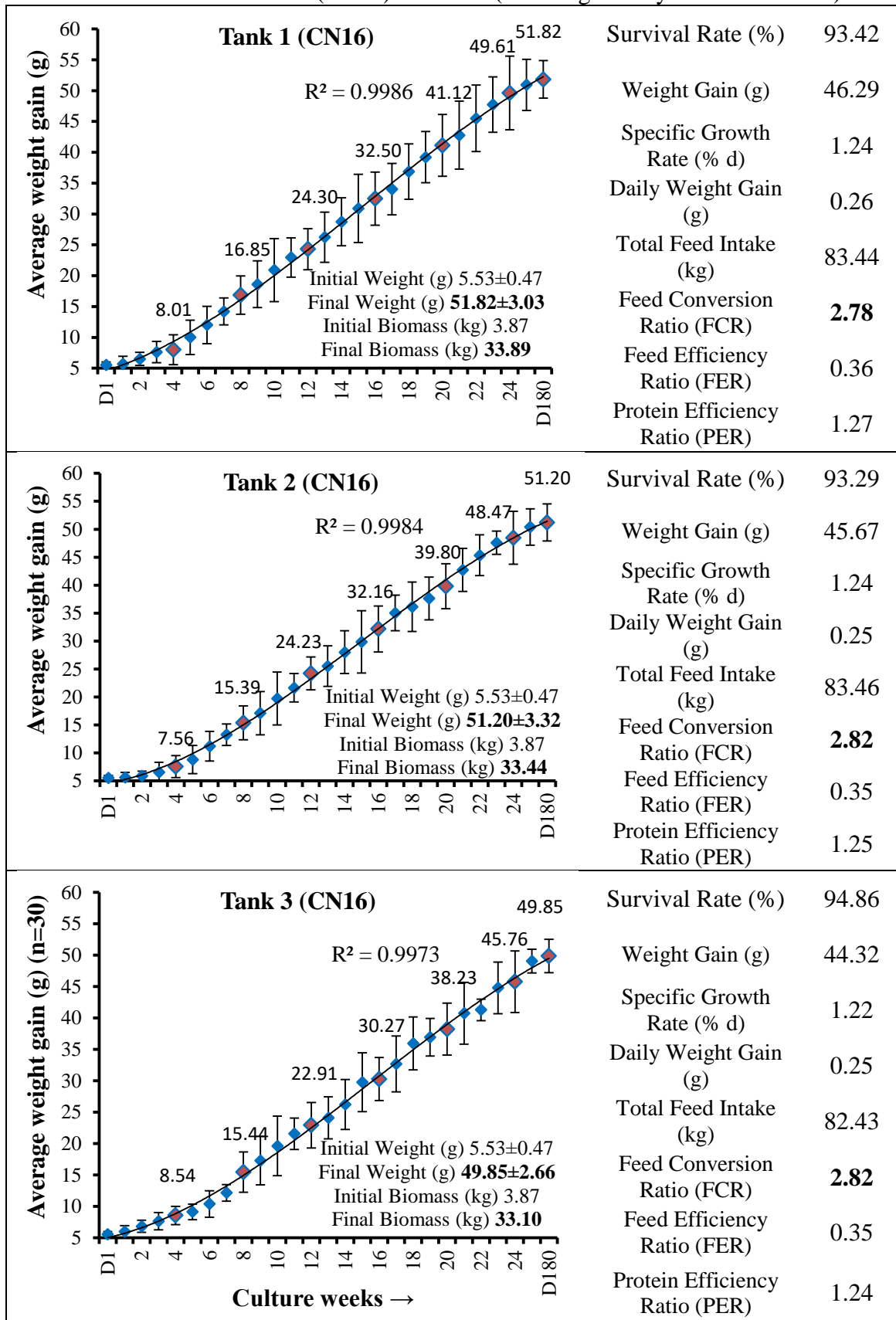


Fig.4.24d. Growth and feed performances of *Heteropneustes fossilis* reared in control tanks without biofloc for 180 d (Stocking density 700 nos./ 1500 l)

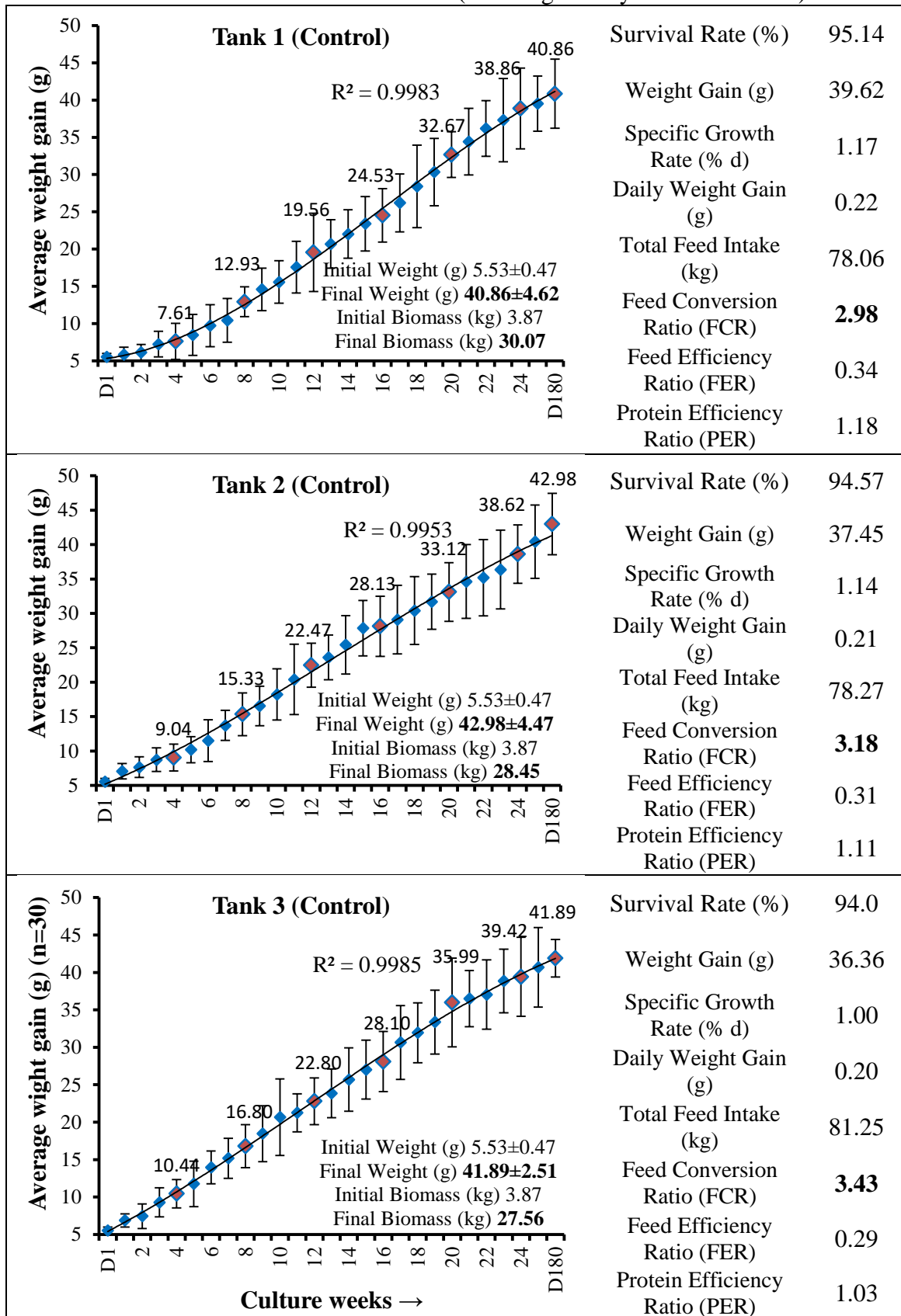


Plate 4.2. Length and weight measurements of Singhi (*Heteropneustes fossilis*) and final harvesting after 180 d culture in biofloc system.



Plate 4.2A



Plate 4.2B



Plate 4.2C



Plate 4.2D



Plate 4.2E



Plate 4.2F



Plate 4.2G



Plate 4.2H



Plate 4.2I



Plate 4.2J

The growth rate of the *H. fossilis* in the present study was found similar with the Shamsuddin *et al.* (2022) where among three different stocking densities they found highest final body weight of approximately 45 g starting from 5.2 g of initial size in low stocking densities of 3.64 kg/m³ during 150 d of culture of *H. fossilis* in BFT system with a C:N of 13:1. Whereas in our experiment the stocking densities were uniformly 3.22 kg/m³ and we achieved the almost same average body weight in CN12 biofloc groups on 150 d during our 180 d experimental period. The low growth rate of *H. fossilis* in control tanks appeared might be associated with higher stocking densities and increased competition for supplementary food and space in the tank. The rate of average body weight gain decreased from 70 d or 10th weeks of culture of *H. fossilis* in control tanks compare to biofloc groups (Fig.4.22). The inverse relationship between the stocking density and the growth rate during culture period was a space limiting effect on the Singhi population (Johnson, 1965).

4.2.5. Feeding performances of Singhi (*Heteropneustes fossilis*)

The lowest feed conversion ratio (FCR) of 2.61±0.06 and highest feed efficiency ratio (FER) of 0.38±0.01 and protein efficiency ratio (PER) of 1.35±0.02 were observed in the *H. fossilis* cultured in CN12 biofloc. Whereas those were not significantly ($p>0.05$) well gained than the *H. fossilis* cultured in CN8 and CN16 biofloc, but significantly ($p<0.05$) superior than control (Table 4.4). Similarly FCR, FER and PER value of fishes of CN8 and were not significantly better ($p>0.05$) than fishes cultured in CN16 but both were found significantly improved ($p<0.05$) than control. That might be due to the significantly better ($p<0.05$) survival rate and eventually more total feed intake by the *H. fossilis* cultured in CN12 followed by CN8 in compare to the *H. fossilis* cultured in CN16 biofloc system. In this study the overall FBM, WG, DWG, SGR, and FBM were at the utmost levels in *H. fossilis* cultured in biofloc systems with maintaining C:N ratio of 12:1 and 8:1 by incorporation of jaggery as an additional carbohydrate was found supportive in terms of better growth and feed utilization and survival of cultured species (Rajkumar *et al.*, 2016). These results were in accord with earlier conclusion that the growth rate and feed utilization enhanced in biofloc system for other species like *O. niloticus* (Azim and Little, 2008 and Mirzakhani *et al.*, 2019), *L. rohita* (Ahmad *et al.*, 2016, Mahanand *et al.*, 2013 and Kamilya *et al.*, 2017), *C. auratus* (Wang *et al.*, 2015 and Yu *et al.*, 2020a), Dybowski, *Opsariichthys kaopingensis* (Yu *et al.*, 2020b) and *C. carpio* (Najdegerami *et al.*, 2016 and Minabi *et al.*, 2020).

Table 4.4. Feeding performances and somatic indices of Singhi (*Heteropneustes fossilis*) reared in control and biofloc system with different carbon to nitrogen (C:N) ratios of 8:1, 12:1, 16:1 for culture period of 180 d in rectangular cement tanks with a stocking densities of 700 nos./ 1500 l.

	CN8	CN12	CN16	Control
Feed Performances				
TFI (kg)	87.67 ^c ±1.74	91.31 ^c ±3.00	83.11 ^b ±0.59	79.19 ^a ±1.78
FCR	2.66 ^b ±0.16	2.61 ^b ±0.06	2.81 ^b ±0.02	3.20 ^a ±0.23
FER	0.38 ^{bc} ±0.02	0.38 ^c ±0.01	0.36 ^b ±0.00	0.31 ^a ±0.02
PER	1.33 ^b ±0.08	1.35 ^b ±0.02	1.25 ^b ±0.01	1.11 ^a ±0.08
Somatic Indices				
GSI (%)	2.29±0.18	2.34±0.08	2.39±0.17	2.34±0.09
HSI (%)	1.11±0.02	1.12±0.01	1.14±0.02	1.15±0.02
CF	0.82±0.02	0.83±0.02	0.79±0.01	0.80±0.01

Values presented as Mean (± SD, n=3) in the same row with different superscripts differ significantly for each parameter between experimental group (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different carbon to nitrogen ratios of 8:1, 12:1 and 16:1; TFI: Total feed intake; FCR: Feed conversion ratio; FER: Feed efficiency ratio; PER: Protein efficiency ratio; GSI: Gastrosomatic index; HSI: Hepatosomatic index; CF: Condition factor]

H. fossilis was an omnivorous during its life cycle (Hossain *et al.*, 2015). The fry were planktivorous, the juveniles fed on crustacea, plant, miscellaneous matter and insects, while the adults fed on insects, detritus and plant matter (Hossain *et al.*, 2015). Our study revealed that BFT system with different C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16) contained the suspended microbial flocs which provided good sources of protein, essential amino acids, polyunsaturated fatty acids and different vitamins and minerals for the *H. fossilis* (Azim and Little 2008, De Schryver *et al.*, 2008, Luo *et al.*, 2014 and Wang *et al.*, 2015). The BFT system also consisted of profuse natural microbes like algae, plankton-like copepods, ciliates, beneficial bacteria, groups of living and dead particulate organic matter (Rajkumar *et al.*, 2016). *H. fossilis* or Asian stinging catfish or singhi were extremely dependent on copepods, fungus, detritus and eggs of other invertebrates for feeding (Shafi and Quddus, 2001), hence it might be possible that microbial flocs could facilitate to achieve an improved growth and feed performances of *H. fossilis* in the present study. However alike our findings about production of Nile tilapia in BFT system here also the results suggested that high carbon to nitrogen ratio (>12:1) in biofloc system could deteriorate the growth of *H. fossilis*. The microorganisms might not be supportive for growth of *H. fossilis* because of the negative effects of the microorganisms especially

the heterotrophic bacterial community on their digestive systems which need more investigation.

In compare to the control groups, a significantly ($p < 0.05$) superior feed conversion ratio was found in the BFT system singhi as because bioflocs contained a plenty of “in situ” microbial proteins such as peptidoglycan and lipopolysaccharide and poly-hydroxybutyrate (PHB), which was probably consumed as feed ingredients by the cultured Shingi in our studies (Anand *et al.*, 2014 and Avnimelech, 2007). Burford *et al.* (2004) demonstrated that the utilization of biofloc immensely increased the feed consumption efficiency with a lowest retention of excreted nutrients and reduced feed conversion ratio and cost of supplementary feed. Our results also proved that *H. fossilis* also fed on floc and the commercial diet simultaneously. Therefore, the singhi fed on only commercial diet without biofloc in control groups exhibited reduce feeding performances (Jimoh *et al.*, 2014 and Shamsuddin *et al.*, 2022).

4.2.6. Somatic indices of Singhi (*Heteropneustes fossilis*)

Both hepatosomatic index and gastrosomatic index was considered as common measurement of the overall condition of fish, hence the growth status of liver and intestine were known as well forecaster of unfavourable health in fish (Ighwela *et al.*, 2014). In this study the hepatosomatic index (HSI) and gastrosomatic index (GSI) of singhi from both BFT system and control were found insignificantly ($p > 0.05$) different at the end of 180 d of culture (Table 4.4). Being a carnivorous fish *Heteropneustes fossilis* had a large and distensible stomach, and a short intestine (Qu *et al.*, 2012). No significant difference ($p > 0.05$) of condition factor (CF) was observed among all BFT treated groups with different carbon to nitrogen ratios and control groups (Table 4.4). However a non significant ($p > 0.05$) lower value of CF (0.79 ± 0.01) in the BFT treated groups of CN16 compare to other two BFT treated group CN12 and CN8 was probably due to the unpleasant water quality for *H. fossilis* (Minabi *et al.*, 2020). Fatma and Ahmed, (2020) found that both hepatosomatic index (HSI) and gastrosomatic index (GSI) of *Heteropneustes fossilis* were influenced by the levels of dietary protein which found in contrast to our observation in biofloc culture system. The overall uniform HSI, GSI and CF of *Heteropneustes fossilis* in both control and biofloc system with different carbon to nitrogen ratios in this study clearly represented the general well-being of fish (Ighwela *et al.*, 2014).

4.3. Objective 3: To investigate the effects of BFT with different C/N ratios on proximate composition of biofloc as well as harvested *Oreochromis niloticus* and *Heteropneustes fossilis*.

4.3.1. Proximate composition of Nile tilapia (*Oreochromis niloticus*) carcass of control and biofloc with different C:N ratios of 15:1, 20:1, 25:1 after 120 d

Various studies revealed that the dietary protein, carbohydrate and lipid content could influence the final carcass composition of fish (Yusni, 2019) and the fish muscle proximate content could be considered as the quintessence of its nutritional value (Mansour and Esteban, 2017). In our study no considerable differences ($p>0.05$) of whole body crude protein contents (% dry matter) of Nile tilapia were noticed between control (54.70 ± 2.79 %) and biofloc treatment (BFT) with different carbon to nitrogen ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) i.e., 54.39 ± 1.97 %, 53.90 ± 1.17 % and 53.67 ± 2.12 %, respectively. The ash content (% dry matter) of fishes reared in BFT system and control did not showed any significant differences ($p>0.05$). Though, the lipid content (% dry matter) of the Nile tilapia whole body, harvested from the BFT system CN20 (25.12 ± 2.66 %) was found significantly higher ($p<0.05$) than control Nile tilapia group (21.73 ± 1.98 %), but not significantly higher ($p>0.05$) than the fishes of CN15 (23.83 ± 1.89 %) and CN25 (23.14 ± 1.83 %) biofloc groups (Table 4.5).

Table 4.5. Moisture content (%) and proximate composition (% dry matter) of Nile tilapia (*O. niloticus*) carcass cultured for 120 d in control and biofloc with different C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25).

	CN15	CN20	CN25	Control
Moisture (%)	74.55 ± 2.40	74.40 ± 1.83	74.61 ± 1.18	76.08 ± 2.22
Crude Protein (DW %)	54.39 ± 1.97	53.90 ± 1.17	53.67 ± 2.12	54.70 ± 2.79
Crude Lipid (DW %)	$23.83^{ab}\pm 1.89$	$25.12^b\pm 2.66$	$23.14^{ab}\pm 1.83$	$21.73^a\pm 1.98$
Ash (DW %)	9.11 ± 0.45	9.26 ± 1.10	9.53 ± 0.47	9.02 ± 1.15

Data are presented as means \pm SD (n=9). Superscripted letters denote significant differences between experimental groups (ANOVA; $p<0.05$).

The present findings were quite similar with observation of Long *et al.* (2015) where biofloc treated Nile tilapia groups showed no significant changes in terms of body crude protein and ash contents than control groups, but lipid content found

significantly higher than control. Yu *et al.* (2020a) did not find any significant differences for proximate component in golden crucian carp, *Carassius auratus* reared in biofloc system with a series of C:N ratios from 5.48:1, 10:1, 15:1, and 20:1 to 25:1. Our results were contrasted with Luo *et al.* (2014) where no substantial variation in the crude lipid content of tilapia back muscles were observed between the biofloc and RAS treatments, which might be differed due to the analysis of back muscle instead of whole body. Xu and Pan (2012) and Izquierdo *et al.* (2006) explained the influence of biofloc on the whole body proximate content of cultured shrimp where the considerable increase of lipid content was noticed. The possible reason behind the elevation of lipid content in biofloc treated fishes were the contribution of essential amino acids, fatty acids (PUFA and HUFA), minerals, vitamins and other nutritional elements present in the BFT system (Dauda *et al.*, 2018 and Minabi *et al.*, 2020). Our findings were also contradictory with the previous findings of Bakhshi *et al.* (2018), Khanjani *et al.* (2017) and Khanjani *et al.* (2021) where they noticed significant increase in all proximate components like protein, lipid and ash in the Nile tilapia reared in the biofloc system.

4.3.2. Proximate composition of 60th d floc collected from biofloc tanks with different C:N ratios of 15:1, 20:1, 25:1 for rearing Nile tilapia

The proximate composition of the biofloc collected on 60th d from the biofloc system with C:N ratios of 15:1, 20:1 and 25:1 were presented in Table 4.6. A significantly higher ($p < 0.05$) increasing trend of crude protein (% dry matter) (38.57 ± 1.17 %, 39.70 ± 0.93 % and 41.34 ± 1.01 %) and ash content (% dry matter) (33.38 ± 0.93 %, 34.17 ± 0.79 % and 35.28 ± 0.70 %) with increased carbon to nitrogen ratio of 15:1 (CN15), 20:1 (CN20) to 25:1 (CN25) were recorded. However it had been previously published that different carbon sources in BFT system could influence the proximate components of microbial flocs (Crab *et al.*, 2010, Ekasari *et al.*, 2015, Bakhshi *et al.*, 2018 and Khanjani *et al.*, 2021) but in our experiment jaggery was used solely as a carbon source. In this study the proximate component of biofloc were influenced by different C:N ratios maintained by single carbon source. Our findings were corroborated with the previous studies of Yu *et al.* (2020a) and Minabi *et al.* (2020). Existence of high quantity of ash in biofloc was also reported by Bauer *et al.* (2012), Hende *et al.* (2014), Neto *et al.* (2015), Long *et al.* (2015), Kamilya *et al.* (2017), Yu *et al.* (2020b) and Minabi *et al.* (2020). Wei *et al.* (2016)

showed that high ash content could lower the digestibility of other ingredients in the diet that resulted poor growth of shrimp. In this study high ash content in higher C:N ratios biofloc groups might decrease the digestibility of the biofloc by the culture Nile tilapia. The crude protein contents were high in BFT systems in this study were sufficient enough for the growth of tilapia as supported by Jauncey (2000) who suggested 25 to 30 % crude protein in diets was suitable for the growth of tilapia. The protein requirements for juveniles and adult tilapia were reported within the range of 30-40% and 20-30%, respectively (Siddiqui *et al.*, 1988).

The lipid content of all the biofloc treated groups were not significantly different ($p>0.05$) and found at very low level (Table 4.6). The lipid contents (DW %) were recorded as 2.72 ± 0.34 %, 2.82 ± 0.32 % and 2.68 ± 0.31 % for the flocs collected from the CN15, CN20 and CN25 biofloc groups, respectively. Our observation was supported by the previous observation of Luo *et al.* (2014), Long *et al.* (2015) and Kamilya *et al.* (2017). The lower crude lipid content of biofloc in the BFT treatment in between range of 2–5% was recorded in other studies of Azim and Little (2008), Crab *et al.* (2010), Ahmed *et al.* (2016), Yu *et al.* (2020b), Menaga *et al.* (2019), Minabi *et al.* (2020) and Khanjani *et al.* (2021). Thus this lipid content was not adequate dietary lipid requirement of 5–12% for tilapia as suggested by Lim *et al.* (2009). Hence supplementary feed was needed for proper growth of Nile tilapia in biofloc system.

Table 4.6. Proximate analysis (% dry matter) of flocs collected on 60th d from biofloc tanks with different C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) for rearing Nile tilapia (*O. niloticus*).

	CN15	CN20	CN25
Crude Protein (DW %)	$38.57^a \pm 1.17$	$39.70^b \pm 0.93$	$41.34^c \pm 1.01$
Crude Lipid (DW %)	2.72 ± 0.34	2.82 ± 0.32	2.68 ± 0.31
Ash (DW %)	$33.38^a \pm 0.93$	$34.17^b \pm 0.79$	$35.28^c \pm 0.70$

Data are presented as means \pm SD (n=9). Superscripted letters denote significant differences between experimental groups (ANOVA; $p<0.05$).

Considering the protein level of biofloc as a dietary protein provided to the Nile tilapia along with supplementary feeding with 28-32%, when the protein utilization by Nile tilapia in terms of growth rates in biofloc system with different C:N ratios were analysed we did not found any significant influence of biofloc protein on efficiency of protein utilization (Fatma and Ahmed, 2020). In spite of significantly

higher ($p < 0.05$) crude protein level in flocs of CN25 followed by CN20 and CN15, higher but not significant growth rate of fish were observed in CN15 groups, followed by CN20 and CN25 BFT treated groups (Table 4.1). In this study the Nile tilapia converted the protein more efficiently as resulted by highest growth obtained in the BFT system of CN15 where the protein content was 38.57 ± 1.17 %. In this study the protein utilization might be decreased in BFT treated Nile tilapia with increasing dietary protein above optimum level requirement (Gullu *et al.*, 2008).

4.3.3. Proximate composition of Singhi (*Heteropneustes fossilis*) carcass of control and biofloc with different C:N ratios of 8:1, 12:1 and 16:1 after 180 d

In the present study, significantly higher ($p < 0.05$) crude protein content (% dry matter) of 66.20 ± 2.84 % was noticed in whole body of *H. fossilis* reared in the BFT system with a C:N of 12:1 compared to the protein content of control fishes (63.27 ± 2.09 %), but not significantly ($p > 0.05$) higher to the protein content found in fishes reared in BFT system of CN8 (64.58 ± 2.88 %) and CN16 (63.47 ± 3.17 %). No significant differences ($p > 0.05$) were observed in respect of dry matter percentage of lipid content and ash content in the *H. fossilis* raised in control group and the BFT system with C:N of 8:1, 12:1 and 16:1 groups (Table 4.7). Our findings were partly similar with Zafar *et al.* (2021) who observed higher significant value of both crude protein and lipid content in *H. fossilis* reared in BFT treatment with C:N of 10:1 in compare to control but no significant differences ($p < 0.05$) for ash and carbohydrate content. The proximate composition ranges of moisture, protein, lipid and ash contents of *H. fossilis* in this study were quite resembled with the findings of Salma *et al.* (2021).

Table 4.7. Moisture content (%) and proximate composition (% dry matter) of Singhi (*H. fossilis*) carcass cultured for 180 d in control and biofloc with different C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16).

	CN8	CN12	CN16	Control
Moisture (%)	75.38 ± 1.65	74.95 ± 1.92	75.40 ± 2.26	75.19 ± 1.92
Crude Protein (DW %)	$64.58^{ab} \pm 2.88$	$66.20^b \pm 2.84$	$63.47^{ab} \pm 3.17$	$63.27^a \pm 2.09$
Crude Lipid (DW %)	15.18 ± 1.60	15.09 ± 1.19	15.37 ± 1.89	15.41 ± 1.09
Ash (DW %)	10.37 ± 1.27	10.51 ± 1.42	11.39 ± 0.82	11.48 ± 0.80

Data are presented as means \pm SD (n=9). Superscripted letters denote significant differences between experimental groups (ANOVA; $p < 0.05$).

4.3.4. Proximate composition of 60th d floc collected from biofloc tanks with different C:N ratios of 8:1, 12:1 and 16:1 for rearing *H. fossilis*

The proximate component of the microbial floc in the biofloc system might be altered due to various factors like the type of carbon source (Khanjani *et al.*, 2021), the carbon to nitrogen ratio (Minabi *et al.*, 2020) and salinity of biofloc water (Khanjani *et al.*, 2020). The proximate composition of biofloc collected on 60th d from the BFT system with different C:N of 8:1 (CN8), 12:1(CN12) and 16:1 (CN16) were also observed during 180 d of *H. fossilis* culture. The crude protein percentage of dry matter in biofloc of BFT system of CN16 (34.98±1.11%) was found significantly higher (p<0.05) than biofloc of CN8 (32.77±0.99%) and not significantly higher (p>0.05) than flocs of CN12 (33.91±1.65%) (Table 4.8). The crude protein content of the flocs in our studies were quite similar to the protein content of the flocs observed by Crab *et al.* (2010) and Mahanand *et al.* (2013). Presence of high quantity of ash and low lipid content in biofloc was also supported by the observation of previous studies done by Bauer *et al.* (2012), Hende *et al.* (2014), Neto *et al.* (2015), Long *et al.* (2015), Kamilya *et al.* (2017), Yu *et al.* (2020b) and Minabi *et al.* (2020). The recommended range of dietary protein level for fry of *H. fossilis* was 40.8-41.8% (Fatma and Ahmed, 2020). Hence the supplementary feeding was urgently required for rearing Singhi in biofloc system. In spite of higher crude protein content in BFT system with C:N of 16:1 (CN16), the highest protein utilization in terms of higher growth rate of *H. fossilis* was observed in BFT system with C:N of 12:1 (Table 4.3). The possible reason for that might be associated with reduction of nutrient absorption by intestine with increasing floc volume in higher carbon to nitrogen ratio.

Table 4.8. Proximate analysis (% dry matter) of flocs collected on 60th d from biofloc tanks with different C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16) for rearing Singhi (*H. fossilis*).

	CN8	CN12	CN16
Crude Protein (DW %)	32.77 ^a ± 0.99	33.91 ^{ab} ± 1.65	34.98 ^b ± 1.11
Crude Lipid (DW %)	1.67 ^a ± 0.30	2.11 ^b ± 0.24	2.63 ^c ± 0.32
Ash (DW %)	29.80 ^a ± 0.79	31.32 ^b ± 0.87	33.84 ^c ± 0.78

Data are presented as means ±SD (n=9). Superscripted letters denote significant differences between experimental groups (ANOVA; p<0.05).

4.4. Objective 4: To study the effects of BFT with different C/N ratios on health issues of *Oreochromis niloticus* and *Heteropneustes fossilis*.

4.4a. Welfare parameters of Nile tilapia (*Oreochromis niloticus*):

All the fish welfare parameters like serum biomarkers for stress (glucose and total protein), liver (Alanine transaminase, Aspartate transaminase and Alkaline phosphatase), ionic balance (Calcium and Chloride), haematological parameters (Total erythrocytes count, Total leucocytes count, Haemoglobin and Haematocrit), innate immune parameters (Respiratory burst activity, Myeloperoxidase assay and serum Lysozyme assay), and antioxidant assays (Superoxide dismutase and Catalase) were measured from the blood and serum samples collected on 60th and 120th d from the control and biofloc treatment (BFT) Nile tilapia (*Oreochromis niloticus*) groups with different carbon to nitrogen ratios (C:N) of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) during the four months experimental period. All the outcomes in connection with welfare parameters of Nile tilapia mentioned in the Table 4.9 to Table 4.14 were showing analysis of variance (ANOVA, $p < 0.05$) for each parameters between control and experimental groups for a particular sampling date. Whereas the statistical comparison (Students' t-test, $p < 0.05$) between two separate sampling date of 60th and 120th d, respectively, for each individual parameter for both control and BFT treated Nile tilapia groups were showed graphically in the Fig.4.25 to Fig.4.30.

4.4a.1. Serum biomarkers for stress of Nile Tilapia (*Oreochromis niloticus*)

Blood biochemistry parameters were the helpful tools to indicate the general state of fish health that can change with water condition factors and nutritional status (Dawood *et al.*, 2015, El Basuini *et al.*, 2016 and 2017). In this study the serum glucose level of all biofloc treatment (BFT) Nile tilapia with different C:N ratios were found significantly lower ($p < 0.05$) from the serum glucose level of Nile tilapia in control group i.e., 83.90 ± 5.52 mg/dl and 139.70 ± 5.60 mg/dl measured on both 60th and 120th d, respectively. No significant differences ($p > 0.05$) of serum glucose level was found in BFT Nile tilapia groups on 60th d of culture but it was found significantly higher ($p < 0.05$) for the fishes of BFT system of CN25 (107.81 ± 2.91 mg/dl) from fishes of BFT system of CN15 (90.87 ± 3.44 mg/dl) and CN20 (93.63 ± 4.22 mg/dl) on 120th d (Table 4.25). Augmentation of glycogenolysis and the synthesis of glucose from extra hepatic tissue proteins and aggravation of the glucose content in blood by

amino acids were considered as stress indicator in animals (Almeida *et al.*, 2001). EL-Khalidi (2010) reported higher serum glucose in Nile tilapia exposed to different kinds of stress. The serum glucose level was also a marker of changeability in the digestion and metabolism of fish subjected to diverse feeding management practices (Li *et al.*, 2016). Along with a general response to stress the glucose content was also considered as an index of mobilization of metabolism to generate an additional energy provides (Bonga, 1997).

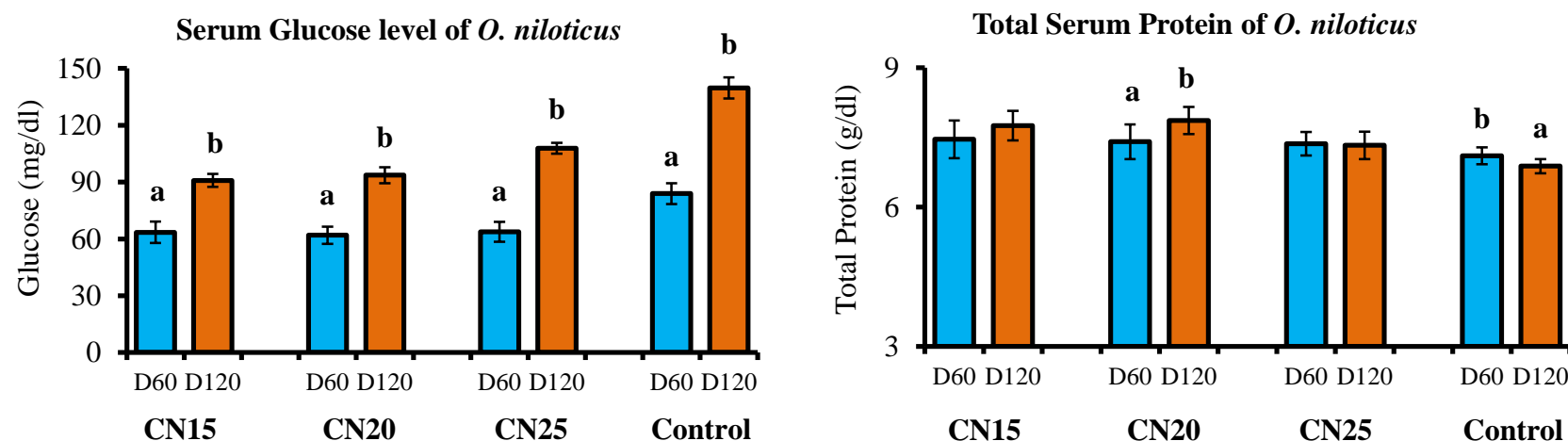
In this experiment serum glucose levels in the BFT Nile tilapia groups were significantly lower than that in the control, and the lowest values were recorded in the CN15 and CN20 (Table 4.25). Liu *et al.* (2018a) also found significant low ($p < 0.05$) glucose level in tilapia cultured in BFT system with C:N ratios of 10:1, 15:1 compare to the C:N ratio of 20:1 and control groups. Haridas *et al.* (2017) also detected a higher glucose level in the genetically improved farmed tilapia group with decreased growth in control groups compare to the BFT groups. The BFT system were able to reduce the physiological stress in Nile tilapia in our study agreed with the other studies where reduced levels of glucose level were reported in fishes reared in biofloc system than to the control, like rohu (Ahmad *et al.*, 2016), tilapia (Menaga *et al.*, 2019). In our study, in spite of same stocking density the possible reason for higher significant ($p < 0.05$) glucose level in the BFT system of CN25 groups in compare to CN15 and CN20 biofloc groups, might be linked with other environmental factors like high TSS level, high biological oxygen demand in higher carbon content biofloc system or too much floc volume that created stress to the aquatic organisms.

Our studies also revealed that increased biomass along with culture duration also produced higher significant ($p < 0.05$) glucose level on 120th d compare to 60th d in Nile tilapia reared in both biofloc and control groups (Fig.4.25). The increased patterns of glucose levels in Nile tilapia in our studies were found to be size and age dependent as described by Laizcarrion *et al.* (2012). Our results indicated that the biofloc system under minimal water exchange did not cause much stress-related problems to the fish even at higher stocking densities up to 625 nos./m³. The results of our study were akin with previous findings in Nile tilapia (Azim and Little 2008, Ahmed and Sadek 2014 and Menaga *et al.*, 2019).

Table 4.9. Serum biomarkers for stress measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

		CN15	CN20	CN25	Control
Serum Glucose (mg/dl)	60 th d	64.49 ^a ± 5.61	61.93 ^a ± 4.55	63.77 ^a ± 5.27	83.90 ^b ± 5.52
	120 th d	90.87 ^a ± 3.44	93.63 ^a ± 4.22	107.81 ^b ± 2.91	139.70 ^c ± 5.60
Total Protein (g/dl)	60 th d	7.46 ^b ± 0.41	7.41 ^{ab} ± 0.37	7.36 ^{ab} ± 0.26	7.10 ^a ± 0.18
	120 th d	7.75 ^c ± 0.32	7.86 ^c ± 0.29	7.33 ^b ± 0.30	6.88 ^a ± 0.15

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1]

Fig.4.25. Serum biomarkers for stress measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [D60: 60th d; D120: 120th d; CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1]

The significant declined ($p < 0.05$) total serum protein content in control Nile tilapia groups from 7.10 ± 0.18 g/dl on 60th d to 6.88 ± 0.15 g/dl on 120th d in the present study might be associated with the conversion of blood and structural proteins to convene the higher energy demands produced during high stocking density stress along with adverse water quality parameters (Fig.4.25). However, some researchers proved that liver disorder and renal excretion was considered as a factor for the reduction of serum protein in fish (Mutlu *et al.*, 2015). The higher significant ($p < 0.05$) level of serum protein level on 120th d in CN15 (7.75 ± 0.32 g/dl), CN20 (7.86 ± 0.29 g/dl) and CN25 (7.33 ± 0.30 g/dl) (Table 4.9) helped to reduce the dietary protein levels of the supplementary feed with the improvement of the nonspecific immune response (Rao *et al.*, 2006).

In this study no significant difference ($p > 0.05$) of serum protein level was found among the biofloc reared tilapia groups on 60th d but significantly lower ($p < 0.05$) serum protein were noticed in CN25 group than other two BFT treated CN15 and CN20 groups on 120th day, but it was significantly higher ($p < 0.05$) than to the control Nile tilapia group (Table 4.9). That might be due to the liver disorder due to excessive heterotrophic bacterial presence, floc volume and TSS level in BFT system with higher carbon to nitrogen ratio. Our findings regarding elevated serum protein level in biofloc groups were supported by Menaga *et al.* (2019), Ahmad *et al.* (2016) but not by Kamilya *et al.* (2017).

4.4a.2. Serum biomarkers for liver of Nile Tilapia (*Oreochromis niloticus*):

Liver condition was a vital pathological sign for identifying injuries caused by nutritional status as its function was to metabolize ingredients passing through the gastrointestinal tract (Abedian *et al.*, 2013). Alanine transaminase (ALT) and Aspartate transaminase (AST) enzymes were the ubiquitous aminotransferases in the mitochondrion of fish which were considered as markers for the deterioration of tissues like liver, kidneys and gills. AST and ALT in liver and serum could be used as prominent indicators of hepatopancreas function and damage (Zhou *et al.*, 2014), and also acted as a stress indicators (Haridas *et al.*, 2017). Elevated concentration of those enzymes in fishes indicated a raise in aspartate and alginate through utilization of sugar to produce the glucose for adjusting and countering the stress status (Gowda *et al.*, 2009). Alteration in Alkaline Phosphatase (ALP) level was a physiological response which associated with immune responses and could be considered as an

indicator of fish health (Liu *et al.*, 2014). A reduce level of this enzyme indicated improved immune system (Khanjani *et al.*, 2021).

In the present study different carbon nitrogen ratios showed the different effects on the Nile tilapia serum ALT and AST level on 60th and 120th d in both BFT and control groups. The ALT (44.66 ± 3.08 IU/l and 67.25 ± 2.52 IU/l), AST (78.66 ± 1.61 IU/l and 103.58 ± 4.66 IU/l) and ALP (45.46 ± 3.39 IU/l and 63.42 ± 3.67 IU/l) of control group were found significantly higher ($p < 0.05$) than the BFT treated Nile tilapia groups with different C:N ratios on both 60th 120th d (Table 4.10). No significant alteration ($p > 0.05$) of those aforesaid enzymes were observed among the BFT treated tilapia groups with C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25) on the 60th d but surprisingly on 120th d significant higher ($p < 0.05$) level of serum ALT, AST and ALP were recorded in CN25 fish group compare to lower C:N groups of CN15 and CN20 (Fig.4.26).

In this study the rose in the concentration of ALT and AST of tilapia might be caused by the fish stress that led to increased internal oxidative stress. That stress altered permeability of cellular membranes and increased enzymatic (AST and ALT) leakage to blood. Hence in all BFT treated and control Nile tilapia groups the ALT, AST and ALP were increased significantly ($p < 0.05$) on 120th d than 60th d (Fig.4.26). The lowest serum ALT, AST and ALP was noticed in BFT treated groups of CN15 and CN20 throughout the experiment (Table 4.10).

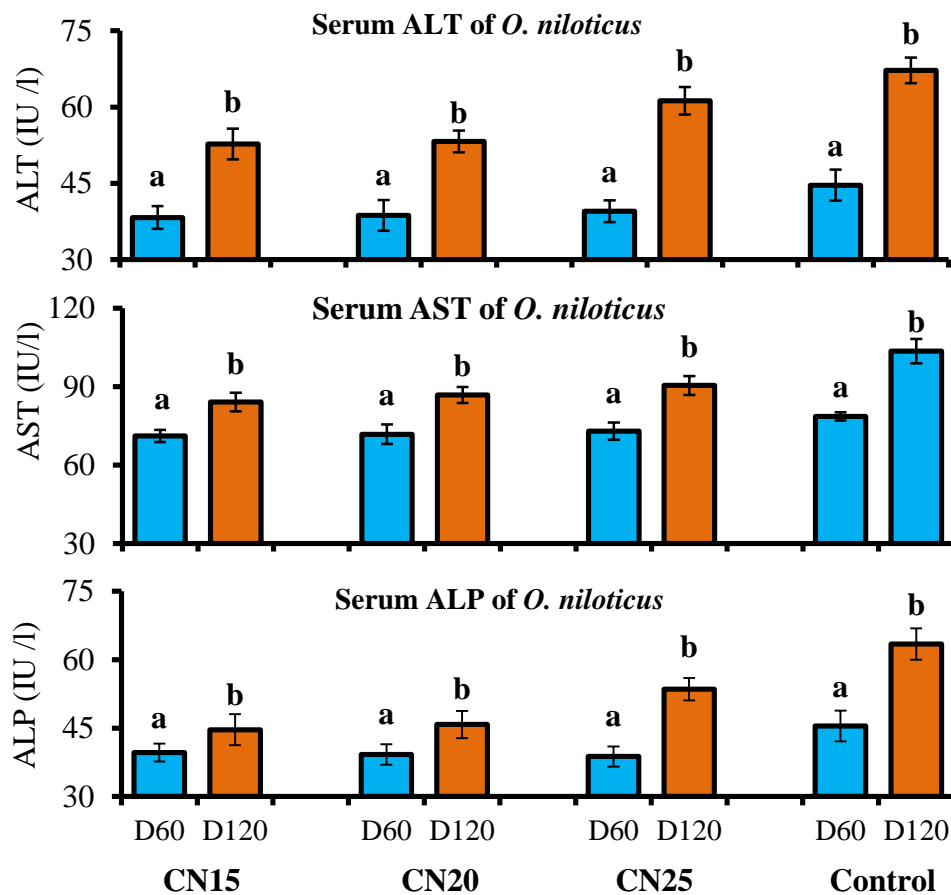
Our studies was consistent with other previous studies where biofloc cultivated *O. niloticus* showed reduced activity of hepatic enzymes (Liu *et al.*, 2018a, Liu *et al.*, 2018b and Khanjani *et al.*, 2021). Our experiment showed that the biofloc could effectively reduce the damage of hepatocytes in Nile tilapia with reduced ALP, AST and ALP level in such intensive culture system. It was also revealed from this study that maintenance of biofloc system with different C:N ratios by incorporation of jaggery as carbon source could produce different effects on the activity of hepatic enzymes in Nile tilapia. That might be due to the positive and beneficial effects of produced biofloc on the liver function of Nile tilapia in those BFT treated groups i.e., CN15 and CN20 in compare to the CN25 and control groups.

Table 4.10. Serum biomarkers for liver measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

			CN15	CN20	CN25	Control
Serum ALT (IU/l)	60 d		38.28 ^a ±2.23	38.72 ^a ±3.02	39.51 ^a ±2.15	44.66 ^b ±3.08
	120 d		52.74 ^a ±3.03	53.25 ^a ±2.16	61.22 ^b ±2.70	67.25 ^c ±2.52
Serum AST (IU/l)	60 d		71.18 ^a ±2.35	71.83 ^a ±3.68	72.96 ^a ±3.32	78.66 ^b ±1.61
	120 d		84.09 ^a ±3.55	86.80 ^a ±3.02	90.45 ^b ±3.64	103.58 ^c ±4.66
Serum ALP (IU/l)	60 d		39.64 ^a ±1.97	39.22 ^a ±2.23	38.76 ^a ±2.23	45.46 ^b ±3.39
	120 d		44.63 ^a ±3.61	45.78 ^a ±3.19	53.53 ^b ±2.58	63.42 ^c ±3.67

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

Fig.4.26. Serum biomarkers for liver measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

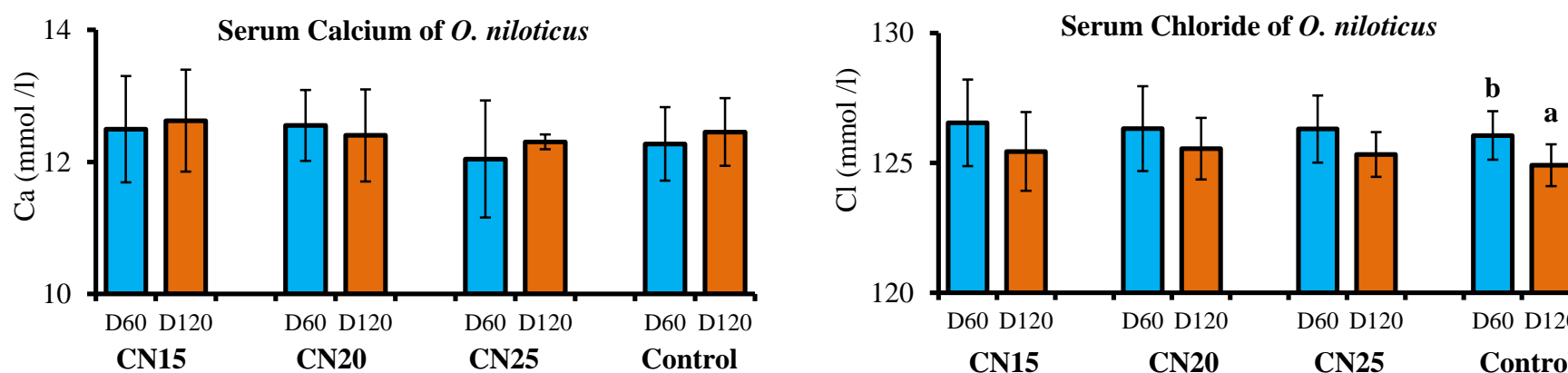


Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [D60: 60th d; D120: 120th d]

Table 4.11. Serum biomarkers for ionic balance measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

		CN15	CN20	CN25	Control
Serum Calcium (mmol/L)	60 th d	12.50 ± 0.80	12.55 ± 0.54	12.04 ± 0.89	12.27 ± 0.56
	120 th d	12.63 ± 0.77	12.40 ± 0.70	12.30 ± 0.11	12.45 ± 0.51
Serum Chloride (mmol/L)	60 th d	126.54 ± 1.66	126.32 ± 1.63	126.30 ± 1.29	126.05 ± 0.93
	120 th d	125.44 ± 1.52	125.55 ± 1.18	125.32 ± 0.86	124.91 ± 0.81

Values presented as Mean (\pm SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, $p < 0.05$). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

Fig.4.27. Serum biomarkers for ionic balance measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

Values are presented as Mean (\pm SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, $p < 0.05$). [D60: 60th d; D120: 120th d; CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

4.4a.3. Serum biomarkers for ionic balance of Nile Tilapia (*Oreochromis niloticus*):

In vertebrates, calcium played an essential function in a variety of biological activities (Srivastav *et al.*, 2021) and serum chloride level was often measured to detect few health conditions like kidney and liver diseases (Handyani *et al.*, 2020). The chloride (Cl⁻) ions in the body fluid also played a critical role for osmoregulation (Kaneko *et al.*, 2008). Till now no information regarding the impact of biofloc on blood electrolytes of Nile tilapia in high stocking density BFT system had been reported. In our studies we did not find any significant differences ($p < 0.05$) in serum calcium and chloride level among BFT treated and control Nile tilapia groups on sampling date of 60th and 120th d which indicated Nile tilapia could effectively adapt the biofloc system (Table 4.11). But the serum chloride level was found significantly lower ($p < 0.05$) on 120th d than 60th d in control, might be due to increased accumulation of nitrogenous toxic elements with increased biomass in control tanks (Fig.4.27).

4.4a.4. Hematological status of Nile Tilapia (*Oreochromis niloticus*):

Hematological status of fish was the basic way to assess the health condition of fish (Harikrishnan *et al.*, 2011 and Ismail *et al.*, 2019). In this study we did not find any significant differences ($p > 0.05$) for hematological parameters like total erythrocytes count (TEC), total leucocytes count (TLC), hemoglobin (Hb) and hematocrit (Ht) values among Nile tilapia reared in control and three BFT systems such as CN15, CN20 and CN25 on the 60th d during 120 d rearing period (Table 4.12). Long *et al.* (2015) also did not find any noticeable effects on RBC, WBC, Hb, or Ht while measured the negative effects of biofloc on the physical status of the tilapia after eight weeks experimental period. Azim and Little (2008) also reported that blood hematocrit level of Nile tilapia did not alter significantly between the biofloc treated and the control group after 12 weeks of experimental period. On the other hand significant changes among hematological parameters were observed among control and biofloc treated Nile tilapia groups on 120th d. The TEC, TLC, Hb and Ht (%) of the all BFT treated CN15, CN20 and CN25 groups were recorded significantly higher ($p < 0.05$) than to the control Nile tilapia group, except TEC of CN25 group which was found not significantly ($p > 0.05$) higher than control (Table 4.12).

Erythrocytes (RBCs) were the markers of diverse types of stress and the higher total erythrocytes concentration in the fish blood could increase the availability of oxygen inside the fish tissues (Rehulka *et al.*, 2002). In addition, leukocytes (WBCs) were the immune cells that concerned about non specific immunity, so the profusion of leukocytes directly indicated the health condition in fish (Guardiola *et al.*, 2019). Hence, the significant lower levels of erythrocytes and leukocytes in the control Nile tilapia group in this study on 120th d, specified the stressful effect of adverse water quality conditions at high biomass in respect of less biomasses on the mid of the culture i.e., 60th d. Besides that age of the fish was often cited as a factor which could affects hematological and blood biochemistry (Lone *et al.*, 2012), hence a significant increase ($p < 0.05$) trend of all the hematological parameters were observed from 60th to 120th d for all BFT treated Nile tilapia (Fig.4.28).

No Significant differences ($p > 0.05$) were observed for TEC, Hb and Ht on 120th d among the BFT treated groups, but the TLC of CN15 ($4.03 \pm 0.26 \times 10^4$ cells/mm³) and CN20 ($3.99 \pm 0.15 \times 10^4$ cells/mm³) were noted statistically higher ($p < 0.05$) than to the TLC of CN25 ($3.79 \pm 0.10 \times 10^4$ cells/mm³). Whereas, the TEC, Hb and Ht of CN25 Nile tilapia group i.e., $1.95 \pm 0.12 \times 10^6$ cells/mm³, 9.67 ± 0.68 g/dl and 28.87 ± 1.00 %, respectively on 120th d were found not significantly ($p > 0.05$) lower compare to CN15 and CN20 fish groups (Table 4.12). All the aforesaid hematological indices of Nile tilapia of CN15 and CN20 groups were found superior on 120th d with a highest recorded value of TEC of $2.05 \pm 0.16 \times 10^6$ cells/mm³ in CN20, followed by $2.02 \pm 0.17 \times 10^6$ cells/mm³ in CN15. On the other hand highest values of TLC, Hb and Ht were noticed in CN15 Nile tilapia group i.e., $4.03 \pm 0.26 \times 10^4$ cells/mm³, 9.95 ± 0.48 g/dl and 29.35 ± 1.71 %, respectively, followed by the CN20 Nile tilapia group (Table 4.12).

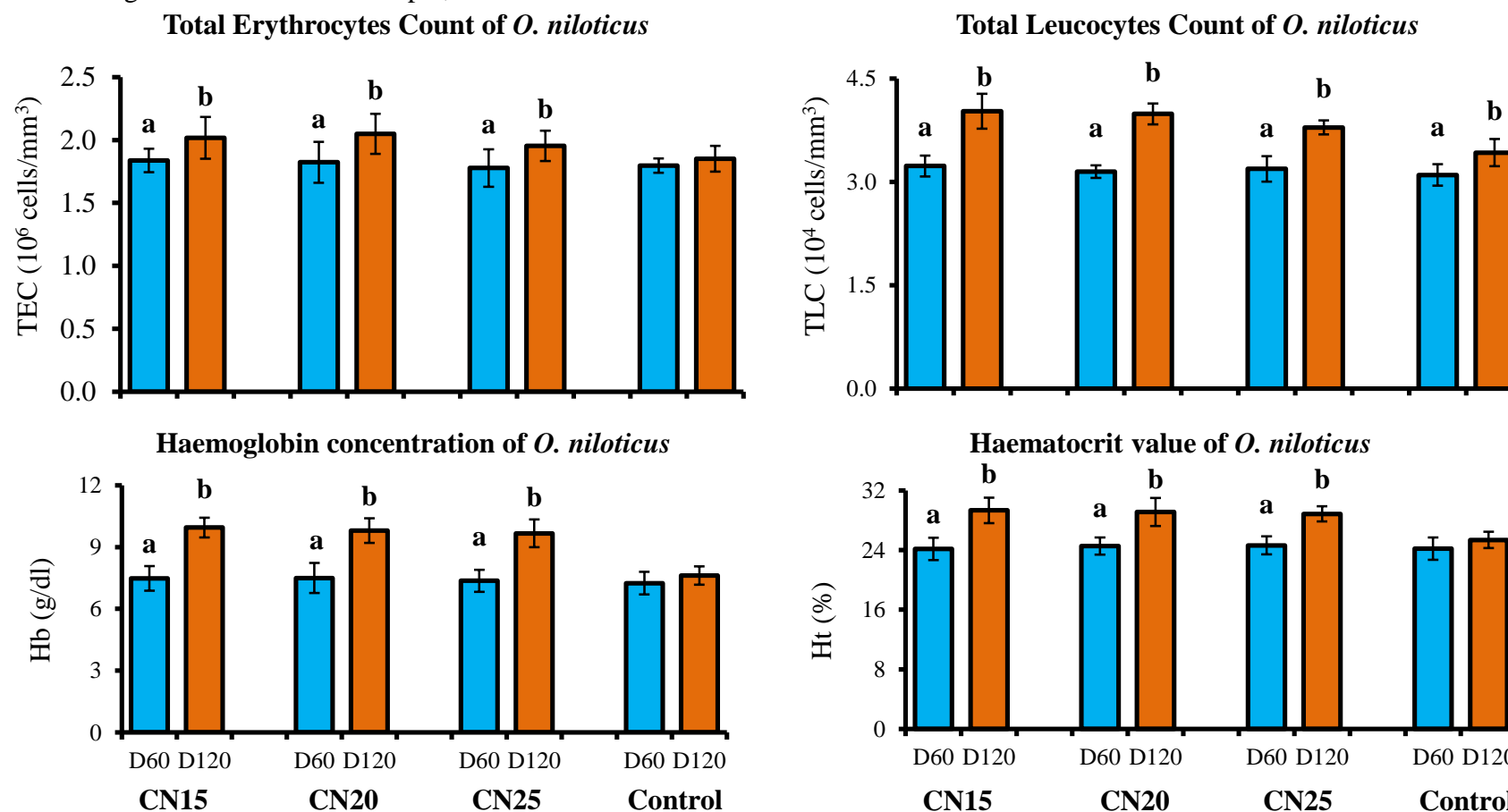
Our results were quite similar with the findings of Elayaraja *et al.* (2020) who found significant differences for the RBC, WBC, Hb and Ht in the biofloc treated Nile tilapia groups reared in different C:N ratios of 12:1, 15:1 and 20:1 compare to the control during 75 d experimental period for assessment of potentiality of jaggery as a carbon source in biofloc. Elayaraja *et al.* (2020) found superior hematological indices of Nile tilapia in C:N of 20:1 groups followed by C:N of 15:1 and 12:1 groups. Xu and Pan (2013) also observed a considerable rise in the blood indices of shrimp cultured in biofloc system.

Table 4.12. Hematological parameters measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

		CN15	CN20	CN25	Control
Total erythrocyte count (TEC) (10 ⁶ cells/mm ³)	60 th d	1.84 ± 0.09	1.82 ± 0.16	1.78 ± 0.15	1.80 ± 0.06
	120 th d	2.02 ^b ± 0.17	2.05 ^b ± 0.16	1.95 ^{ab} ± 0.12	1.85 ^a ± 0.10
Total leucocytes count (TLC) (10 ⁴ cells/mm ³)	60 th d	3.23 ± 0.15	3.15 ± 0.09	3.19 ± 0.18	3.10 ± 0.16
	120 th d	4.03 ^c ± 0.26	3.99 ^c ± 0.15	3.79 ^b ± 0.10	3.43 ^a ± 0.20
Haemoglobin (Hb) (g/dl)	60 th d	7.48 ± 0.60	7.50 ± 0.73	7.36 ± 0.54	7.25 ± 0.55
	120 th d	9.95 ^b ± 0.48	9.79 ^b ± 0.60	9.67 ^b ± 0.68	7.62 ^a ± 0.44
Haematocrit (Ht) (%)	60 th d	24.18 ± 1.50	24.54 ± 1.15	24.63 ± 1.21	24.20 ± 1.50
	120 th d	29.35 ^b ± 1.71	29.13 ^b ± 1.89	28.87 ^b ± 1.00	25.37 ^a ± 1.10

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

Fig.4.28. Haematological parameters measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d



Values are presented as Mean (\pm SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, $p < 0.05$). [D60: 60th d; D120: 120th d; CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

4.4a.5. Non-specific immunity of Nile Tilapia (*Oreochromis niloticus*):

Numbers of positive experiments had been conducted so far on fish nutrition in respect with deliberate incorporation of super complex diet for obtaining optimal growth and improving the immunological, antioxidant, and physiological status of fish (Li *et al.*, 2019). In our studies we found considerable changes ($p < 0.05$) while measured non specific immune parameters on 60th and 120th d of sampling in control and BFT treated Nile tilapia groups with different C:N ratios such as of CN15, CN20 and CN25. Significant higher ($p < 0.05$) level of respiratory burst activity, as considered by the Nitroblue tetrazolium (NBT) assay (OD_{620}) was noticed in all BFT fish groups than control fish on both 60th and 120th d, which were 0.254 ± 0.017 and 0.269 ± 0.025 , respectively in control (Table 4.13). No significant changes ($p > 0.05$) in NBT (OD_{620}) assesses were observed among BFT treated groups of CN15 (0.293 ± 0.035), CN20 (0.289 ± 0.012) and CN25 (0.294 ± 0.020) on 60th d but on 120th d significantly superior ($p < 0.05$) level of NBT (OD_{620}) in the CN15 (0.357 ± 0.027) and CN20 (0.359 ± 0.034) groups were recorded in compare to CN25 (0.324 ± 0.015) (Table 4.13). In all of the BFT treated Nile tilapia groups significant higher ($p < 0.05$) levels of NBT (OD_{620}) were measured on 120th d in contrast to the previous measurement on 60th d (Fig.4.29).

The increased values of NBT with the advancement of culture duration in biofloc were also reported in *R. rohita* (Ahmad *et al.*, 2016). Enhancement of NBT activity in biofloc treated fishes were also reported in tilapia (Haridas *et al.*, 2017 and Menaga *et al.*, 2019), rohu (Kamilya *et al.*, 2017). In this study the respiratory burst activity in terms of NBT assay of Nile tilapia in BFT system with different carbon to nitrogen ratios revealed a superior performance in CN15 and CN20 compare to the CN25 and control groups. That might be associated with the better intake of biofloc by the Nile tilapia in CN15 and CN20 groups. As a result biofloc boosted the fish nutrition and stimulated the cellular defence system by means of respiratory burst activity (Xu and Pan, 2014).

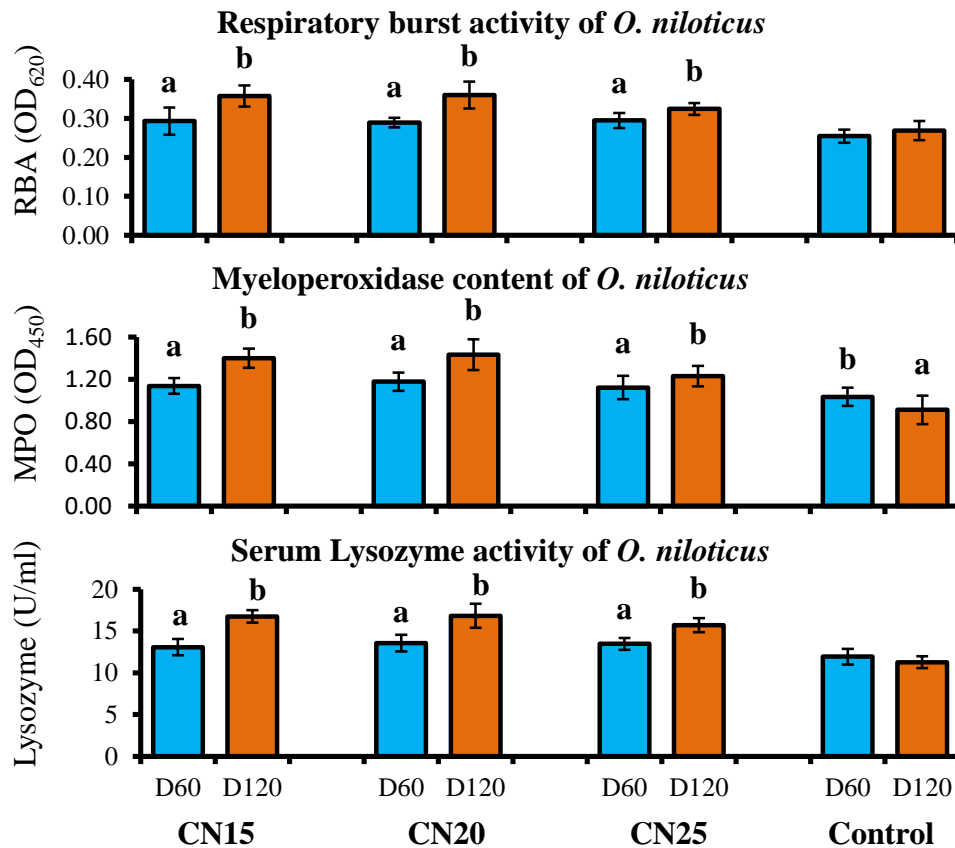
Myeloperoxidase (MPO) was an important enzyme having antimicrobial activity that played by utilizing oxidative radicals to produce hypochlorous acid. The primary azurophilic granules of neutrophils were the key storage of myeloperoxidase enzymes and it utilized the peroxide throughout respiratory burst to generate hypochlorous acid (Dalmo *et al.*, 1997). Alike, NBT assay we observed the same pattern of MPO assay (OD_{450}) in BFT treated and control Nile tilapia groups.

Table 4.13. Non specific immune parameters assessed on 60th and 120th d in control and biofloc systems with different C:N ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

		CN15	CN20	CN25	Control
Respiratory burst activity (RBA) (OD ₆₂₀)	60 D	0.293 ^b ±0.035	0.289 ^b ±0.012	0.294 ^b ±0.020	0.254 ^a ±0.017
	120 D	0.357 ^c ±0.027	0.359 ^c ±0.034	0.324 ^b ±0.015	0.269 ^a ±0.025
Serum Myeloperoxidase (MPO) (OD ₄₅₀)	60 D	1.113 ^b ±0.109	1.179 ^b ±0.086	1.122 ^b ±0.111	1.035 ^a ±0.086
	120 D	1.402 ^c ±0.091	1.433 ^c ± 0.146	1.231 ^b ±0.097	0.911 ^a ±0.134
Serum Lysozyme activity (U/ml)	60 D	13.06 ^b ±0.98	13.54 ^b ± 0.99	13.45 ^b ±0.70	11.92 ^a ±0.95
	120 D	16.74 ^c ±0.76	16.82 ^c ± 1.42	15.68 ^b ±0.85	11.26 ^a ±0.72

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

Fig.4.29. Non specific immune parameters assessed on 60th and 120th d in control and biofloc systems with different C:N ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d



Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [D60: 60th d; D120: 120th d; CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

Considerable higher ($p < 0.05$) level of MPO (OD_{450}) activities were recorded in all BFT system Nile tilapia groups compare to control on both 60th and 120th d, i.e., 1.035 ± 0.086 and 0.911 ± 0.134 , respectively (Table 4.13). No significant changes ($p > 0.05$) in MPO (OD_{450}) assay were observed among BFT treated groups CN15 (1.113 ± 0.109), CN20 (1.179 ± 0.086) and CN25 (1.122 ± 0.111) on 60th d but on 120th d noticeably superior ($p < 0.05$) level of MPO (OD_{450}) were recorded in the CN15 (1.402 ± 0.091) and CN20 (1.433 ± 0.146) compare to CN25 (1.231 ± 0.097) Nile tilapia groups (Table 4.13). In all the BFT treated Nile tilapia groups statistically higher ($p < 0.05$) levels of MPO (OD_{450}) were recorded on 120th d compare to the prior measurement on 60th d, whereas, for control group it was found reverse (Fig.4.29).

The increased values of NBT with the progress of culture period in biofloc were also reported in *R. rohita* (Ahmad *et al.*, 2016). Improvement of MPO (OD_{450}) activity in biofloc treated fishes were reported in tilapia (Haridas *et al.*, 2017 and Menaga *et al.*, 2019), rohu (Ahmad *et al.*, 2016). The MPO (OD_{450}) assay of Nile tilapia reared in BFT system with CN15 and CN20 showed better performance in compare to the CN25 and control groups. It also indicated that the immunostimulatory effects of biofloc in those aforesaid particular carbons to nitrogen ratios influenced the Nile tilapia for better immune status.

Lysozyme was one of an important factor for immune defense mechanisms of fish. The key role of lysozyme was antibacterial activity against Gram positive and to some extent against Gram negative bacteria. It was also considered an important enzyme in blood that aggressively lysed bacteria within the fish body. An elevated lysozyme activity pointed out a well natural protective system in fish (Saurabh and Sahoo, 2008). While assessing the serum lysozyme activity (U/ml) we found the same pattern like NBT and MPO assay, where the serum lysozyme activity (U/ml) were found significantly lower ($p < 0.05$) in control Nile tilapia groups on both 60th d (11.92 ± 0.95) and 120th d (11.26 ± 0.72) compare to the BFT treated fish groups (Table 4.13). The lysozyme activity of control Nile tilapia was declined on 120th d whereas it was increased in all BFT treated groups. No significant differences ($p > 0.05$) of serum lysozyme activities (U/ml) were observed on 60th d among Nile tilapia reared in different carbon to nitrogen ratio groups, i.e., CN15 (13.06 ± 0.98), CN20 (13.54 ± 0.99) and CN25 (13.45 ± 0.70) (Table 4.13). Lysozyme activities of CN15 (16.74 ± 0.76) and CN20 (16.82 ± 1.42) were recorded statistically higher ($p < 0.05$) compare to CN25 (15.68 ± 0.85) Nile tilapia groups, which corroborated with the previous studies of Liu

et al. (2018a). In the present study, augmented serum lysozyme activities were observed in CN15 and CN20 biofloc systems indicated that the non specific immune parameters were possibly influenced by the quality and composition of floc produced by jaggery as a carbon source which was consumed by Nile tilapia. Elevated lysozyme activity of tilapia in biofloc system were also supported by the previous experiments (Long *et al.*, 2015, Haridas *et al.*, 2017, Liu *et al.*, 2018a, Liu *et al.*, 2018b and Elayraja *et al.*, 2020). Alike two previously stated innate immune parameters the serum lysozyme activities were also noticed statistically higher ($p < 0.05$) on 120th d than to 60th d in all BFT treated Nile tilapia groups (Fig.4.29).

In this study the superior NBT, MPO and lysozyme activities in biofloc system with the carbon to nitrogen ratio of 15:1 and 20:1 indicated the immune stimulatory effects of the natural probiotics were existed in the biofloc, whereas the decline values in control tilapia groups might be caused by adverse water condition along with crowding stress. Biofloc system could develop the immune systems of cultured fishes but it might not be fully triggered till encountered by a pathogen (Panigrahi *et al.*, 2017). Avnimelech (2007) reported that biofloc system could trim down the pathogenic bacteria load compared to clear water condition because of the competitive action among the pathogen and heterotrophic bacteria might control the pathogens. In this study we did not observed any external lesion, scale loss, fin erosion or hemorrhagic condition in Nile tilapia reared in both biofloc system and control groups, hence no attempt were taken for isolation and identification of pathogens from diseased or moribund fishes. Mortalities in Nile tilapia reared in control tanks might be associated with crowding stress, hypoxia, compete for feed and changes in water qualities. The initial mortalities in BFT system up to 7th weeks were due to sudden elevation of total ammonia nitrogen and nitrite which was almost became nil afterwards.

4.4a.6. Serum antioxidant assay of Nile Tilapia (*Oreochromis niloticus*):

All the immune parameters were always correlated with the antioxidant potentialities of animals and various types of antioxidants were required to sustain the complex immune mechanisms of fish (Martinez-Alvarez *et al.*, 2005). The pro oxidant species like free radicals and reactive oxygen species (ROS) were constantly produced under both usual and stressful environment. Fish acquired enzymatic antioxidant defence system such as superoxide dismutase (SOD) and catalase (CAT)

to repair the damage in tissues caused by free radicals and ROS (Liu *et al.*, 2018b). The imbalance between pro-and antioxidant species was the key factors for oxidative stress in fish Tan *et al.* (2018). Antioxidant ability in fish includes both enzymatic and non-enzymatic antioxidant activities. Antioxidant enzymes constituted the first line of enzymatic defence against free radicals in organisms. SOD and CAT mainly involved in defence against lipid peroxidation inside the body and played key role for conversion of active oxygen molecules into non-toxic compounds. SOD catalyzed the dismutation of superoxide radicals to oxygen molecule and hydrogen peroxide, which was later removed by CAT to prevent lipid peroxidation (Hoseinifar *et al.*, 2021 and Shourbela *et al.*, 2021). A reduction in SOD and CAT indicated the weaker cell function and tissue injury due to accumulation of free radicals (Wang *et al.*, 2015).

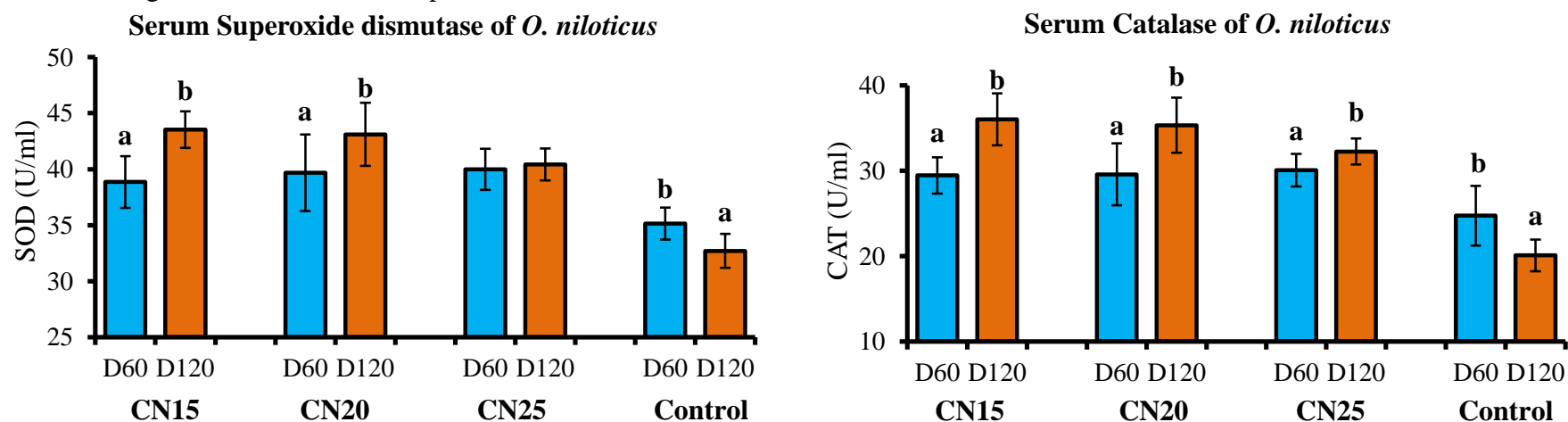
Our study confirmed that the fish reared under biofloc system with different carbon to nitrogen ratios of 15:1 (CN15), 20:1(CN20) and 25:1 (CN25) by incorporation of jaggery as carbon source exhibited an amazing significantly elevated ($p<0.05$) antioxidants capacities in terms of superoxide dismutase (SOD) and catalase (CAT) compare to the Nile tilapia maintained in control group throughout the culture (Table 4.14). The elevated level of antioxidant enzymes in the Nile tilapia reared in biofloc revealed the fish welfare and reduced oxidative stress in the BFT system. Better antioxidant properties in BFT treated Nile tilapia compare to control were also reported by Mansour and Esteban (2017), Liu *et al.* (2018b), Menaga *et al.* (2019) and Shourbela *et al.* (2021).

Among the biofloc system no significant differences ($p>0.05$) of serum SOD and serum CAT were noticed in the Nile tilapia reared in BFT system of CN15, CN20 and CN25 on 60th d. But on 120th d considerable ($p<0.05$) higher SOD (U/ml) was recorded for CN15 (43.53 ± 1.64 U/ml) and CN20 (43.11 ± 2.81 U/ml) compare to CN25 (40.42 ± 1.42 U/ml) Nile tilapia group (Table 4.14). Similarly, statistically higher CAT (U/ml) values were found on 120th d for the Nile tilapia of CN15 (36.03 ± 3.05) and CN20 (35.34 ± 3.23) compare to CN25 (32.27 ± 1.53) fish group (Table 4.14). Significant increased ($p<0.05$) level of both SOD and CAT were found in 120th d compare to 60th d in BFT treated CN15 and CN20 Nile tilapia while it was observed significantly reversed ($p<0.05$) for control Nile tilapia (Fig.4.30).

Table 4.14. Serum antioxidant assay measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

		CN15	CN20	CN25	Control
Superoxide dismutase (SOD) (U/ml)	60 th d	38.86 ^b ± 2.30	39.69 ^b ± 3.42	39.99 ^b ± 1.83	35.15 ^a ± 1.42
	120 th d	43.53 ^c ± 1.64	43.11 ^c ± 2.81	40.42 ^b ± 1.42	32.70 ^a ± 1.52
Catalase (CAT) (U/ml)	60 th d	29.47 ^b ± 2.12	29.59 ^b ± 3.63	30.06 ^b ± 1.91	24.75 ^a ± 3.49
	120 th d	36.03 ^c ± 3.05	35.34 ^c ± 3.23	32.27 ^b ± 1.53	20.10 ^a ± 1.86

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1.]

Fig.4.30. Serum antioxidant assay measured on 60th and 120th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Nile Tilapia, *Oreochromis niloticus* for 120 d

Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [D60: 60th d; D120: 120th d; CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1]

Our observation regarding the effects of different carbon to nitrogen ratios on the serum antioxidants level in Nile tilapia reared in BFT system was supported by the previous experiments of Elayraja *et al.* (2020) and Liu *et al.* (2018a) where considerable changes were noticed in antioxidant properties with alteration of carbon nitrogen ratios. Liu *et al.* (2018a) observed significant increase superoxide dismutase level in Nile tilapia reared in BFT system with C:N ratio of 15:1 followed by C:N ratios of 10:1 and 20:1. Whereas, Elayraja *et al.* (2020) observed significant higher antioxidant activities in Nile tilapia reared in BFT system with C:N ratio of 20:1 followed by 15:1 and 12:1. Our results were found contradictory with the findings of Dilmi *et al.* (2021) where no significant differences of SOD and CAT were observed in Nile Tilapia cultured in BFT system with C:N ratios of 14:1, 17:1, 20:1 and control.

4.4b. Welfare parameters of Singhi (*Heteropneustes fossilis*):

Similarly during 180 d culture of *Heteropneustes fossilis* or Asian stinging catfish or Singhi in jaggery added biofloc system with different carbon to nitrogen (C:N) ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16) along with control (without addition of jaggery), all the fish welfare parameters alike our previous experiment on Nile tilapia were measured from the fish blood and serum samples collected on mid of the culture i.e., 90th d and on the date of harvesting i.e., 180th d. All the welfare parameters results of Singhi were mentioned in the Table 4.15 to Table 4.20 regarding analysis of variance (ANOVA, $p < 0.05$) for each parameter between control and experimental groups for a particular sampling date. Whereas, the statistical comparison (Students' t-test, $p < 0.05$) in between two separate sampling date i.e., 90th and 180th d, respectively, of individual parameter for both control and BFT treated Singhi groups were expressed graphically in the Fig.4.31 to Fig.4.36.

4.4b.1. Serum biomarkers for stress of Singhi (*Heteropneustes fossilis*):

Amplification of glycogenolysis and the synthesis of extra glucose from hepatic tissue proteins followed by aggravation of the glucose content in blood by amino acids were considered as stress indicator in animals (Almeida *et al.*, 2001). Along with a general response to stress the glucose content was also considered as an index of mobilization of metabolism to generate an additional energy provides (Bonga, 1997). BFT system were able to reduce the physiological stress in *H. fossilis* in our studies agreed with the other studies where reduced levels of glucose level were

reported in fishes reared in biofloc system than to the control, like rohu (Ahmad *et al.*, 2016), tilapia (Menaga *et al.*, 2019).

The serum glucose content of Singhi or *Heteropneustes fossilis* in control were measured significantly high ($p < 0.05$) on both 90th d and 180th d i.e., 84.59 ± 3.65 and 114.16 ± 3.71 mg/dl, respectively in compare to the all BFT treated CN8, CN12 and CN16 Singhi groups. Among BFT systems the CN16 group showed significantly greater ($p < 0.05$) level of serum glucose 78.75 ± 2.95 mg/dl and 95.38 ± 4.95 mg/dl on 90th and 180th d, respectively in compare to Singhi groups of CN12 and CN8. The lowest glucose level of 75.06 ± 3.35 mg/dl and 86.90 ± 2.65 mg/dl were recorded in CN12 Singhi groups on 90th and 180th d, respectively (Table 4.15). The serum glucose level increased significantly ($p < 0.05$) from mid (90th d) to end date (180th d) of culture period for all the BFT treated and control Singhi groups might be associated with increased biomasses and water quality issues (Fig.4.31). No previous studies on serum biochemistry of biofloc reared Singhi were reported however this study were supported by the positive effects of biofloc system on other aquatic species like rohu (Ahmad *et al.*, 2016), tilapia (Liu *et al.*, 1018a and Menaga *et al.*, 2019).

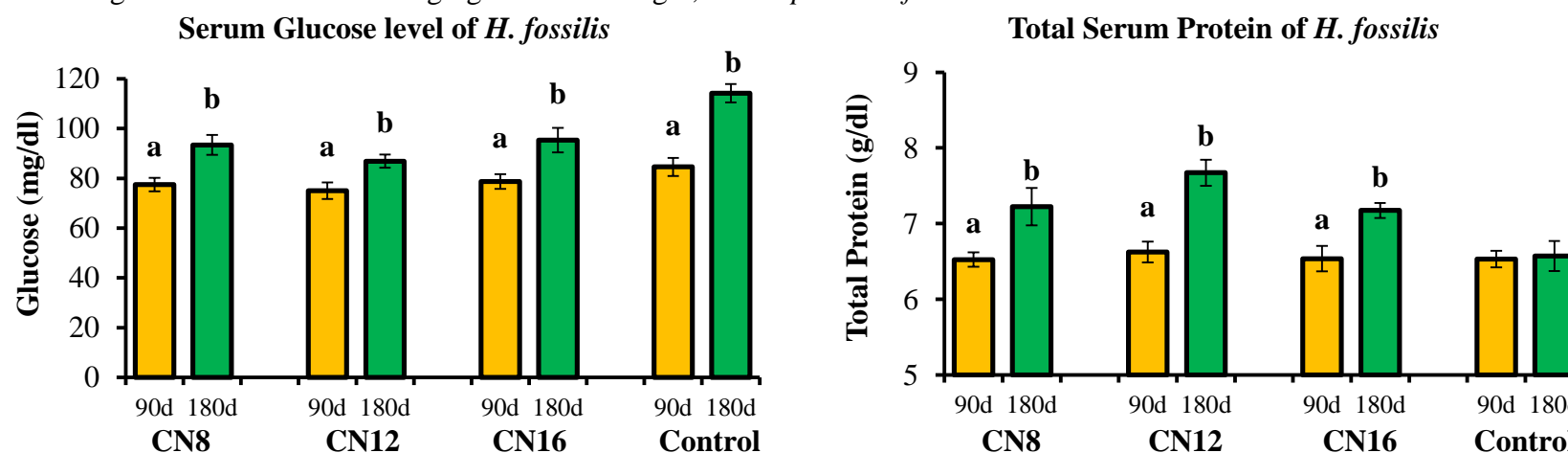
In this study, the possible reason for higher significant ($p < 0.05$) glucose level in the *H. fossilis* of CN16 biofloc group in compare to CN12 and CN8 were might be due to the environmental stressors like higher level of TSS, biological oxygen demand or too much floc volume. Our study also revealed that increased biomass along with culture duration in biofloc also produced higher significant ($p < 0.05$) glucose level on 180th d compare to 90th d in Singhi reared in both biofloc and control groups (Fig.4.31). The increased patterns of glucose levels in Singhi in this study were found to be size and age dependent as described by Laizcarrion *et al.* (2012). The results in this study indicated that the biofloc system under minimal water exchange did not cause much stress-related problems to the *H. fossilis* even at stocking densities up to 467 nos./m³.

Total serum protein was used to examine the welfare issues related with liver and kidney function (Banaee, 2013). Total serum protein level of all the BFT treated and control Singhi or *Heteropneustes fossilis* groups were found not significantly ($p > 0.05$) different on 90th d but considerable differences ($p < 0.05$) were noticed on 180th d.

Table 4.15. Serum biomarkers for stress measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d

		CN8	CN12	CN16	Control
Serum Glucose (mg/dl)	90 th d	77.50 ^{ab} ± 2.69	75.06 ^a ± 3.35	78.75 ^b ± 2.95	84.59 ^c ± 3.65
	180 th d	93.43 ^b ± 3.99	86.90 ^a ± 2.65	95.38 ^b ± 4.95	114.16 ^c ± 3.71
Total Protein (g/dl)	90 th d	6.52 ± 0.09	6.62 ± 0.14	6.54 ± 0.17	6.53 ± 0.11
	180 th d	7.22 ^b ± 0.25	7.67 ^c ± 0.17	7.17 ^b ± 0.10	6.57 ^a ± 0.20

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1.]

Fig.4.31. Serum biomarkers for stress measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi, *Heteropneustes fossilis* for 180 d

Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [90d: 90th d; 180d: 180th d]

Higher serum protein level of 7.67 ± 0.17 g/dl was recorded in CN12 group compare to CN8 (7.22 ± 0.25 g/dl), CN16 (7.17 ± 0.10 g/dl) which were again significantly higher ($p < 0.05$) than control (6.57 ± 0.20 g/dl) Singhi group (Table 4.15). The higher significant ($p < 0.05$) level of serum protein level on 180th d in BFT groups of CN12, CN8 and CN16 (Table 4.15) helped to reduce the dietary protein levels of the supplementary feed with the improvement of the nonspecific immune response (Rao *et al.*, 2006). The serum protein level of all BFT treated groups significantly increased ($p < 0.05$) on 180th d compare to the 90th d indicated that in spite of higher glucose level matured fishes were able to achieve its normal trends of higher total serum protein level simultaneously, while it was not observed in control (Fig.4.31).

The lower total serum protein content in the control Singhi group compare to the BFT treated Singhi groups at the end of 180 d culture period, in the present study might be due to conversion of structural proteins to filled up the higher energy demands on exposure to stress for accumulation of higher nitrogenous toxic substances. The reduction of total serum protein levels indicated the shortage of synthesis and loss of plasma proteins (hypoproteinemia) as described by Hedayati *et al.* (2011) in yellowfin sea bream, *Acanthopagrus latus*.

4.4b.2. Serum biomarkers for liver of Singhi (*Heteropneustes fossilis*):

Alanine Transaminase (ALT) and Aspartate Transaminase (AST) content in serum could be used as prominent indicators of hepatopancreas function and damage (Zhou *et al.*, 2014), and also acted as a stress indicators (Haridas *et al.*, 2017). Elevated concentration of those enzymes in fishes indicated a raise in aspartate and alginate through utilization of sugar to produce the glucose for adjusting and countering the stress status (Gowda *et al.*, 2009). Alteration in Alkaline phosphatase (ALP) level was a physiological response associated with immune responses and could be considered as an indicator of fish health (Liu *et al.*, 2014). A reduce level of this enzyme indicated improved immune system (Khanjani *et al.*, 2021).

Serum ALT, AST and ALP of the control Singhi group were found significantly higher ($p < 0.05$) compare to the biofloc treatment Singhi groups of CN8, CN16 and CN16 on 180th d. Serum AST and ALP of control Singhi groups measured on 90th d also showed significantly higher ($p < 0.05$) level than BFT treated Singhi groups except the CN16 group. Serum ALT, AST and ALP did not differs significantly ($p > 0.05$) among BFT treated groups on 90th d but considerable greater

($p < 0.05$) levels were recorded for CN16 group than CN8 and CN12 groups on 180th d of sampling. Overall lowest level of serum ALT, AST and ALP were noticed in CN12 group throughout the experiment had pointed out the better liver condition compare to others BFT treated groups (Table 4.16). Significant increases ($p < 0.05$) of serum ALT, AST and ALP were observed in all BFT treated and control Singhi groups on 180th d compare to the 90th d were associated with the higher stress level at the end of culture that might affect the permeability of hepatic cell membranes followed by leakage of AST, ALT in blood stream (Fig.4.32).

The lowest serum ALT, AST and ALP was noticed in CN12 group throughout the experiment (Table 4.16). Our experiment showed that the biofloc could effectively reduce the damage of hepatocytes in Singhi by reducing the ALP, AST and ALP level in biofloc culture system. It was also revealed from this study that maintenance of biofloc system with C:N ratio of 12:1 by addition of jaggery as carbon source could produce better controls on the activity of those said hepatic enzymes in Singhi in compare to the other BFT treated CN8 and CN16 Singhi groups. That might be due to the positive and optimal beneficial effects of produced biofloc on the liver function of Singhi in CN12 biofloc treatment group.

4.4b.3. Serum biomarkers for ionic balance of Singhi (*Heteropneustes fossilis*):

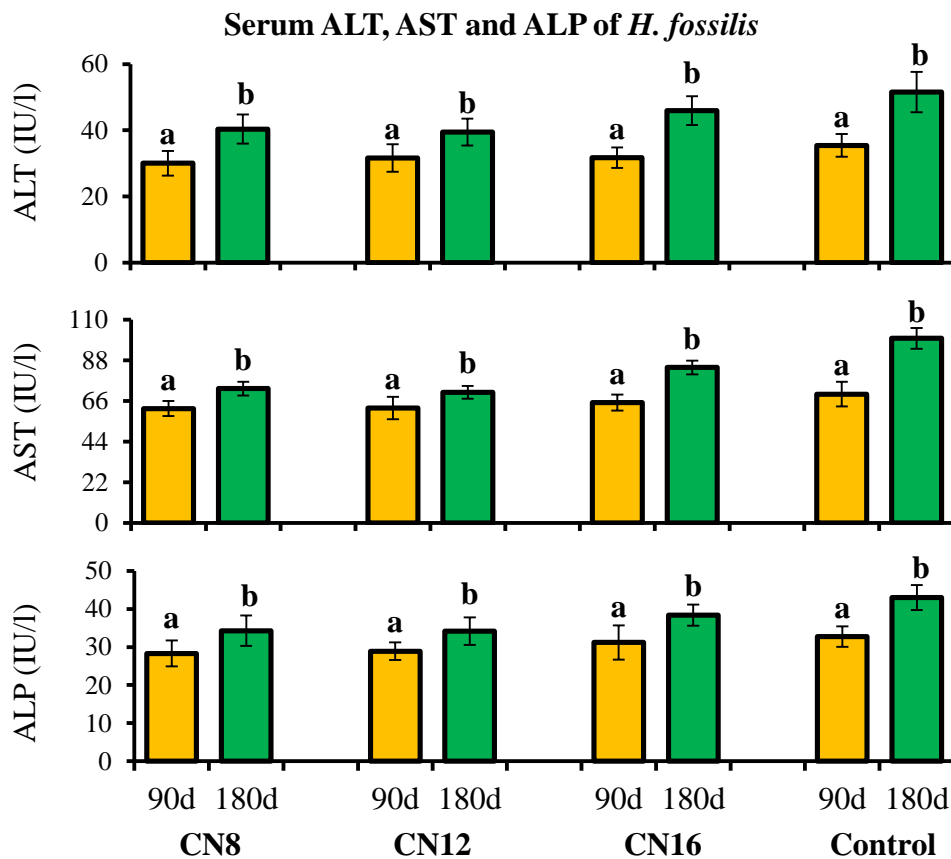
In vertebrates, calcium played an essential function in a variety of biological activities (Srivastav *et al.*, 2008) and serum chloride level was often measured to detect few health conditions like kidney and liver diseases (Handyani *et al.*, 2020). The chloride (Cl^-) ions in the body fluid also played a critical role for osmoregulation (Kaneko *et al.*, 2008). Till now no information regarding the impact of biofloc on blood electrolytes of Singhi in high stocking density BFT system had been reported. In this study no observation of significant ($p > 0.05$) differences for serum calcium and chloride levels among different BFT treatment and control Singhi groups measured on both 90th and 180th d, indicated Singhi could adapt the biofloc system (Table 4.17). The same time the both serum chloride and calcium level was found lower but not significant ($p < 0.05$) on 180th d than 90th d in all BFT treated and control Singhi groups might be due to stress resulted by increased biomass (Fig.4.33).

Table 4.16. Serum biomarkers for liver measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d

			CN8	CN12	CN16	Control
Serum ALT (IU/l)	90 th d		30.02 ^a ±3.71	31.64 ^a ±4.16	31.72 ^a ±3.13	35.41 ^b ±3.44
	180 th d		40.32 ^a ±4.40	39.44 ^a ±4.08	45.91 ^b ±4.36	51.53 ^c ±6.08
Serum AST (IU/l)	90 th d		61.94 ^a ±4.02	62.26 ^a ±6.09	65.20 ^{ab} ±4.32	69.69 ^b ±6.66
	180 th d		72.73 ^a ±3.73	70.73 ^a ±3.42	84.18 ^b ±3.73	99.93 ^c ±5.60
Serum ALP (IU/l)	90 th d		28.32 ^a ±3.42	28.90 ^a ±2.31	31.19 ^{ab} ±4.47	32.73 ^b ±2.67
	180 th d		34.28 ^a ±3.98	34.17 ^a ±3.65	38.42 ^b ±2.78	43.02 ^c ±3.28

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1.]

Fig.4.32. Serum biomarkers for liver measured on 90th and 180th d in control and biofloc systems with different C:N ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d



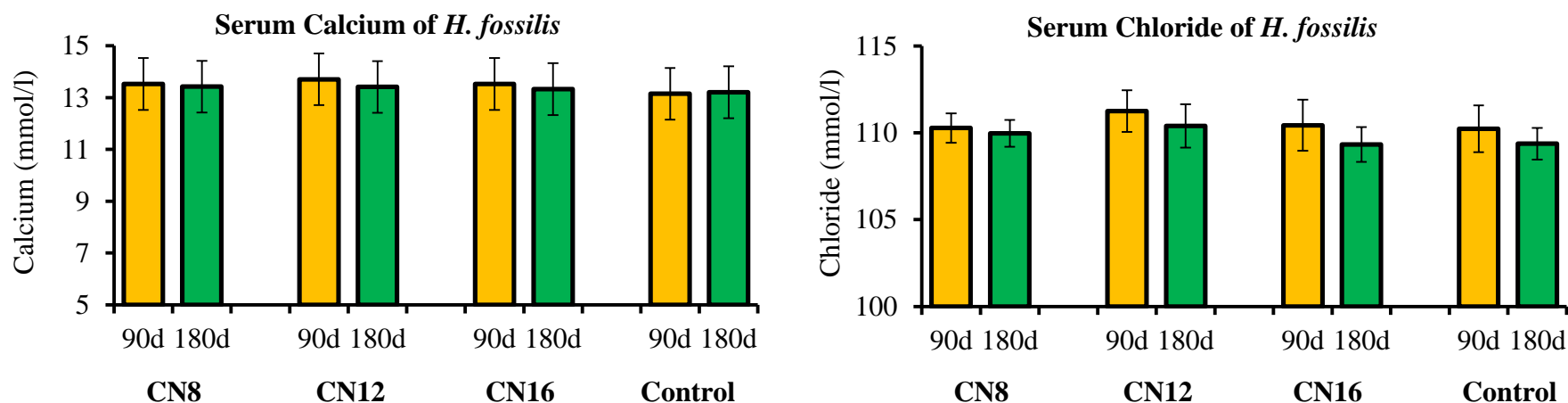
Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [90d: 90th d; 180d: 180th d]

Table 4.17. Serum biomarkers for ionic balance measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d

		CN8	CN12	CN16	Control
Serum Calcium (m mol/l)	90 th d	13.52 ± 0.71	13.71 ± 0.72	13.53 ± 0.80	13.15 ± 0.72
	180 th d	13.42 ± 0.83	13.41 ± 0.57	13.53 ± 0.50	13.21 ± 0.71
Serum Chloride (m mol/l)	90 th d	110.28 ± 0.84	111.26 ± 1.20	110.44 ± 1.47	110.24 ± 1.34
	180 th d	109.97 ± 0.77	110.40 ± 1.25	109.33 ± 1.01	109.38 ± 0.92

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1]

Fig.4.33. Serum biomarkers for ionic balance measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d



Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [90d: 90th d; 180d: 180th d; CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1]

4.4b.4. Hematological status of Singhi (*Heteropneustes fossilis*):

Hematological status of fish was the basic way to assess the health condition of fish (Ismail *et al.*, 2019). Erythrocytes (RBCs) were the markers of diverse types of stress and the higher total erythrocytes concentration in the fish blood could increase the availability of oxygen inside the fish tissues (Rehulka *et al.*, 2002). In addition, leukocytes (WBCs) were the immune cells that concerned about non specific immunity in fish and the profusion of leukocytes directly indicated the health condition in fish (Guardiola *et al.*, 2019).

During the hematological assessment like Total erythrocyte count (TEC), Total leucocytes count (TLC), Hemoglobin (Hb) and Hematocrit (Ht) in blood samples collected from *H. fossilis* on 90th and 180th d, higher significant ($p < 0.05$) values of TEC ($2.54 \pm 0.29 \times 10^6$ cells/mm³), TLC ($1.60 \pm 0.26 \times 10^4$ cells/mm³), Hb (13.36 ± 0.98 g/dl) and Ht (36.11 ± 2.65 %) were recorded in CN12 Singhi compare to the control and other BFT treated CN8 and CN16 groups on 180th d only, except the Hb (12.16 ± 1.71 g/dl) level of CN16 on 180th d (Table 4.18). Any other statistically significant ($p < 0.05$) changes in haematological parameters were not found between control and other biofloc treatment CN8 and CN16 groups, though a non significant ($p > 0.05$) lower haematological properties were observed in control Singhi groups compare to BFT treated CN8 and CN16 Singhi groups throughout the experiment (Table 4.18).

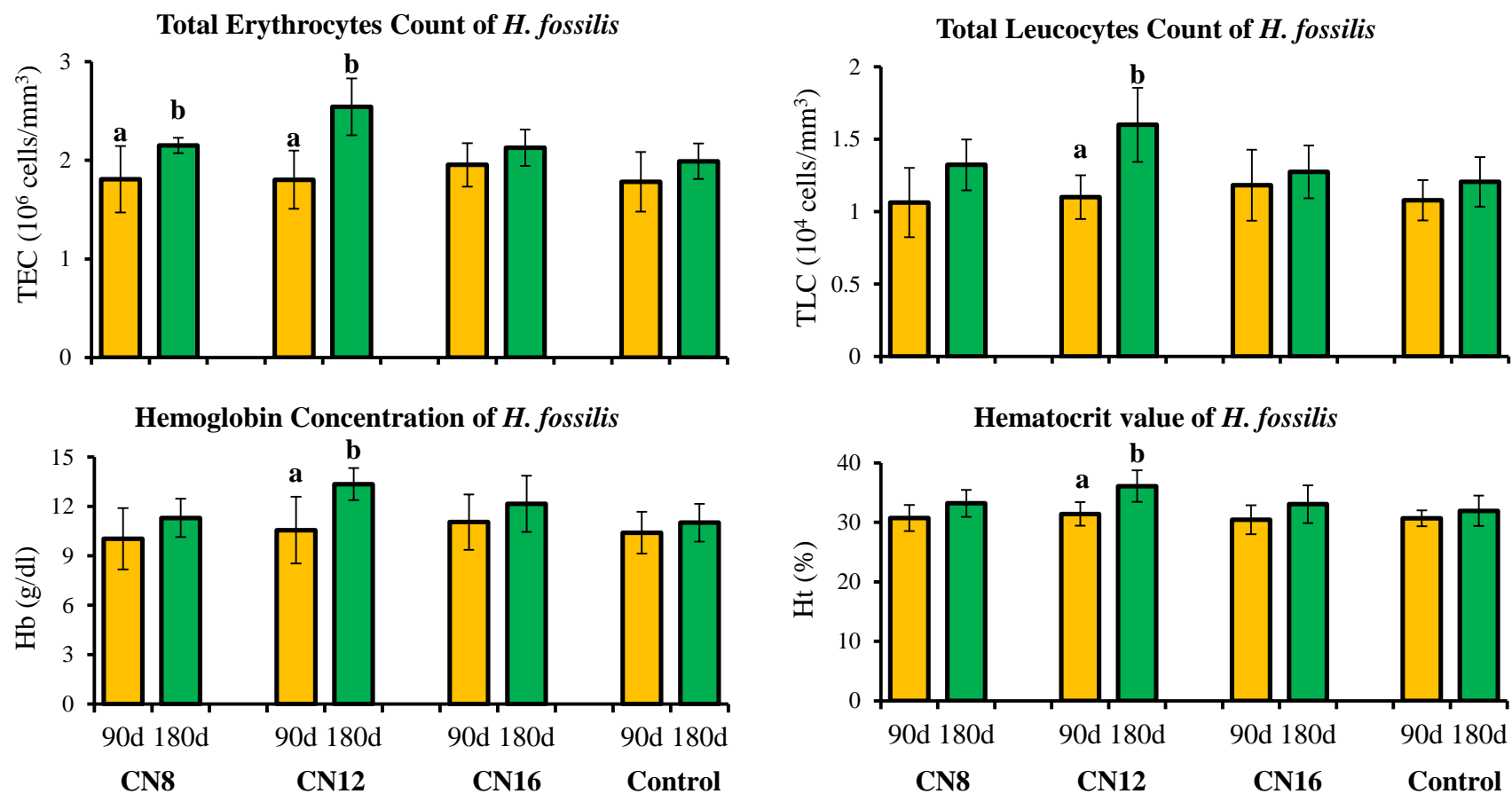
Our findings corroborated with the previous experiments of Zafar *et al.* (2021) where significant higher ($p < 0.05$) hematological properties were recorded in *H. fossilis* reared in biofloc system for 120 d with C:N ratio of 10:1. In previous studies Azim and Little (2008) and Long *et al.* (2015) did not found any noticeable effects on haematological properties in the tilapia reared in biofloc. Hematological indices of *H. fossilis* of CN12 were found significantly superior ($p < 0.05$) on 180th d compare to 60th d indicated better health in higher biomasses compare to the control and other BFT groups (Fig.4.34). The possible reasons for such probably due to development of comparatively favourable aquatic condition in CN12 biofloc tanks for *H. fossilis* which were discussed in section 4.6.

Table 4.18. Hematological parameters measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d

		CN8	CN12	CN16	Control
Total erythrocyte count (TEC) (10 ⁶ cells/mm ³)	90 th d	1.81 ± 0.34	1.80 ± 0.29	1.95 ± 0.22	1.78 ± 0.30
	180 th d	2.15 ^a ± 0.08	2.54 ^b ± 0.29	2.13 ^a ± 0.18	1.99 ^a ± 0.18
Total leucocytes count (TLC) (10 ⁴ cells/mm ³)	90 th d	1.06 ± 0.24	1.10 ± 0.15	1.18 ± 0.24	1.08 ± 0.14
	180 th d	1.32 ^a ± 0.18	1.60 ^b ± 0.26	1.28 ^a ± 0.18	1.21 ^a ± 0.17
Hemoglobin (Hb) (g/dl)	90 th d	10.04 ± 1.87	10.56 ± 2.02	11.05 ± 1.69	10.41 ± 1.27
	180 th d	11.30 ^a ± 1.16	13.36 ^b ± 0.98	12.16 ^{ab} ± 1.71	11.02 ^a ± 1.14
Hematocrit (Ht) (%)	90 th d	30.73 ± 2.19	31.43 ± 1.96	30.46 ± 2.44	30.67 ± 1.34
	180 th d	33.20 ^a ± 2.27	36.11 ^b ± 2.65	33.07 ^a ± 3.17	31.95 ^a ± 2.54

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1.]

Fig.4.34. Hematological parameters measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d



Values are presented as Mean (\pm SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, $p < 0.05$). [90d: 90th d; 180d: 180th d; CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1]

4.4b.5. Non-specific immunity of Singhi (*Heteropneustes fossilis*):

Assessment of innate immune status of *H. fossilis* reared in biofloc treatment with different carbon to nitrogen ratio of 8:1 (CN8), 12:1 (CN12) and 16:1 (16:1) and control water were also made with blood and serum samples collected on 90th and 180th d. In this study we found considerable changes ($p < 0.05$) in non specific immune parameters measured on 90th and 180th d among control and BFT treated Nile tilapia with different C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16). The Respiratory burst activity (RBA) in terms of NBT assay (OD_{620}) showed higher significant ($p < 0.05$) value on 90th and 180th d i.e., 0.304 ± 0.028 and 0.329 ± 0.013 , respectively for Singhi of CN12 group than control and other BFT treated CN8 and CN16 groups. The NBT assay (OD_{620}) of CN8 and CN16 were also found significantly higher ($p < 0.05$) on 180th d, while it was not significantly ($p > 0.05$) higher on 90th d than control Singhi (Table 4.19). Only BFT treated CN12 group showed significantly higher ($p < 0.05$) gain of RBA on 180th d than 90th d whereas, it was not found statistically higher ($p > 0.05$) for other BFT treated and control Singhi groups (Fig.4.35).

The increased values of NBT with the advancement of culture duration in biofloc were also reported in *R. rohita* (Ahmad *et al.*, 2016). Enhancement of NBT activity in biofloc treated fishes were also reported in tilapia (Haridas *et al.*, 2017 and Menaga *et al.*, 2019), rohu (Kamilya *et al.*, 2017). In our study the respiratory burst activity in terms of NBT assay of Singhi in BFT system with different carbon to nitrogen ratio revealed a superior performance in CN12 compare to the CN8, CN16 and control groups. That might be associated with the better intake of biofloc by the *H. fossilis* in CN12 group. As a result biofloc boosted the fish nutrition and stimulated the cellular defence system of cultured fish by means of respiratory burst activity (Xu and Pan, 2014).

Myeloperoxidase (MPO) was an important enzyme having antimicrobial activity that played by utilizing oxidative radicals to produce hypochlorous acid. The primary azurophilic granules of neutrophils were the key storage of myeloperoxidase enzymes and it utilized the peroxide throughout respiratory burst to generate hypochlorous acid (Dalmo *et al.*, 1997). The Serum Myeloperoxidase (MPO) activities (OD_{450}) of BFT treated CN12 on 90th and 180th d i.e., 1.116 ± 0.066 and 1.314 ± 0.126 , respectively were found significantly higher ($p < 0.05$) form other BFT and control groups, except non significantly ($p > 0.05$) higher than CN16 Singhi group

on 90th d. The remaining BFT treated CN8 and CN16 fish groups also showed higher MPO values compare to control but that were found statistically superior ($p < 0.05$) on 180th d only. No perceptible difference between CN8 and CN16 were noticed in terms of MPO assay (Table 4.19). The BFT treated CN12 and CN8 Singhi groups exhibited statistically higher ($p < 0.05$) levels of MPO (OD_{450}) on 180th d in compare to the prior measurement on 90th d (Fig.4.35). The increased values of NBT with the progress of culture period in biofloc were also reported in *R. rohita* (Ahmad *et al.*, 2016). Improvement of MPO (OD_{450}) activity in biofloc treated fishes were reported in tilapia (Menaga *et al.*, 2019), rohu (Ahmad *et al.*, 2016). The MPO (OD_{450}) assay of Nile tilapia reared in CN12 showed better performance in compare to the CN8 and CN16 and control groups. It also indicated that the immunostimulatory effects of biofloc in carbon to nitrogen ratio of 12:1 could influence the Singhi for better immune status.

Lysozyme was one of an important factor for immune defense mechanisms of fish. It was also considered an important enzyme in blood that aggressively lysed bacteria within the fish body. An elevated lysozyme activity pointed out a well natural protective system in fish (Saurabh and Sahoo, 2008). While assessing the serum lysozyme activity (U/ml) of BFT treated and control Singhi on 90th and 180th d, we found significantly elevated ($p < 0.05$) lysozyme activity (U/ml) of 15.61 ± 0.61 U/ml and 17.44 ± 1.44 U/ml in BFT treated CN12 group on both 90th and 180th d, respectively, compare to control and other BFT treated CN8 and CN16 Singhi groups. The difference of lysozyme activity in between the BFT treated CN8 and CN16 and control groups were found statistically insignificant ($p > 0.05$) except the result of CN16 on 180th d which was found significantly higher ($p < 0.05$) than control but not from CN8 groups (Table 4.19).

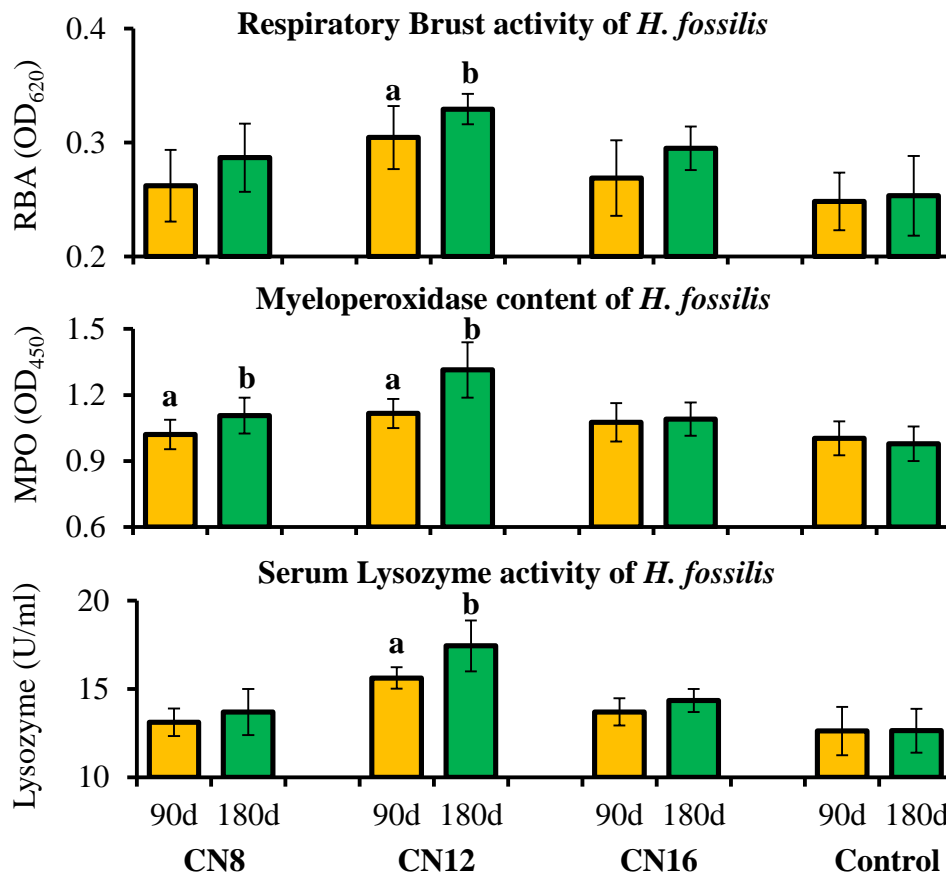
Alike two previously stated innate immune parameters the serum lysozyme activities were also noticed statistically higher ($p < 0.05$) on 180th d than to 90th d in BFT treated CN12 Singhi group (Fig.4.35). Augmentation of serum lysozyme activity observed in biofloc system with C:N of 12:1 indicated that the innate immunity was possibly influenced by the quality and composition of floc produced by jaggery as a carbon source in that particular carbon to nitrogen ratio which was consumed by Singhi. Elevated lysozyme activities of fish in biofloc culture were also supported by the previous experiments on other fishes (Liu *et al.*, 2018a; Liu *et al.*, 2018b and Elayraja *et al.*, 2020).

Table 4.19. Non specific immune parameters assessed on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d

		CN8	CN12	CN16	Control
Respiratory burst activity (RBA) (OD ₆₂₀)	90 d	0.262 ^a ±0.031	0.304 ^b ±0.028	0.269 ^a ±0.033	0.244 ^a ±0.029
	180 d	0.287 ^b ±0.030	0.329 ^c ±0.013	0.295 ^b ±0.019	0.253 ^a ±0.035
Serum Myeloperoxidase (OD ₄₅₀)	90 d	1.020 ^a ±0.067	1.116 ^b ±0.066	1.076 ^{ab} ±0.087	1.003 ^a ±0.077
	180 d	1.106 ^b ±0.082	1.314 ^c ±0.126	1.090 ^b ±0.042	0.978 ^a ±0.079
Serum Lysozyme activity (U/ml)	90 d	13.11 ^a ±0.77	15.61 ^b ±0.61	13.69 ^a ±0.77	12.61 ^a ±1.37
	180 d	13.69 ^{ab} ±1.30	17.44 ^c ±1.44	14.34 ^b ±0.65	12.63 ^a ±1.25

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N ratios of 8:1, 12:1 and 16:1]

Fig.4.35. Non specific immune parameters assessed on 90th and 180th d in control and biofloc systems with different C:N ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d



Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [90d: 90th d; 180d: 180th d; CN8, CN12 and CN16: Biofloc groups with different C:N ratios of 8:1, 12:1 and 16:1.]

Effects of biofloc system on innate immunity properties of *H. fossilis* were not reported yet. In this study the superior NBT, MPO and lysozyme activities in biofloc system with the carbon to nitrogen ratio of 12:1 indicated the immune stimulatory effects of the optimal natural probiotics present in the biofloc in that particular carbon to nitrogen ratio in contrast with lower (8:1) and higher (16:1) carbon to nitrogen ratios, whereas the decline values in control tilapia groups might be caused by adverse water condition along with crowding stress.

In this study we did not observed any external lesion, fin erosion or hemorrhagic condition in *H. fossilis* reared in both biofloc system and control groups, hence no attempt were taken for isolation and identification of pathogens from diseased or moribund fish. All the mortalities in treatment and control tanks might be associated with crowding stress, hypoxia and/or alteration of water qualities.

4.4b.6. Serum antioxidant assay of Singhi (*Heteropneustes fossilis*):

All the immune parameters were always linked with the antioxidant potentialities of animals and various types of antioxidants played crucial role to sustain the complex immune mechanisms of fish (Martinez-Alvarez *et al.*, 2005). The free radicals and reactive oxygen species (ROS) were constantly produced under both usual and stressful environment, hence fishes had to acquire enzymatic antioxidant defence systems like superoxide dismutase (SOD) and catalase (CAT) to repair the damage in tissues caused by free radicals and ROS (Liu *et al.*, 2018b). The imbalance between pro and antioxidant species was the key factors for oxidative stress in fish Tan *et al.* (2018). SOD and CAT were mainly involved in defence against lipid peroxidation inside the body (Hoseinifar *et al.*, 2021 and Shourbela *et al.*, 2021). A reduction in SOD and CAT indicated the weaker cell function and tissue injury due to accumulation of free radicals (Wang *et al.*, 2015).

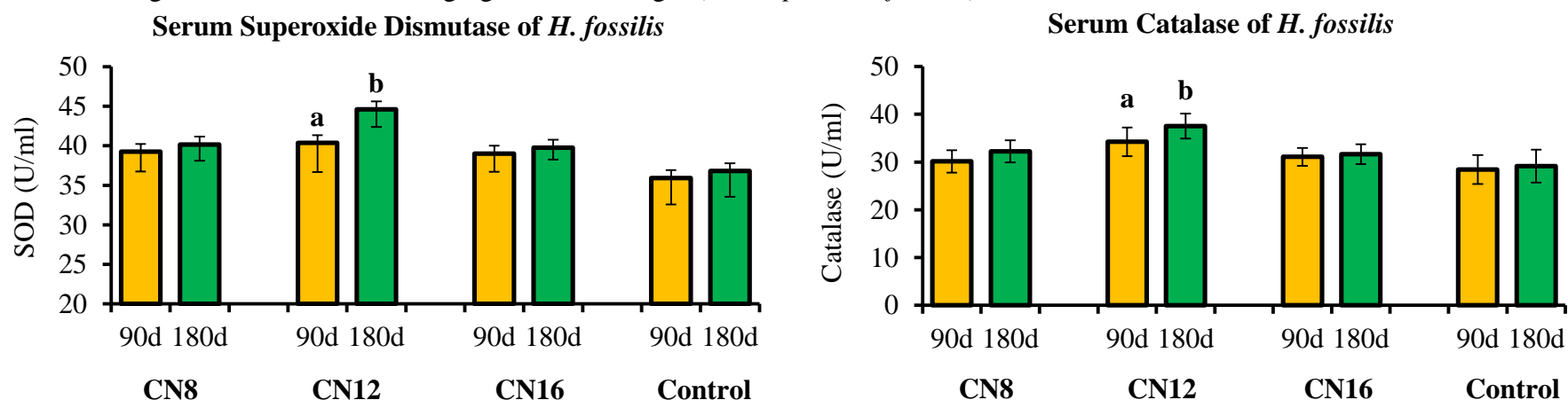
Effects of biofloc treatments on antioxidant properties of *H. fossilis* had not been published yet. Our study confirmed that the fish reared under biofloc system with different carbon to nitrogen ratio of 8:1 (CN8), 12:1(CN12) and 16:1 (CN16) by incorporation of jaggery as carbon source exhibited considerable higher ($p<0.05$) antioxidants capacities in terms of superoxide dismutase (SOD) and catalase (CAT) compare to the Singhi maintained in control groups throughout the culture (Table 4.20).

Table 4.20. Serum antioxidant assay measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d

		CN15	CN20	CN25	Control
Superoxide dismutase (SOD) (U/ml)	90 th d	39.24 ^b ± 2.49	40.35 ^b ± 3.68	39.02 ^b ± 2.32	35.92 ^a ± 3.35
	180 th d	40.15 ^b ± 2.06	44.61 ^c ± 2.22	39.77 ^b ± 1.53	36.80 ^a ± 3.24
Catalase (CAT) (U/ml)	90 th d	30.13 ^b ± 2.35	34.22 ^b ± 3.03	31.08 ^b ± 1.88	28.45 ^a ± 3.03
	180 th d	32.24 ^b ± 2.32	37.54 ^c ± 2.62	31.65 ^{ab} ± 2.08	29.15 ^a ± 3.44

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (ANOVA, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1.]

Fig.4.36. Serum antioxidant assay measured on 90th and 180th d in control and biofloc systems with different carbon to nitrogen (C:N) ratios during the culture of Asian stinging catfish or Singhi (*Heteropneustes fossilis*) for 180 d



Values are presented as Mean (± SD, n=9) in the same treatment group with different superscripts differ significantly between two sampling date (Students' t-test, p<0.05). [90d: 90th d; 180d: 180th d; CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1.]

Significantly higher ($p < 0.05$) serum antioxidant activities in terms of superoxide dismutase (SOD) and catalase (CAT) were found in BFT treated groups in compare to the control groups strongly proved that the jaggery as carbon source could be effective for biofloc culture of Singhi as the antioxidants played a crucial role to maintain the complex immune mechanisms of fish (Martinez-Alvarez *et al.*, 2005).

The BFT system of CN12 group showed the considerable superior ($p < 0.05$) level of SOD (44.61 ± 2.22 U/ml) and CAT (37.54 ± 2.62 U/ml) on 180th d compare to the control and other BFT treated CN8 and CN16 fish groups, while no significant differences ($p > 0.05$) of SOD and CAT were noticed between all BFT treated groups on 90th d. The CN8 and CN16 biofloc treatment groups also showed significantly higher ($p < 0.05$) level of serum SOD and CAT activities than control on both 90th and 180th d, except the CAT values of CN16 on 180th d, which was not found significantly higher ($p > 0.05$) than control Singhi group (Table 4.20).

Significantly higher ($p < 0.05$) gain of SOD and CAT activities on 180th d compare to 90th d was observed for only BFT treated CN12 group (Fig.4.36). It might be possible due to optimal benefit of biofloc system was gained in C:N ratio of 12:1 than other two C:N ratios in this study. Our observation regarding the effects of different carbon to nitrogen ratios on the serum antioxidants level in Singhi reared in BFT system was supported by the previous experiments on other fish (Elayraja *et al.*, 2020, Liu *et al.*, 2018a, Mansour and Esteban, 2017, Liu *et al.*, 2018b, Menaga *et al.*, 2019 and Shourbela *et al.*, 2021).

4.5. Objective 5: To find out the effects of BFT with different C/N ratios on histological alteration in liver and intestine of *Oreochromis niloticus* and *Heteropneustes fossilis*.

4.5.1. Histological studies of liver of Nile tilapia (*Oreochromis niloticus*) reared in control and biofloc system with C:N ratios of 15:1, 20:1 and 25:1

Liver was considered the most important organ in nutrient metabolism and hence the histological changes of liver were considered to be a dependable marker for evaluation of nutritional status in fish (Caballero *et al.*, 2003, Ostaszewska *et al.*, 2005, Raskovic *et al.*, 2011 and Bakhshi *et al.*, 2018). The results obtained in this study were found in contrast with previous finding by Najdegerami *et al.* (2016) and Bakhshi *et al.* (2018) where no apparent histological changes were recorded in biofloc treated fishes. However Najdegerami *et al.* (2016) reported that BFT system improved quantification and qualification of hepatic cell because of the good nutritional condition and surplus energy supply from biofloc treatment. Bakhshi *et al.* (2018) also reported that BFT treatments of tilapia did not alter the gut histo construction and liver histopathology. In this experiment better histological properties were observed in the liver and intestine of Nile tilapia reared in the biofloc treatment with carbon to nitrogen ratio of 15:1 (CN15) and 20:1 (CN20) in compare to the Nile tilapia reared in the BFT system with carbon to nitrogen ratio of 25:1 (CN25) and control tanks with no additional carbon sources.

The healthy tilapia liver possessed normal irregular shaped hepatocytes with centrally placed prominent nuclei (Kaewamatawong *et al.*, 2012). Liver was involved with numerous essential functions like absorption, bile secretion, detoxification and excretion of detoxified and harmful substances, synthesis of various components of blood plasma, glycogen storage, and release of glucose as well as manage over common metabolism (Bruslé and Anadon, 2017). Fish liver also played a major role for maintaining the body metabolic homeostasis which involves processing of proteins, carbohydrate, fat and vitamins (Genten *et al.*, 2009 and Jobling and Roberts, 2012). Though the fish liver had no direct contact with the external aquatic environment in both biofloc and control tanks, but it could have been indirectly affected and prone to several structural changes and metabolic instabilities because of its direct contact with blood (Bruslé and Gemma, 2017). In our study the histological findings of both BFT treated and control fish liver sections stained with H&E exposed

an apparent histological effect on hepatocytes of Nile tilapia after 120 d culture. Three major histological changes were noticed i.e., vacuoles in cytoplasm (CV), degeneration of cytoplasm (CD) and cellular hypertrophy (CH) in these histological slides and accordingly the visual scores were given by observing nine slides originating from nine fish for each treatment including control for Kruskal-Wallis non parametric test at 95% significant level. The score were given starting from 1 to 5, based on percentage of every change in each slide. Score 1 indicated 0-5% changes, score 2: 5-15% changes; score 3: 15-25% changes; score 4: 25-50% changes and score 5 indicated 50-100% changes. Liver section of Nile tilapia of BFT treated groups of CN15 and CN20 showed highest number of uniform, normal-shaped hepatocytes with centrally located nuclei (Plate 4.3A and 4.3B).

Whereas significant higher ($p<0.05$) amount of cytoplasmic vacuole, degeneration of cytoplasm and cellular hypertrophy were observed in liver sections of Nile tilapia in control group (Plate 4.3D) compare to the all biofloc treated groups (Table 4.21). That might be due to nutritional deficiency in comparison to the higher stocking density at the end of culture. While comparing the liver histology of Nile tilapia among biofloc treatment groups all the aforesaid histological changes were found significantly higher ($p<0.05$) in CN25 Nile tilapia liver section in compare to CN15 and CN20 Nile tilapia group might be due to lower nutrient utilization in too much dense floc environment (Plate 4.3C). Increased vacuolization of the hepatocytes indicated the degenerative process towards metabolic damages in adverse condition (Pacheco and Santos, 2002). The increased numbers of vacuoles in cytoplasm probably resulted by glycogen and/or lipid storage which were associated with common metabolic function of the fish liver (Myers *et al.*, 1987). The glycogen vacuoles often broke down to release glucose in blood resulted high serum glucose level. In this study we found significant higher ($p<0.05$) serum glucose level in control groups followed by CN25, CN20 and CN15 fish groups might be due to the aforesaid histological findings. Lower significant ($p<0.05$) serum glucose level in CN25 group compare to the control Nile tilapia group most probably associated with storage of glycogen in liver instead of being released as glucose in the blood circulation. Liver was also responsible for synthesis of plasma protein like albumin and globulin (Taddese, 2014). In this study we observed lower level of serum protein in the control Nile tilapia probably due to lower protein synthesis.

Aminotranferases enzymes like AST and ALT catabolised amino acids and were used to demonstrate the liver damage (Gholami *et al.*, 2013). In normal condition the activity of ALT and AST were very low. AST localized predominantly in mitochondria and ALT localized in the cytosol of liver cells. Hepatocytes degeneration indicated the release of aminotranferases into the blood stream (Al-Habori *et al.*, 2002). Observation of significant ($p<0.05$) elevated level of serum ALT and AST of Nile tilapia in control group followed by CN25, CN20 and lowest in CN15 in our study were strongly established by the histopathological investigations of degeneration of cytoplasm.

Significant higher ($p<0.05$) cellular hypertrophy in control Nile tilapia followed by CN25 compare to CN15 and CN20 fish group were also reflected in this study, where significant higher ($p<0.05$) hepatosomatic index (HSI %) were observed in CN25 and control Nile tilapia group due to enlargement of the liver size (Table 4.21). Swelling of hepatocytes due to hypertrophy caused narrowing of sinusoids and perisinusoidal spaces that located between hepatocytes and sinusoids. Hepatocytes got blood and oxygen through sinusoids from hepatic portal vein and artery. The blood reached to hepatocytes from sinusoids through perisinusoidal spaces. Hence contraction of sinusoids and perisinusoidal spaces due to hypertrophy could create hindrance of blood and oxygen flow to hepatocytes (Hwang, 2011). Higher nutrient inflow and obstructed blood and oxygen supply might suppress the metabolizing ability of hepatocytes in BFT system with higher carbon to nitrogen ratio of 25:1 (CN25) in compare to lower C:N ratios of 15:1 and 20:1. Over nutrient or overfeeding could cause hypertrophy of fish hepatocytes was observed by Taddese *et al.* (2014). The hypertrophy in hepatocytes directly influenced the hepatosomatic index. At the same time the hepatosomatic index (HSI) was influenced by the protein level and the lowest level of digestible protein showed the highest HSI (Gallagher, 1999). So in case of lower availability of dietary protein the body need to be adapted for growing the liver volume to metabolize more protein as possible (Durigon *et al.*, 2020). That was the major cause of highest significant level ($p<0.05$) of hypertrophy in control tilapia group in this study.

Plate 4.3A. Histological features of the liver of *Oreochromis niloticus* reared in BFT with C:N ratio of 15:1 (CN15) showing normal hepatocytes (NH) with less presence of vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD) and cellular hypertrophy (CH) ×200 H&E staining.

Plate 4.3B. Histological features of the liver of *Oreochromis niloticus* reared in BFT with C:N ratio of 20:1 (CN20) showing normal hepatocytes with less presence of vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD), cellular hypertrophy (CH) and karyorrhexis (K) ×200 H&E staining.

Plate 4.3C. Histological features of the liver of *Oreochromis niloticus* reared in BFT with C:N ratio of 25:1 (CN25) showing normal hepatocytes with presence of vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD), cellular hypertrophy (CH) and glycogen vacuole (GV) ×200 H&E staining.

Plate 4.3D. Histological features of the liver of *Oreochromis niloticus* reared in control without biofloc showing normal hepatocytes with presence of vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD), cellular hypertrophy (CH) and glycogen vacuole (GV) ×200 H&E staining.

Plate 4.3. Histological sections of liver of Nile tilapia (*Oreochromis niloticus*) reared in biofloc system with C:N ratios of 15:1, 20:1 and 25:1, and in without biofloc as control

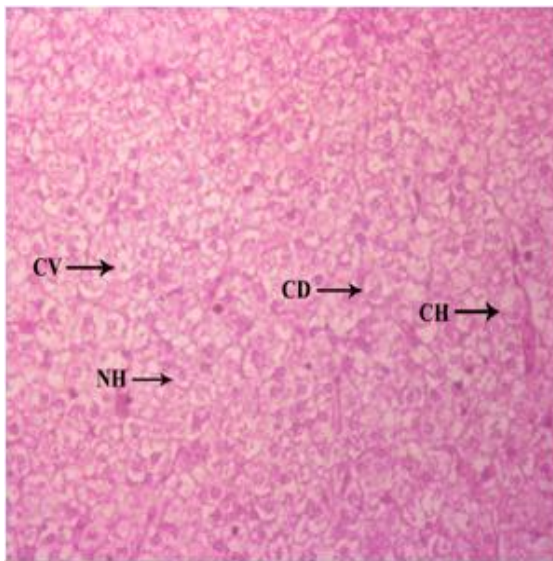


Plate 4.3A

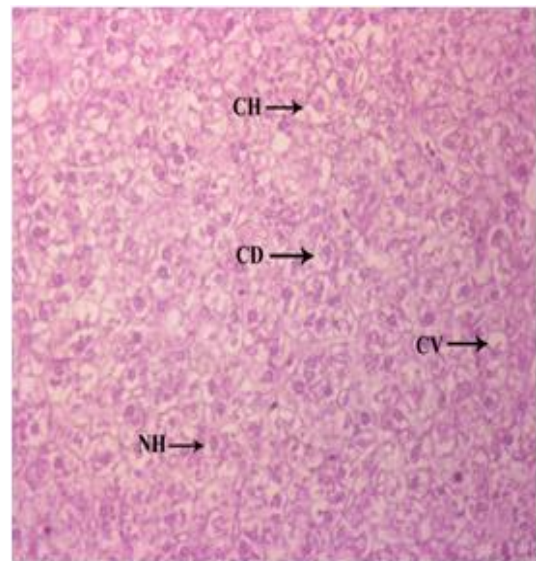


Plate 4.3B

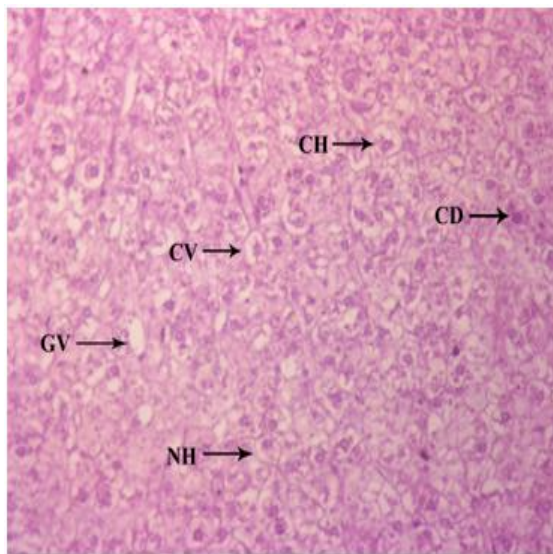


Plate 4.3C

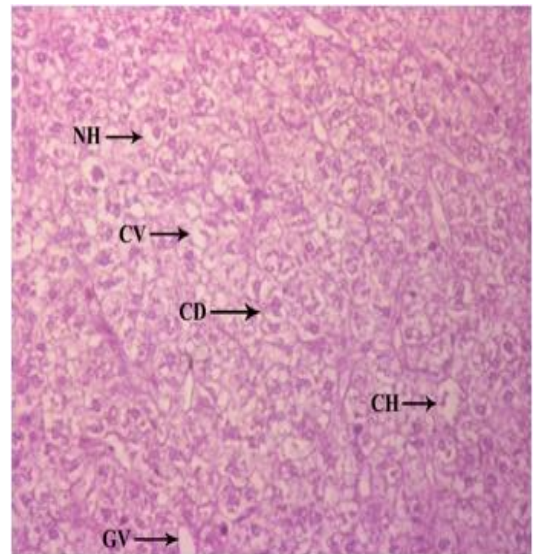


Plate 4.3D

4.5.2. Histological studies of intestine of Nile tilapia (*O. niloticus*) reared in control and biofloc system with C:N ratios of 15:1, 20:1 and 25:1

Intestine was a major organ of fish that interacted with the environment and played a decisive role in growth, nutrition and also survival under stressful environmental condition like higher stocking densities in this study. Besides that fish intestine was the major site for nutrient absorption, hence the histological study was urgently required to assess its' efficiency to absorb nutrients (da Silva *et al.*, 2012). Any adverse anatomical results could reduce its efficiency to absorb digestive nutrient (Hu *et al.*, 2007 and Rašković *et al.*, 2011). Being an omnivore's fish which ate greater amounts of both animals and plants in their diet, Nile tilapia had a large stomach and a longer intestine (Smith, 2000). In normal condition fish intestine sections could be stained entirely, and its structure of the villi, mucosal epithelial cells, goblet cell, lamina propria and muscularis should be visible properly. In this study the histological observations of Nile tilapia intestine sections stained with H&E exposed apparent histological changes in intestines of Nile tilapia reared in control and BFT system of different carbon to nitrogen ratios after 120 d culture period. Five major histological observations were recorded i.e., swollen lamina propria (SLP), mucinous degeneration (MD), loss of absorptive vessels (LV), degeneration of epithelium (DE) and necrotized area (NA). Similarly visual scores were given by observing nine slides originating from nine fishes of each treatment groups including control for Kruskal-Wallis non parametric test ($p \leq 0.05$) as stated earlier.

Comparatively significant higher ($p < 0.05$) observation of swollen lamina propria, mucinous degeneration, loss of absorptive vessels, degeneration of epithelium and necrotized area were noticed in the control Nile tilapia followed by CN25 group than CN15 and CN20 fish groups (Table 4.21). The overall observation of Nile tilapia intestine section of CN15 and CN20 fish groups showed constructive muscularis and lamina propria, however few minute aforesaid changes were observed probably due to the effects of biofloc for prolonged period over 120 d (Plate 4.4A – 4.4F). Appearance of mucinous degeneration in fish intestines with higher stocking densities was reported by Aly *et al.* (2009). Higher histological changes in intestine of CN25 Nile tilapia group also suggested that higher floc volume might cause damage to the intestine (Plate 4.4G – 4.4I). Highest histological changes in control Nile tilapia group probably due to the amino acids and/or fatty acids deficiency in comparison to the BFT treatment groups for a long duration over 120 d (Plate 4.4J – 4.4L). Significant

increased ($p < 0.05$) swollen lamina propria could have been associated with higher significant ($p < 0.05$) gastroscopic index (GSI %) in control and CN25 Nile tilapia group in compare to the CN15 and CN20 fish groups discussed in the section 4.2.3. Any adverse effects of carbon nitrogen ratio in biofloc system on fish intestine histology were not reported previously.

Table 4.21. Histological changes of liver and intestine Nile Tilapia (*Oreochromis niloticus*) in control and BFT system with different carbon to nitrogen (C:N) ratios of 15:1, 20:1, 25:1 after 120 d rearing in circular tarpaulin tanks with a stocking densities of 2500 nos./4000 l

	CN15	CN20	CN25	Control
<i>Histological changes in liver hepatocytes</i>				
Vacuoles in cytoplasm (CV)	1.17 ^b ±0.41	1.00 ^a ±0.00	1.83 ^c ±0.41	2.00 ^d ±0.00
Degeneration of cytoplasm (CD)	1.17 ^a ±0.41	1.17 ^a ±0.41	1.83 ^b ±0.41	2.00 ^c ±0.00
Cellular hypertrophy (CH)	1.33 ^a ±0.52	1.33 ^a ±0.52	2.33 ^b ±0.52	2.50 ^c ±0.55
<i>Histological changes in intestine</i>				
Swollen lamina propria (SLP)	1.17 ^b ±0.41	1.00 ^a ±0.00	2.67 ^c ±0.52	3.83 ^d ±0.41
Mucinous degeneration (MD)	1.33 ^a ±0.52	1.67 ^b ±0.52	2.00 ^c ±0.00	3.50 ^d ±0.55
Loss of absorptive vacuole (LV)	1.33 ^a ±0.52	1.83 ^b ±0.41	2.33 ^c ±0.52	3.67 ^d ±0.82
Degeneration of epithelium (DE)	1.50 ^a ±0.55	1.50 ^a ±0.55	2.67 ^b ±0.82	3.83 ^c ±0.41
Necrotized area (NA)	1.00 ^a ±0.00	1.00 ^a ±0.00	1.50 ^b ±0.55	3.00 ^c ±0.63

Values presented as Mean (\pm SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups (Kruskal-Wallis, $p < 0.05$). [CN15, CN20 and CN25: Biofloc groups with different C:N of 15:1, 20:1 and 25:1]

Plate 4.4A. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 15:1 (CN15) showing constructive muscularis (CM), well structured lamina propria (LP) with few degeneration of epithelium (DE) ×200 H&E staining.

Plate 4.4B. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 15:1 (CN15) showing well structured lamina propria (LP) and abundant of goblet cells (G) with degeneration of epithelium (DE) ×200 H&E staining.

Plate 4.4C. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 15:1 (CN15) showing presence of degeneration of epithelium (DE), mucinous degeneration (MD) and swollen lamina propria (SLP) ×200 H&E staining.

Plate 4.4D. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 20:1 (CN20) showing well structured lamina propria (LP) with presence of degeneration of epithelium (DE), mucinous degeneration (MD), loss of absorptive vacuole (LV) and swollen lamina propria (SLP) ×200 H&E staining.

Plate 4.4E. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 20:1 (CN20) showing well structured lamina propria (LP) and abundant of goblet cells (G) with degeneration of epithelium (DE) ×200 H&E staining.

Plate 4.4F. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 20:1 (CN20) showing degeneration of epithelium (DE), loss of absorptive vacuole (LV) with goblet cells (G) and presence of vacuoles (V) ×200 H&E staining.

Plate 4.4. Histological sections of intestine of Nile tilapia (*Oreochromis niloticus*) reared in biofloc systems with C:N ratios of 15:1, 20:1 and 25:1, and in a without biofloc system as control

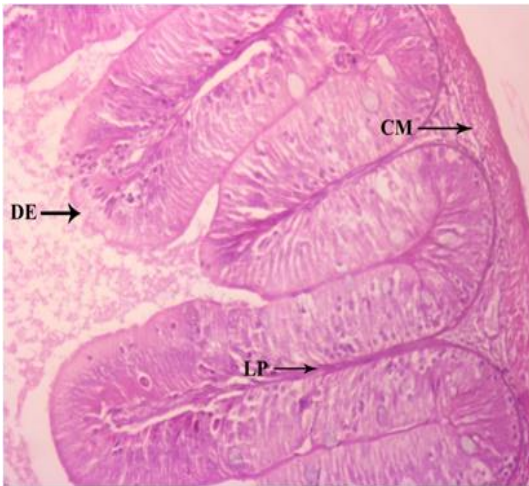


Plate 4.4A

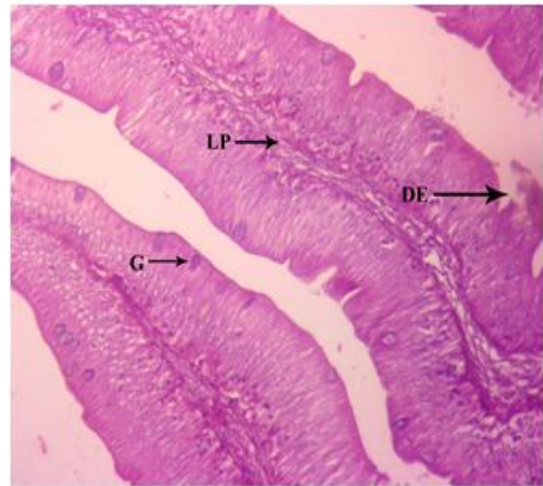


Plate 4.4B

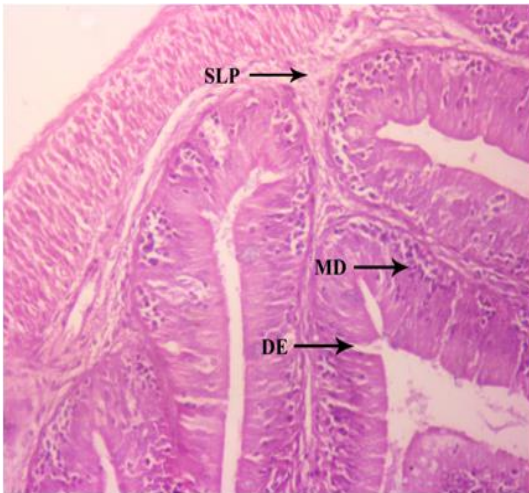


Plate 4.4C

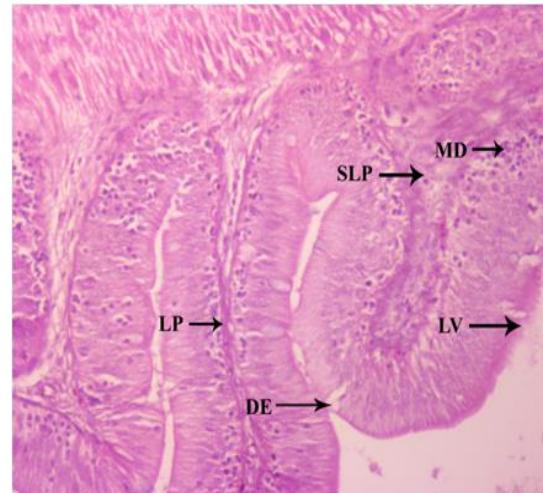


Plate 4.4D

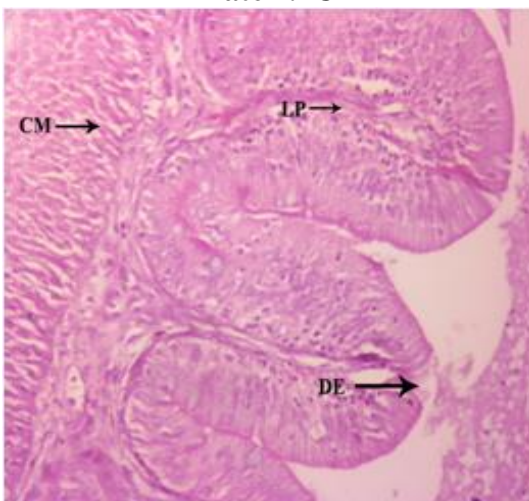


Plate 4.4E

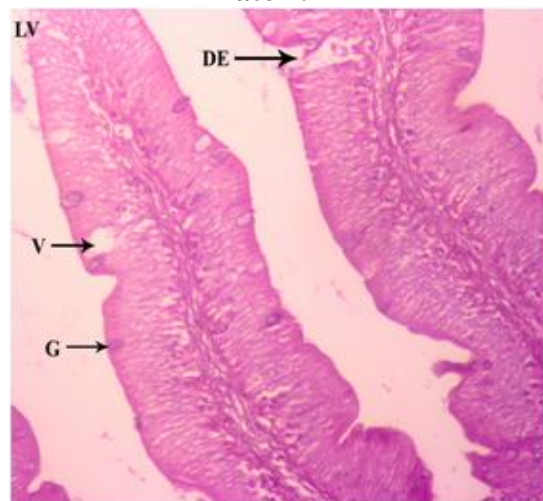


Plate 4.4F

Plate 4.4G. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 25:1 (CN25) showing degeneration of epithelium (DE), mucinous degeneration (MD), loss of absorptive vacuole (LV) and swollen lamina propria (SLP) ×200 H&E staining.

Plate 4.4H. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 25:1 (CN25) showing degeneration of epithelium (DE), mucinous degeneration (MD), necrotized area (NE) and swollen lamina propria (SLP) ×200 H&E staining.

Plate 4.4I. Histological features of the intestine of *Oreochromis niloticus* reared in BFT with C:N ratio of 25:1 (CN25) showing presence of goblet cells (G) with degeneration of epithelium (DE), loss of absorptive vacuole (LV), necrotized area (NE) ×200 H&E staining.

Plate 4.4J. Histological features of the intestine of *Oreochromis niloticus* reared in control without biofloc showing presence of goblet cells (G) with degeneration of epithelium (DE), mucinous degeneration (MD), loss of absorptive vacuole (LV), swollen lamina propria (SLP) and necrotized area (NE) ×200 H&E staining.

Plate 4.4K. Histological features of the intestine of *Oreochromis niloticus* reared in control without biofloc showing presence of degeneration of epithelium (DE), mucinous degeneration (MD), loss of absorptive vacuole (LV) and swollen lamina propria (SLP) ×200 H&E staining.

Plate 4.4L. Histological features of the intestine of *Oreochromis niloticus* reared in control without biofloc showing presence of degeneration of epithelium (DE), mucinous degeneration (MD), loss of absorptive vacuole (LV), swollen lamina propria (SLP) and necrotized area (NE) ×200 H&E staining.

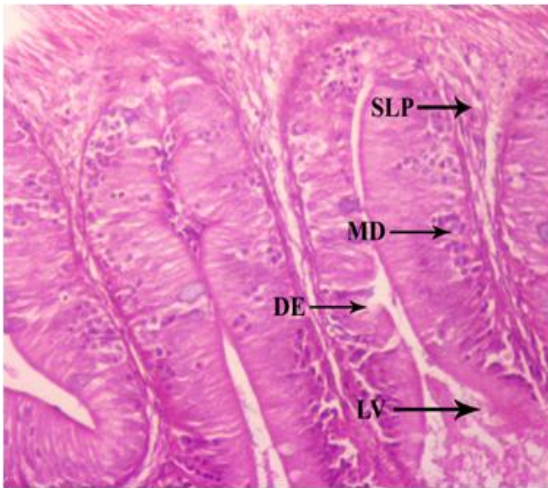


Plate 4.4G

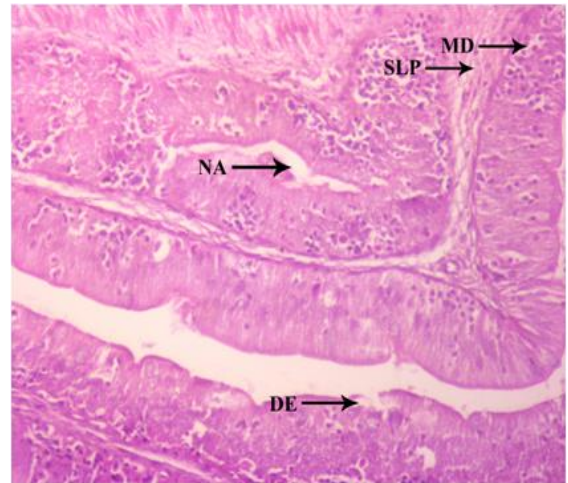


Plate 4.4H

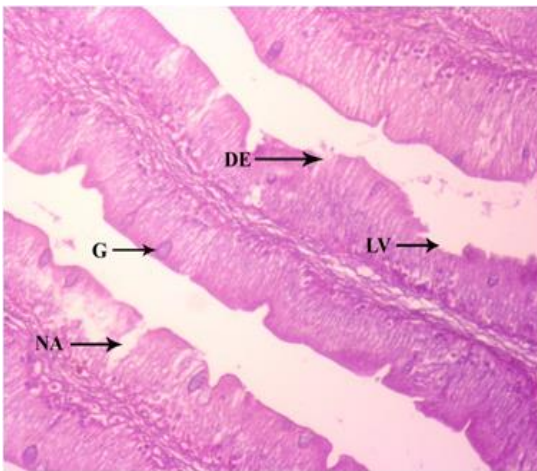


Plate 4.4I



Plate 4.4J

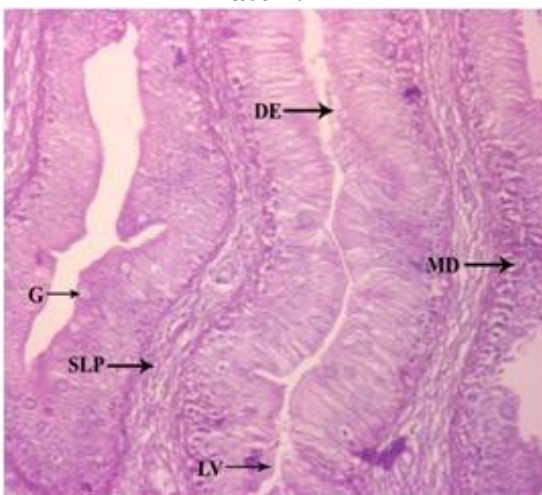


Plate 4.4K

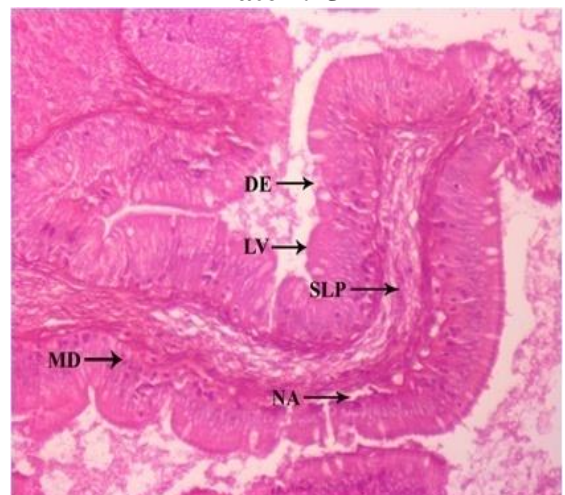


Plate 4.4L

4.5.3. Histological studies of liver of Singhi (*Heteropneustes fossilis*) reared in control and biofloc system with C:N ratios of 8:1, 12:1 and 16:1

Hepatocytes of *H. fossilis* liver of all experimental biofloc and control groups exhibited distinct centrally placed nucleus with heavily stained chromatin. However, the liver sections of *Heteropneustes fossilis* stained with H&E also exposed few histological changes on hepatocytes after 180 d of culture in biofloc system with carbon to nitrogen ratio of 8:1 (CN8), 12:1 (CN12), 16:1 (CN16) and control. Alike Nile tilapia the vacuoles in cytoplasm (CV), degeneration of cytoplasm (CD), cellular hypertrophy (CH) were found out by observing nine slides from nine fishes for each treatment including control for Kruskal-Wallis non parametric test ($p \leq 0.05$) after giving visual score as stated above.

Apparently the H&E liver section of all treatments and control *H. fossilis* showed well structured hepatocytes but statistically significant ($p < 0.5$) higher vacuoles in cytoplasm, degeneration of cytoplasm and cellular hypertrophy were noticed in control fish group followed by CN16 group compare to CN8 and CN12 Singhi groups after 180 d culture. Those could be due to the adverse effects of high floc volume in biofloc system of CN16 and nutritional deficiency in control *H. fossilis* group (Plate 4.5A to Plate 4.5D).

Cytoplasmic vacuole formation in Singhi hepatocytes might be due to the glycogen and lipid accumulation where glycogen vacuoles often broke down to release glucose. Hence significant higher serum glucose levels were found in the control group followed by CN16 *H. fossilis* group. Significant higher ($p < 0.05$) degeneration of cytoplasm in hepatocytes of control Singhi group followed by CN16, CN8 and CN12 fish groups also resulted by significant higher ($p < 0.05$) level of serum ALT and AST as stated in fish welfare section 4.4b.2. Considerable higher ($p < 0.05$) level of cellular hypertrophy were observed in control and CN16 Singhi groups than CN8 and CN12 fish groups, but that minute changes were not found enough to enlarge liver or increase hepatosomatic index (HSI %) significantly (Table 4.4) in control and CN16 fish group.

Plate 4.5A. Histological features of the liver of *Heteropneustes fossilis* reared in BFT with C:N ratio of 8:1 (CN8) showing normal hepatocytes with presence of few vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD) and cellular hypertrophy (CH) ×200 H&E staining.

Plate 4.5B. Histological features of the liver of *Heteropneustes fossilis* reared in BFT with C:N ratio of 12:1 (CN12) showing normal hepatocytes with presence of few vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD) and cellular hypertrophy (CH) ×200 H&E staining.

Plate 4.5C. Histological features of the liver of *Heteropneustes fossilis* reared in BFT with C:N ratio of 16:1 (CN16) showing normal hepatocytes with presence of few vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD) and cellular hypertrophy (CH) ×200 H&E staining.

Plate 4.5D. Histological features of the liver of *Heteropneustes fossilis* reared in control without biofloc showing normal hepatocytes with presence of few vacuoles in cytoplasm (CV), degeneration of hepatocytes (CD) and cellular hypertrophy (CH) ×200 H&E staining.

Plate 4.5. Histological sections of liver of Singhi (*Heteropneustes fossilis*) reared in biofloc systems with C:N ratios of 8:1, 12:1 and 16:1, and in a without biofloc system as control for 180 d

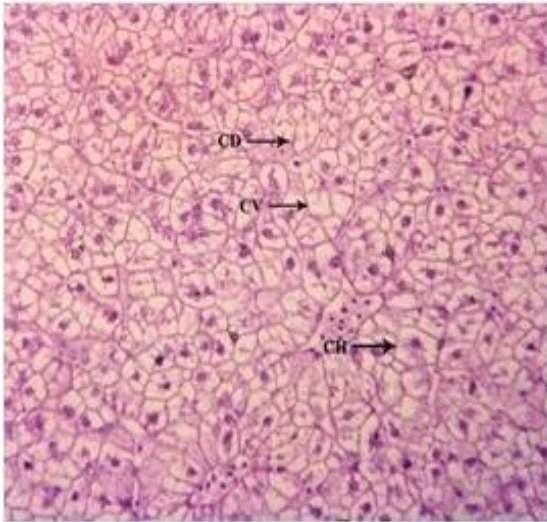


Plate 4.5A

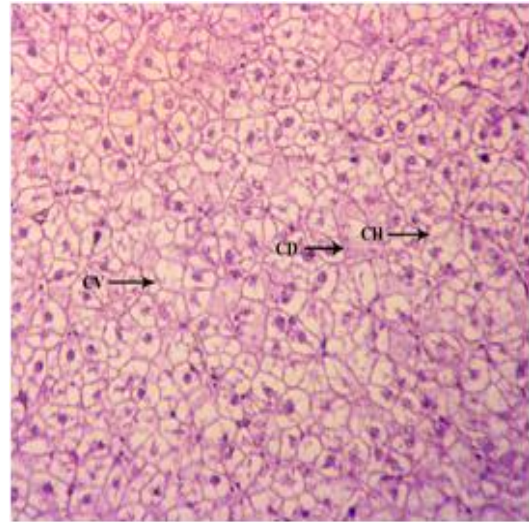


Plate 4.5B

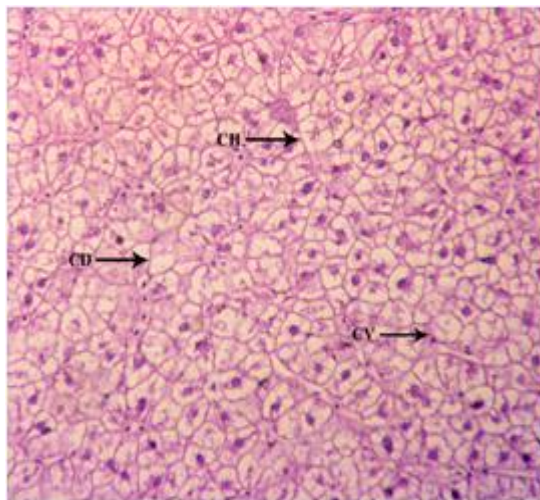


Plate 4.5C

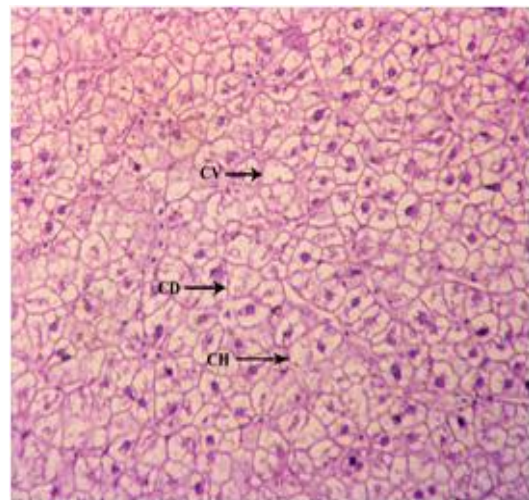


Plate 4.5D

4.5.4. Histological studies of intestine of Singhi (*Heteropneustes fossilis*) reared in control and biofloc system with C:N ratios of 8:1, 12:1 and 16:1

Being a carnivorous fish *Heteropneustes fossilis* had a large and distensible stomach, and a short intestine (Qu *et al.*, 2012). Mucosa, submucosa, muscularis and thin serosa were the four layers possessed by the intestine of *Heteropneustes fossilis* in all BFT treated and control fish group (Samanta *et al.*, 2016). Mucosa was made up of simple and elongated finger like villi, which was supported by absorptive columnar epithelial cells along with basally or centrally located nucleus. A thin top plate of brush borders embraced the villi. Intestinal mucosa was supported by several mucous cells. Lamina propria of submucosa was constructed by loose connective tissues and serosa layer was provided with compact network of blood vessels. All the BFT treated and control *H. fossilis* groups showed well structured intestine during histological studies of intestine sections stained with H&E, however, three minute but noticeable histological changes were observed in *H. fossilis* which were damage in lamina propria (DL), degeneration of epithelium (DE) and loss of brush border structure (LB) (Plate 4.6A to Plate 4.6H).

Damage in tip of mucosal layer and intestinal vacuoles were observed in only control groups might be due to intestinal inflammation for nutritional deficiency (Plate 4.6G – 4.6H). Similarly the visual scores were given by observing nine slides from nine fishes of each treatment and control groups for Kruskal-Wallis non parametric test ($p \leq 0.05$) as stated earlier.

Highest significant ($p < 0.05$) changes of damage in lamina propria, distortion in columnar epithelial cells and loss of brush border structure were observed in both control and BFT treated CN16 Singhi in compare to BFT treated CN12 and CN8 Singhi intestine (Table 4.22). All those epithelial degeneration observed in intestine of Singhi of CN16 BFT treatment and control was indicating the increased formation of free radicals which might cause damage to the intestinal tract including gastric mucosa (Olaley *et al.*, 2007).

No previous reports on histopathological studies of Singhi intestine were available till date. Kumari and Kumar (1997) reported the necrosis, degeneration in mucosa and submucosal layers and proliferation of the exterior portion of intestinal villi during the histological observation in *Heteropneustes fossilis* taken from polluted water. Hence it can be concluded that BFT system with higher carbon to nitrogen ratio could affects on the intestinal structure of *Heteropneustes fossilis*. The adverse

histological changes in the intestine of Singhi might be due to the insufficient nutrient for a prolonged period over 180 d because balanced diets containing essential nutritional requirements were the major issues for maintaining intestinal fitness in fish (Dawood and Koshio, 2020).

Table 4.22. Histological changes of liver and intestine of Singhi (*Heteropneustes fossilis*) in control and BFT system with different carbon to nitrogen (C:N) ratios of 8:1, 12:1, 16:1 after 180 d rearing in rectangular cement tanks with a stocking densities of 700 nos./1500 l

	CN8	CN12	CN16	Control
<i>Histological changes in liver hepatocytes</i>				
Vacuoles in cytoplasm (CV)	1.50 ^a ±0.55	1.50 ^a ±0.55	2.00 ^b ±0.00	1.83 ^c ±0.41
Degeneration of cytoplasm (CD)	1.50 ^b ±0.55	1.33 ^a ±0.52	1.83 ^c ±0.41	2.00 ^d ±0.89
Cellular hypertrophy (CH)	1.67 ^a ±0.52	1.67 ^a ±0.52	2.17 ^b ±0.41	2.50 ^c ±0.55
<i>Histological changes in intestine</i>				
Damage in lamina propria (DL)	1.17 ^b ±0.41	1.00 ^a ±0.00	1.50 ^c ±0.55	1.50 ^c ±0.55
Degeneration of epithelium (DE)	1.33 ^a ±0.52	1.33 ^a ±0.52	2.00 ^b ±0.00	2.00 ^b ±0.00
Loss of brush border structure (LB)	1.50 ^a ±0.55	1.50 ^a ±0.55	1.83 ^b ±0.41	2.17 ^c ±0.41

Values presented as Mean (± SD, n=9) in the same row with different superscripts differ significantly for each parameter between experimental groups for a given time (Kruskal-Wallis, p<0.05). [CN8, CN12 and CN16: Biofloc groups with different C:N of 8:1, 12:1 and 16:1]

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Plate 4.6A. Histological features of the intestine of *Heteropneustes fossilis* reared in BFT with C:N ratio of 8:1 (CN8) showing constructive muscularis (CM), well structured lamina propria (LP) with few loss of brush border structure (LB) ×200 H&E staining.

Plate 4.6B. Histological features of the intestine of *Heteropneustes fossilis* reared in BFT with C:N ratio of 8:1 (CN8) showing damage in lamina propria (DL) and presence of vacuoles (V) ×200 H&E staining.

Plate 4.6C. Histological features of the intestine of *Heteropneustes fossilis* reared in BFT with C:N ratio of 12:1 (CN12) showing presence of degeneration of epithelium (DE), damage in lamina propria (DL) and loss of brush border structure (LB) ×200 H&E staining.

Plate 4.6D. Histological features of the intestine of *Heteropneustes fossilis* reared in BFT with C:N ratio of 12:1 (CN12) showing constructive muscularis (CM), goblet cells (G) with presence of damage in lamina propria (DL) ×200 H&E staining.

Plate 4.6. Histological sections of intestine of Singhi (*Heteropneustes fossilis*) reared in biofloc systems with C:N ratios of 8:1, 12:1 and 16:1, and in a without biofloc system as control for 180 d

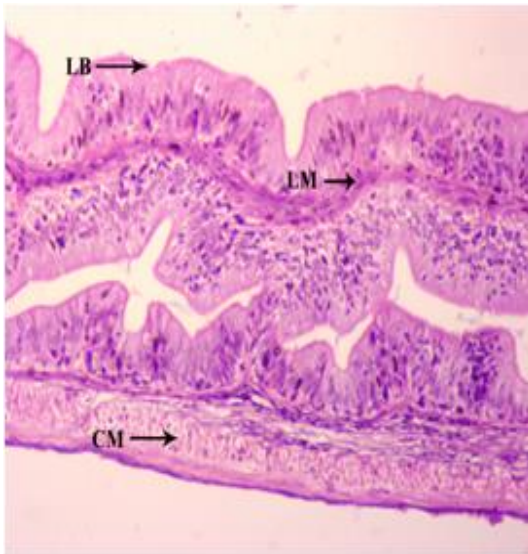


Plate 4.6A

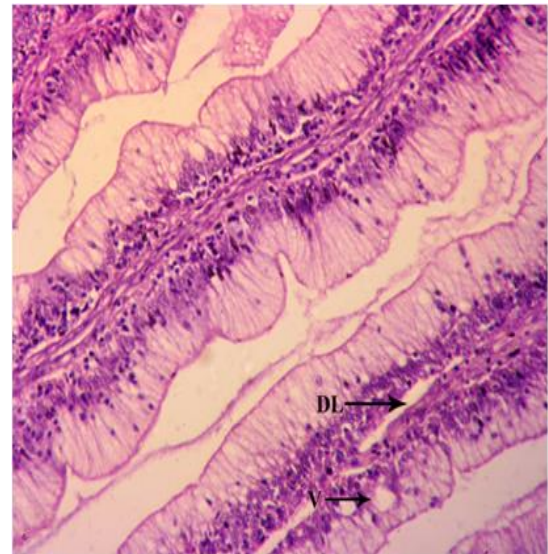


Plate 4.6B

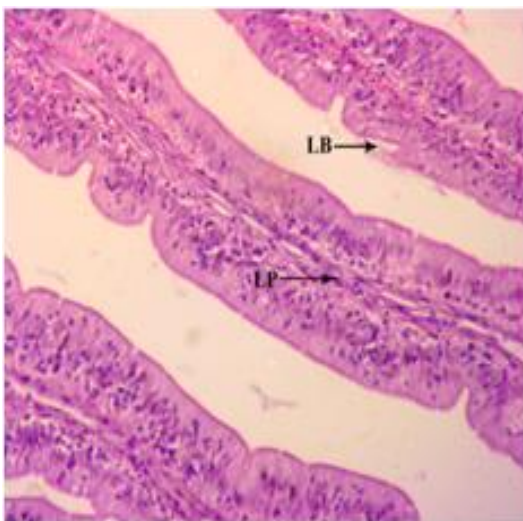


Plate 4.6C

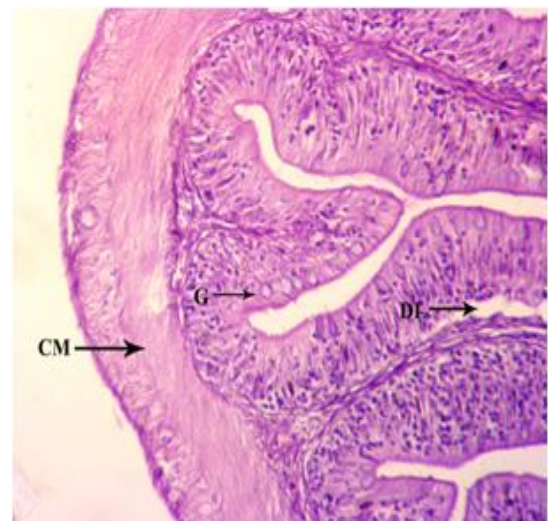


Plate 4.6D

Plate 4.6E. Histological features of the intestine of *Heteropneustes fossilis* reared in BFT with C:N ratio of 16:1 (CN16) showing constructive muscularis (CM) with damage in lamina propria (DL) and degeneration of epithelium (DE)×200 H&E staining.

Plate 4.6F. Histological features of the intestine of *Heteropneustes fossilis* reared in BFT with C:N ratio of 16:1 (CN16) showing constructive muscularis (CM) with degeneration of epithelium (DE) and loss of brush border structure (LB) ×200 H&E staining.

Plate 4.6G. Histological features of the intestine of *Heteropneustes fossilis* reared in control without biofloc showing presence of degeneration of epithelium (DE), damage in lamina propria (DL) and loss of brush border structure (LB) ×200 H&E staining.

Plate 4.6H. Histological features of the intestine of *Heteropneustes fossilis* reared in control without biofloc showing presence of degeneration of epithelium (DE) and damage in lamina propria (DL) ×200 H&E staining.

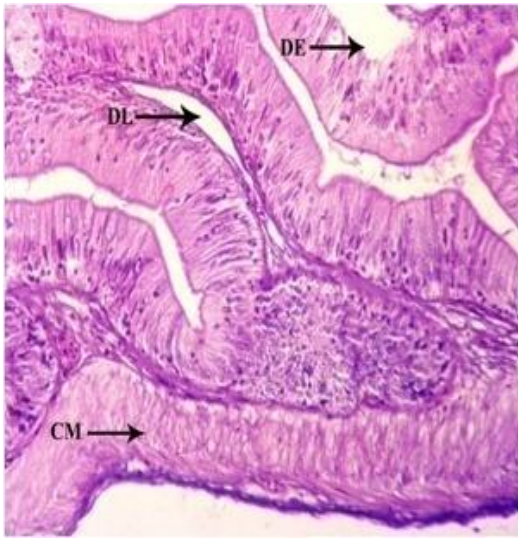


Plate 4.6E

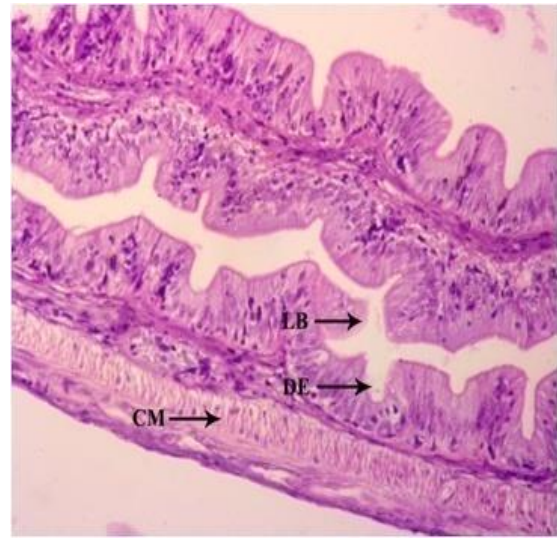


Plate 4.6F

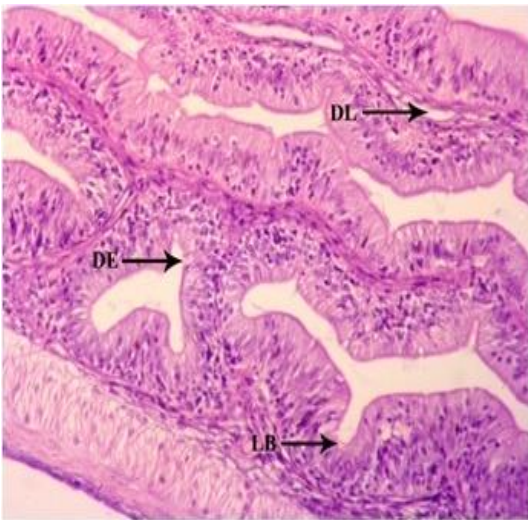


Plate 4.6G

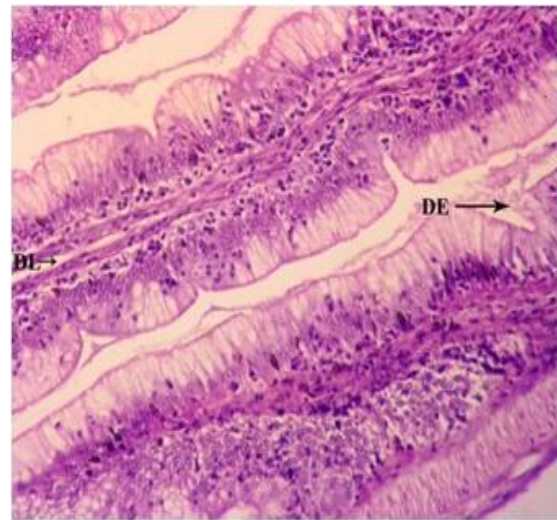


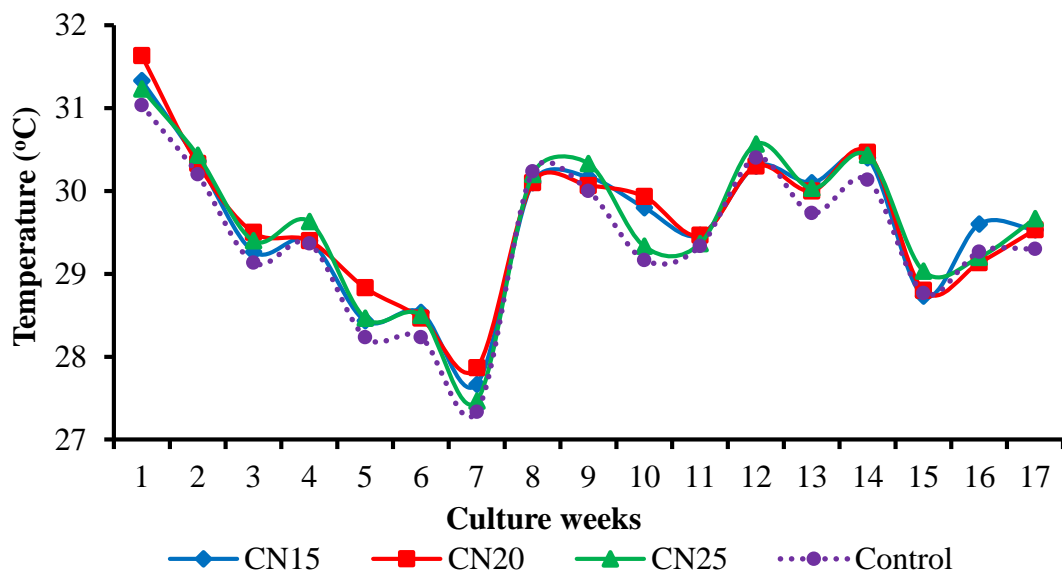
Plate 4.6H

4.6. Objective 6: To assess the effects of BFT with different C/N ratios on water quality parameters during culture of *Oreochromis niloticus* and *Heteropneustes fossilis*.

4.6.1. Temperature measurements in control and biofloc system during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

In this study the rearing of Nile tilapia under biofloc system with different carbon to nitrogen ratios of 15:1 (CN15), 20:1 (CN20), 25:1 (CN25) along with control (without biofloc) were started on dated 2th June, 2021. The culture were continued for 120 d and ended on dated 30th September, 2021. The culture period was extended from summer to rainy season hence the water temperatures range were recorded between 27.3–31°C for control and 27.7–31.3°C, 27.9–31.6°C and 27.5–31.2°C for BFT system of CN15, CN20 and CN25, respectively. The indoor experimental setup was facilitated of diffuse sunlight though transparent asbestos sheet in day time and well natural air circulation through iron fence windows. The water temperatures were measured at 7 am daily where uniform deviations of temperature in all BFT treated and control tanks were observed throughout 120 d (Fig.4.37).

Fig.4.37. Mean(n=3) temperature recorded in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*) for 120 d



No significant differences ($p>0.05$) of water temperature were observed among BFT treated and control tanks throughout the experiment, where the mean

Results and Discussion

water temperature for 120 d were recorded as 29.4 ± 0.2 , 29.6 ± 0.1 , 29.6 ± 0.1 and 29.6 ± 0.2 for the control and BFT system of CN15, CN20 and CN25, respectively (Table 4.23). The mean water temperature and the temperature ranges during 120 d culture in all BFT treatments and control tanks were recorded almost same with negligible variations. All the experimental tanks were maintained in the same condition but an insignificant ($p > 0.05$) decline temperature were recorded in control tanks might be due to the daily exchange of 15-20% water. This experiment was conducted within the standard thermal comfort zone for tilapia rearing which was considered $27-31^\circ\text{C}$ (Elayaraja *et al.*, 2020). Temperature was one of the important influencing factors for biofloc formation. In the said experiment the temperature was measured within the proper range of floc development (Hostins *et al.*, 2015).

Table 4.23. Mean (\pm SD, $n=3$) values of water quality parameters recorded in control and biofloc tanks with different C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25), for rearing of Nile tilapia (*O. niloticus*) for 120 d

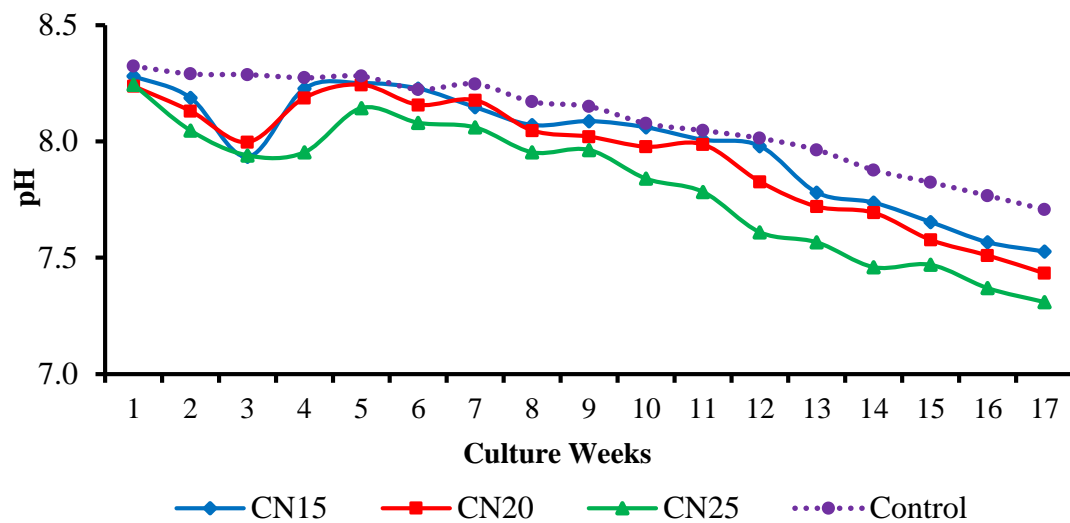
	CN15	CN20	CN25	Control
Temperature ($^\circ\text{C}$)	29.6 ± 0.1	29.6 ± 0.1	29.6 ± 0.2	29.4 ± 0.2
at 7.00 am	(27.7 – 31.3)	(27.9 – 31.6)	(27.5 – 31.2)	(27.3 – 31.0)
pH at 5.00 pm	7.98 ^c	7.94 ^b	7.81 ^a	8.09 ^d
	(7.53 – 8.28)	(7.43 – 8.24)	(7.31 – 8.24)	(7.71 – 8.32)
Total Alkalinity (mg/l)	$177.54^c \pm 0.51$	$173.67^b \pm 1.94$	$167.58^a \pm 0.40$	$180.49^d \pm 1.28$
	(165–185.33)	(155.67–184.33)	(152.33–179.33)	(172.07–188.50)
Dissolve Oxygen (mg/l) 7.00 am	$7.03^b \pm 0.03$	$6.99^b \pm 0.03$	$6.89^a \pm 0.02$	$7.04^b \pm 0.01$
	(6.73 – 7.23)	(6.66 – 7.24)	(6.52 – 7.18)	(6.60 – 7.29)
Total Ammonia Nitrogen (mg/l)	$0.33^c \pm 0.01$	$0.28^b \pm 0.01$	$0.24^a \pm 0.02$	$0.69^d \pm 0.01$
	(0.09 – 1.20)	(0.05 – 1.13)	(0.05 – 1.03)	(0.43 – 0.85)
Nitrite (NO_2) (mg/l)	$0.11^c \pm 0.00$	$0.08^b \pm 0.00$	$0.07^a \pm 0.00$	$0.20^d \pm 0.01$
	(0.05 – 0.27)	(0.03 – 0.23)	(0.03 – 0.20)	(0.11 – 0.26)
Nitrate (NO_3) (mg/l)	$6.86^b \pm 0.69$	$5.44^a \pm 0.38$	$4.83^a \pm 0.45$	$12.63^c \pm 0.23$
	(0.22 – 9.68)	(0.57 – 8.83)	(0.45 – 7.54)	(1.11 – 22.49)
Total Suspended Solids (mg/l)	$319.40^b \pm 8.25$	$352.65^c \pm 6.98$	$376.88^d \pm 8.16$	$30.57^a \pm 1.79$
	(180.03–454.68)	(181.36–545.51)	(183.04–581.40)	(13.96–41.62)
Biofloc volume (ml)	$19.22^b \pm 0.87$	$24.90^c \pm 0.12$	$29.94^d \pm 0.56$	$0.94^a \pm 0.02$
	(7.83 – 25.93)	(9.13 – 33.10)	(9.47 – 39.57)	(0.20 – 1.60)

Values in the same row with different letters are significantly different ($p < 0.05$).

4.6.2. Measurements of pH in control and biofloc tanks during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

A significantly lower trend ($p < 0.05$) of pH were recorded in all biofloc rearing tanks by increasing C:N ratios. The highest and lowest most pH value of all BFT treated and control tanks were found at the 1st and last week of culture, respectively. However the declining patterns were found different in BFT systems and control. The significant highest ($p < 0.05$) mean (\pm SD) pH measured at 5.00 pm in control Nile tilapia group than all BFT tanks throughout 120 d was recorded as 8.09 that varied between 7.71–8.32. A consistent declination of pH without any abrupt changes was found in control rearing tanks (Fig.4.38). The mean (\pm SD) pH during 120 d were noticed significantly different ($p < 0.05$) among BFT treated tanks with different C:N ratios i.e., 8.00, 7.94 and 7.81 in CN15, CN20 and CN25 groups, respectively (Table 4.23). The pH value of the biofloc system tanks such as CN15, CN20 and CN25 were varied between 7.53–8.28, 7.43–8.24 and 7.31–8.24, respectively.

Fig.4.38. Mean ($n=3$) pH in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*) for 120 d



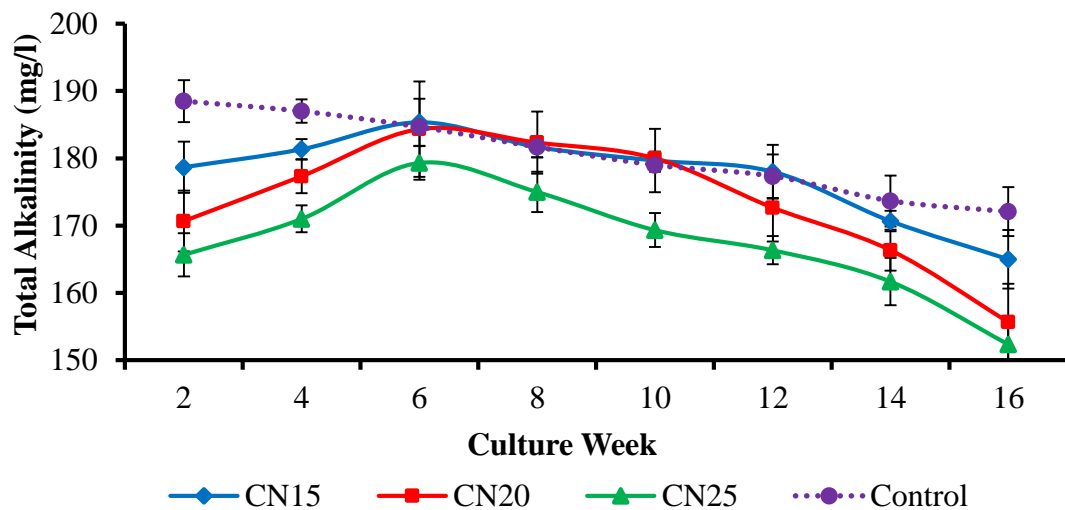
Though the pH value of all BFT rearing tanks declined from beginning to end, however, a sudden drop of pH value was noticed till 3rd week of culture which again rose till 5th week and then again continuously declined overtime till the date of harvesting on 120th d (Fig.4.38). That sudden depletion of pH in biofloc tanks could be associated with abrupt increase of total ammonia nitrogen and nitrite concentration at the beginning of culture which affects buffering capacity of system. Highest level of pH in control along with decrease of pH in biofloc system with increased C:N

ratios were also reported by the Panigrahi *et al.* (2017), Mirzakhani *et al.* (2019) and Minabi *et al.* (2020). The lowering of pH in biofloc system were reported by Kamillya *et al.* (2017), Menaga *et al.* (2019) and Aghabarari *et al.* (2021) which might be connected with the loses of buffering capacity in BFT system (Azim and Little, 2008).

4.6.3. Total alkalinity levels while rearing of Nile tilapia (*Oreochromis niloticus*) in control and biofloc systems for 120 d

Alike pH the mean value of total alkalinity measured at fortnight were also found significantly higher ($p < 0.05$) in control (180.49 ± 1.28 mg/l) followed by BFT system of CN15 (177.54 ± 0.51 mg/l), CN20 (173.67 ± 1.94 mg/l) and CN25 (167.58 ± 0.40 mg/l). A lowering trend of total alkalinity was observed from beginning to the end in control tanks within a range of 172.07-188.50 mg/l. While an increasing trend of total alkalinity from 2nd to 6th week and then decreasing trend from 6th to 16th week were found common in all BFT system tanks such as CN15, CN20 and CN25 where results varied between 165–185.33 mg/l, 155.67–184.33 mg/l and 152.33–179.33 mg/l, respectively (Table 4.23 and Fig.4.39).

Fig.4.39. Mean(\pm SD, n=3) total alkalinity in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*) for 120 d



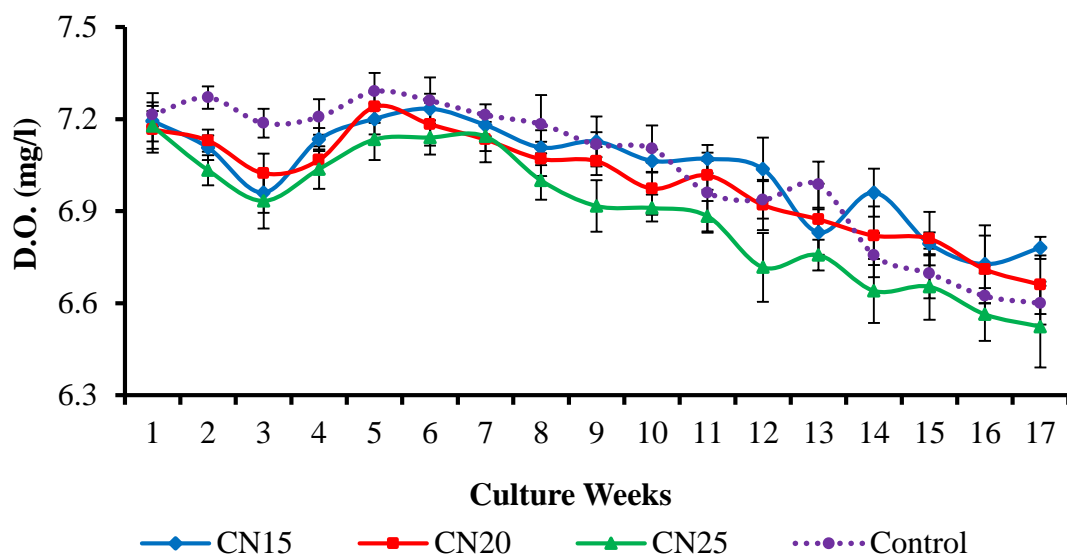
The fluctuation of total alkalinity in BFT tanks were found synchronizing with the changes of pH level in this study indicated the buffering capacity of the biofloc system could highly influence the pH level which needed to be adjusted frequently by addition of $\text{Ca}(\text{HCO}_3)_2$ or NaHCO_3 (Azim and Little, 2008). Reduced level of both pH and alkalinity in the biofloc treatment groups compared to the control were due to

the use of inorganic carbon by heterotrophic bacteria (Ebeling *et al.*, 2006). Our finding about reduced total alkalinity in BFT was supported by Panigrahi *et al.* (2017), Menaga *et al.* (2019). Dilmi *et al.* (2022) also observed lower trend of total alkalinity with increasing C:N ratios while culturing Nile tilapia in biofloc system with C:N ratios of 14:1, 17:1 and 20:1.

4.6.4. Dissolve oxygen measurements in control and biofloc system for Nile tilapia (*Oreochromis niloticus*) culture for 120 d

The dissolve oxygen (DO) measured at 7 am of all BFT treated and control tanks showed a lowering tendency during 120 d rearing period (Fig.4.40). The higher oxygen consumptions by the bacteria and other microorganisms in the higher C:N ratio BFT system was the reason behind it (Long *et al.*, 2015). The weekly measured mean DO values of control (7.04±0.01 mg/l) and BFT system of CN15 (7.03±0.03 mg/l) and CN20 (6.99±0.03 mg/l) were not found significantly different (p>0.05) but those were significantly higher (p<0.05) in compare to mean DO value of BFT treated CN25 (6.89±0.02 mg/l) tanks. The DO value of the control and biofloc system tanks such as CN15, CN20 and CN25 were varied between 6.60–7.29, 6.73–8.28, 7.43–8.24 and 7.31–8.24, respectively (Table.4.23).

Fig.4.40. Mean(±SD, n=3) dissolve oxygen in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*) for 120 d



A sudden drop of DO up to 3rd week and again increasing till 6th week were noticed in all BFT treatments tanks, whereas in control tanks no such rapid lowering

of DO was found (Fig.4.40). That sudden drop of DO during starting of culture along with depletion of pH and total alkalinity might be due to the initial rise of total ammonia nitrogen and nitrite concentration in biofloc tanks. Our results in connection with DO were supported by Panigrahi *et al.* (2017), Liu *et al.* (2018a), Mirzakhani *et al.* (2019) and Minabi *et al.* (2020) who described the lowering trend of DO with increasing carbon to nitrogen ratios in their experiments. The significant lower DO in biofloc system compare to control due to rapid consumption by heterotrophic bacteria to neutralize the toxic nitrogenous components were also reported by the previous studies of Kamillya *et al.* (2017), Liu *et al.* (2018a) and Dilmi *et al.* (2022). The rate of aeration were same for all treatment and control tanks to maintain the desired DO for Nile tilapia culture, however the possible reason for higher DO in control tank might be due to the regular water exchange up to 15-20% and absence of heterotrophic bacteria (Elayaraja *et al.*, 2020).

4.6.5. Total ammonia nitrogen (TAN) and Nitrite (NO₂) measurements in control and biofloc during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

The weekly observed mean total ammonia nitrogen (TAN) and nitrite (NO₂) values of control tanks were found significantly higher ($p < 0.05$) i.e., 0.69 ± 0.01 mg/l and 0.20 ± 0.01 mg/l, respectively compare to all BFT treatment tanks during 120 d rearing of Nile tilapia. Among BFT treatments groups the CN15 tanks showed significantly higher mean TAN (0.33 ± 0.01 mg/l) and NO₂ (0.11 ± 0.00 mg/l), followed by CN20 tanks (TAN- 0.28 ± 0.01 mg/l; NO₂- 0.08 ± 0.00 mg/l) and CN25 tanks (TAN- 0.24 ± 0.02 mg/l; NO₂- 0.07 ± 0.00 mg/l) (Table 4.23).

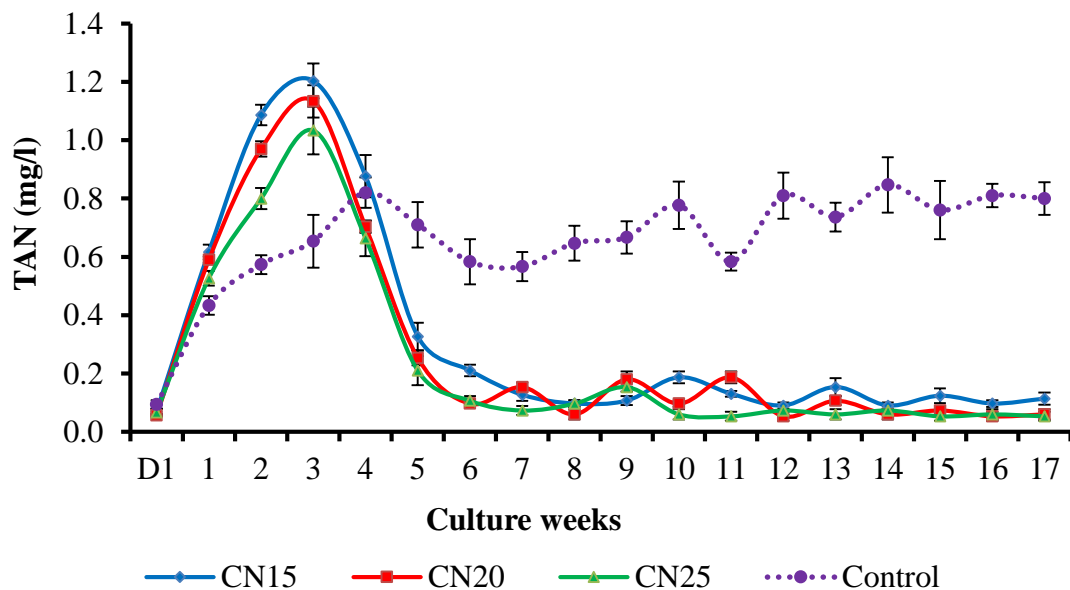
Within BFT system of CN15, CN20 and CN25 the TAN range were found between 0.90–1.20 mg/l, 0.05–1.13 mg/l and 0.05–1.03 mg/l, respectively. The NO₂ levels were recorded between 0.05–0.27 mg/l, 0.03–0.33 mg/l and 0.03–0.20 mg/l for the CN15, CN20 and CN25, respectively (Table 4.23). The TAN level of all biofloc system tanks rose suddenly after commencement of culture and reached at peak level on 3rd week which was abruptly decreased below 0.20 mg/l within next three weeks and did not rose again till the end of the 17th week of culture duration (Fig.4.41).

The weekly measured NO₂ values of all biofloc system tanks also showed the similar unique pattern like TAN level where the elevated NO₂ were observed within first 5th week and after that it became far below than normal permissible limits of 0.25 ppm those were maintained for rest of culture period (Fig.4.42). All the BFT system

tanks with different C:N ratios, could effectively control the TAN and nitrite level throughout the 120 d rearing of Nile tilapia, however among BFT groups, the higher C:N ratio group CN25 most effectively reduced those toxic nitrogenous components followed by CN20 and CN15 (Table 4.23) were supported by the previous studies of Panigrahi *et al.* (2017), Yu *et al.* (2020b) and Dilmi *et al.* (2022).

In control Nile tilapia rearing tanks no such unique pattern of weekly measured TAN and nitrite level were found during 120 d (Fig.4.41 and Fig.4.42). In spite of regular water exchange of 15-20% and continuous aeration a higher but within permissible limits of 1 mg/l of TAN and 0.25 mg/l of NO₂ level were noticed during 17th week of culture. The TAN and NO₂ level of control tanks were varied between 0.43–0.85 mg/l and 0.11–0.26 mg/l (Table 4.23).

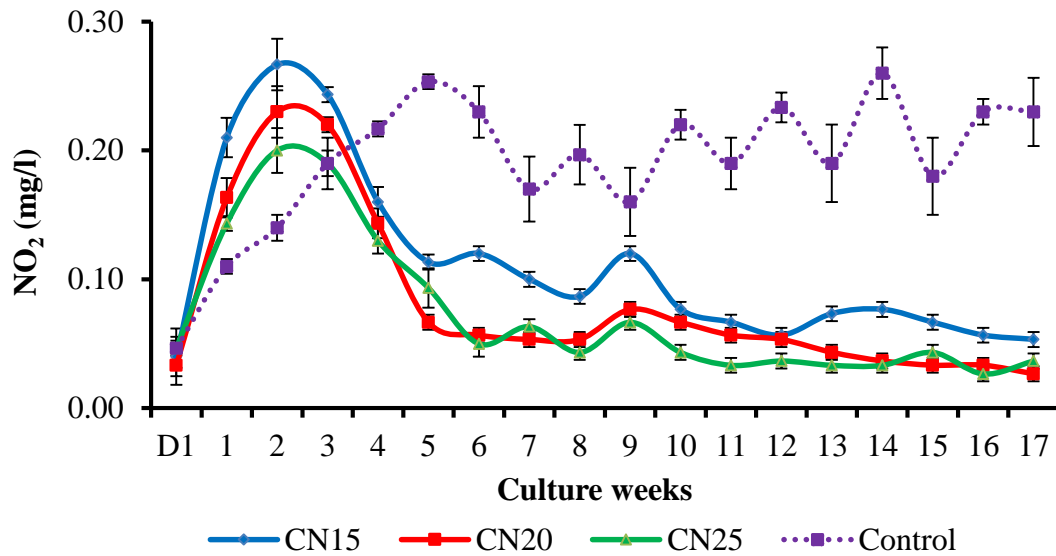
Fig.4.41. Mean (\pm SD, n=3) total ammonia nitrogen in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*)



The increasing trends of TAN and NO₂ at beginning followed by a lower tendency till the end at biofloc system were also described by previous studies of Luo *et al.* (2014), Long *et al.* (2015), Minabi *et al.* (2020) and Dilmi *et al.* (2022). In our experiment the main reason behind rapid decrease of TAN was due to the absorption of decomposed organic matters like faecal and dead cells by rapid growing heterotrophic bacterial community as resulted by addition of jaggery as a carbon source. The nitrogen accumulation in heterotrophic bacterial cells did not last longer as those destroyed very fast and release the inorganic nitrogen to be decomposed by newly growing heterotrophic bacterial community within the biofloc system. Thus the

TAN was circulated within ammonia and floc throughout the rest of the culture period and could not rise after the well establishment of floc development procedure.

Fig.4.42. Mean (\pm SD, n=3) nitrite (NO₂) concentration in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*)



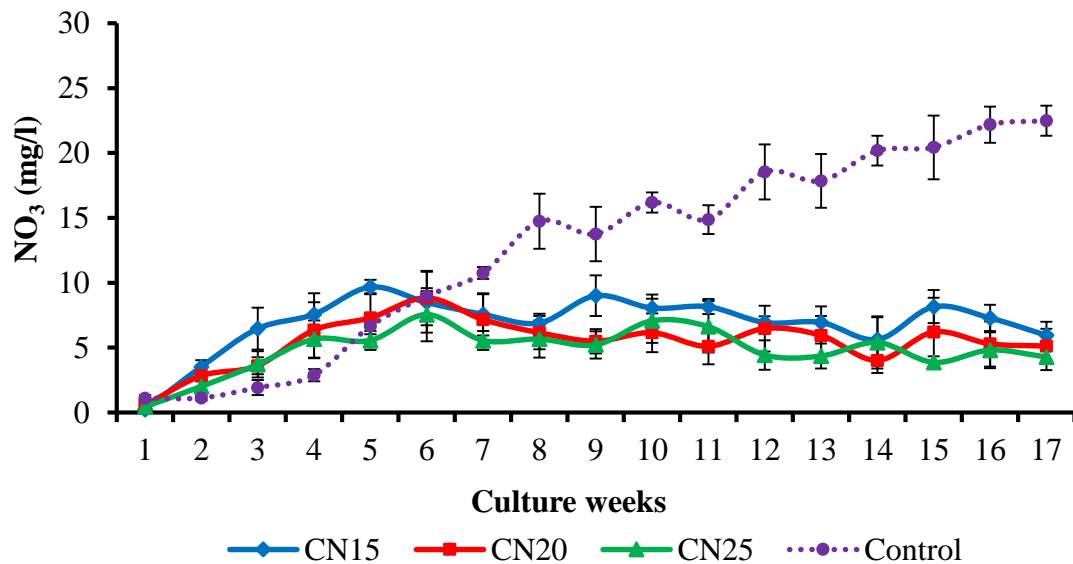
Whereas, the reason behind initial upwards of intermediate inorganic nitrogen NO₂ level were the existence of autotrophic nitrifying bacteria along with heterotrophic bacteria at starting period. Once the heterotrophic bacteria became dominant, the conversion of TAN to nitrite reduced drastically and continued till the end. In this study a mild fluctuation in the nitrite level were noticed in CN15 tanks (Fig.4.42) might be due to occurrence of chemoautotrophic nitrifying bacteria which might almost nil in higher C:N ratios biofloc tanks. Our experiments not corroborated with the findings of Liu *et al.* (2018a) and Kamilya *et al.* (2017) where fluctuations of TAN and NO₂ were observed several times in biofloc culture system due to less dominance of heterotrophic bacteria. The TAN and NO₂ levels of biofloc tanks in our study were within the permissible limits and significant ($p < 0.05$) reduction with increasing carbon to nitrogen ratios were found similar with Elayaraja *et al.* (2020), however the inorganic nitrogen dynamics within the biofloc system was too much complicated which need more investigation.

4.6.6. Nitrate (NO₃) measurements in control and biofloc systems during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

The nitrate (NO₃) accumulation in control tanks (12.63 ± 0.23 mg/l) during 120 d were found significantly higher ($p < 0.05$) than all BFT system tanks. Among BFT

system considerably higher ($p < 0.05$) level of NO_3 accumulation were found in CN15 tanks (6.86 ± 0.69 mg/l) in compare to CN20 tanks (5.44 ± 0.38 mg/l) and CN25 tanks (4.83 ± 0.45 mg/l). The nitrate concentration of all BFT tanks showed an increasing tendency till 5th to 6th week then it became consistent till end (Fig.4.43). The NO_3 level were varied between 1.11–22.49 mg/l for control tanks and 0.22–9.68 mg/l, 0.57–8.83 mg/l and 0.45–7.54 mg/l for BFT system tanks of CN15, CN20 and CN25 groups, respectively (Table 4.23). The lower nitrate values in the CN25 group compared to the CN20 and CN15 groups might be due to the higher immobilization of inorganic nitrogenous compound in higher C:N ratios group.

Fig.4.43. Mean (\pm SD, $n=3$) nitrate (NO_3) concentration in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*)



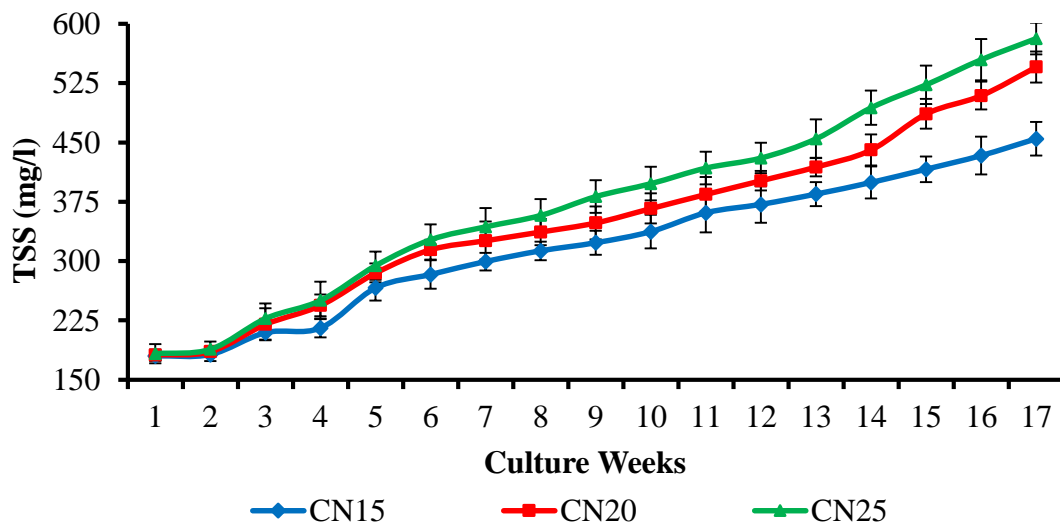
Nitrate (NO_3) was considered as comparatively safer inorganic nitrogen compound with a permissible limit of below 10 mg/l for BFT system (Emerenciano *et al.*, 2017). Nitrate was the end product of inorganic nitrogen conversion and its formation from nitrite could be possible in presence of nitrifying bacteria only. Dominance of heterotrophic bacteria in biofloc tanks prohibited such conversion of inorganic nitrogen. Low nitrate level in BFT system with higher C:N ratios might be due to the lower availability of ammonia nitrogen for oxidation by nitrifying bacteria (Khanjani *et al.*, 2021). In this study the NO_3 levels of all BFT tanks were found very stable throughout the experiment, might be due to utilization of inorganic nitrogen by heterotrophic bacteria to synthesize more bacteria (Liu *et al.* 2018a), however less but existence nitrate level in BFT tanks might be resulted by regular feed input and microbial respiration (Chen *et al.*, 2018). In control tanks the regular exchange of 15-

20% water was not found to be enough to reduce nitrite accumulation which was found almost nil in previous studies of Ahmad *et al.* (2016), Liu *et al.* (2018a) and Liu *et al.* (2018b) where up to 30-40% water in control tanks were exchanged daily. Our findings in respect of higher NO₃ level in control followed by biofloc system with lower C:N ratios were found quite similar with the previous experiments on culturing common carp (Minabi *et al.*, 2020), Nile tilapia (Elayaraja *et al.*, 2020), Golden crucian carp (Yu *et al.*, 2020a), *O. kaopingensis* Dybowski (Yu *et al.*, 2020b).

4.6.7. Total suspended solids (TSS) measurements in control and biofloc systems during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

The total suspended solids (TSS) below 500 mg/l were considered as highest limits for fish culture (Elayaraja *et al.*, 2020) because higher concentration of TSS could have various negative effects on aquatic life as well as water quality parameters. In this study the mean value of TSS measured on every week was found significantly higher ($p < 0.05$) in BFT system with highest C:N ratio of 25:1 (CN25) followed by lower C:N ratios of 20:1 (CN20) and 15:1 (CN15), which were recorded 376.88 ± 8.16 mg/l, 325.65 ± 6.98 mg/l and 310.40 ± 8.25 mg/l, respectively (Table 4.23). An increasing trend of weekly measured total suspended solids (TSS) with higher C:N ratios were observed throughout the 17th week of rearing of Nile tilapia in biofloc system which were recorded highest of 454.68 mg/l, 545.51 mg/l and 581.40 mg/l for CN15, CN20 and CN25 tanks, respectively, on 17th week (Table 4.23 and Fig.4.44).

Fig.4.44. Mean (\pm SD, n=3) total suspended solids (TSS) in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*)

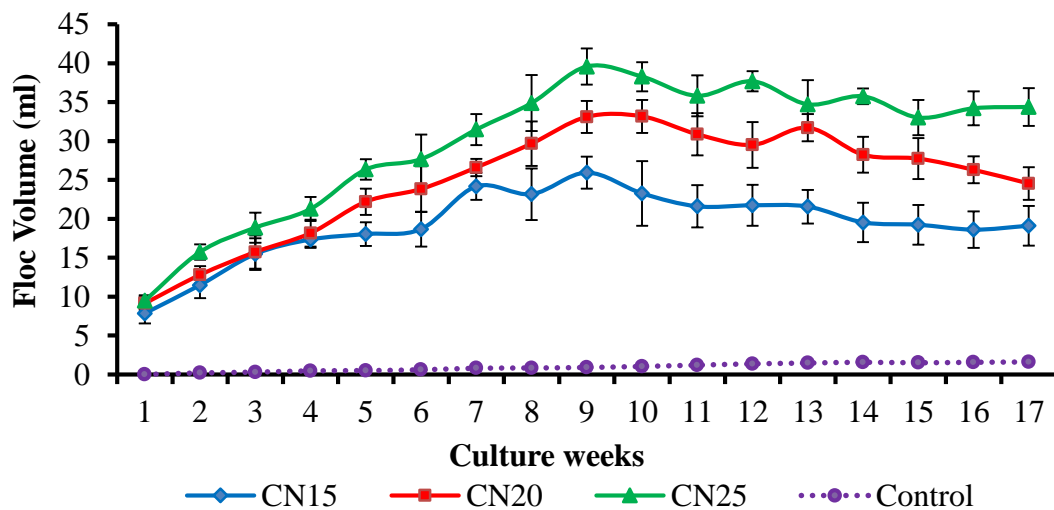


The mean TSS levels of control tanks during 17th week were found negligible of 30.57 ± 1.79 mg/l within a range between 13.96–41.62 mg/l could not be considered as an important factor. The gradual elevation of TSS throughout the culture with directly proportional to the higher C:N ratios were also found to be similar with previous studies of Minabi *et al.* (2020), Elayaraja *et al.* (2020) and Dilmi *et al.* (2022). The increasing trend of TSS level with higher C:N ratios were due to the higher density of microbial abundance in biofloc system with higher C:N ratios which had been discussed next.

4.6.8. Biofloc volume measurements in control and biofloc systems during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

Bioflocs were the combinations of algae, bacteria, protozoan and particulate organic matters like feces and uneaten feed (Hargreaves, 2013). The floc or biofloc volume of our experimental biofloc tanks measured in every week was found to be directly proportional to the higher carbon to nitrogen ratios. Significantly higher ($p < 0.05$) floc volume of 29.94 ± 0.56 ml was noticed in CN25 tanks followed by 24.90 ± 0.12 ml in CN20 tanks and 19.22 ± 0.87 ml in CN15 tanks throughout the culture period. The floc volume of BFT system tanks were varied between 7.83–25.93 ml, 9.13–33.10 ml and 9.47–39.57 ml for CN15, CN20 and CN25 groups, respectively (Table 4.23).

Fig.4.45. Mean (\pm SD, n=3) biofloc volume in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*)



The peak levels of biofloc volume in all BFT tanks were noticed in 9th week of culture which was remained almost constant with little fluctuation in rest of the culture period (Fig.4.45). In biofloc system the volume of suspended flocs was considered as a crucial limiting factor for productivity next to the dissolve oxygen (Ebeling *et al.*, 2006). Avnimelech, (1999) suggested that maintaining floc volume of 25–50 ml/l offered superior biofloc systems for tilapia as bioflocs was considered as good source of vitamins and minerals and also might have probiotic effects. Higher biofloc volume with increasing C:N ratios in BFT system were also reported previously by Panigrahi *et al.* (2017), Mianbi *et al.* (2020) and Elayaraja *et al.* (2020). The increased floc volume in higher C:N ratios biofloc system might be due to more microbial biomass synthesis resulted by daily input of jaggery along with fish feeds (Kamilya *et al.*, 2017). The type of cultured fishes also could be determining factor for floc volume in BFT system (De Schryver *et al.*, 2008). In this study the floc volume could not rise after 9th week might be due to higher consumption of floc by fast growing Nile tilapia in the BFT system.

4.6.9. Total heterotrophic counts (THC) in control and biofloc systems during culture of Nile tilapia (*Oreochromis niloticus*) for 120 d

The total heterotrophic count (THC) of BFT tanks in this study were also found to be highly influenced by higher carbon to nitrogen ratios. The THC counts of all BFT tanks were measured in 1st week, 2nd week and fortnightly after wards. The mean THC value during the culture period was found significantly different ($p < 0.05$) among BFT systems with different C:N ratios, which was recorded 8.0×10^6 cfu/ml (6.90 ± 0.04 log cfu/ml), 1.08×10^7 cfu/ml (7.02 ± 0.05 log cfu/ml) and 2.1×10^7 cfu/ml (7.25 ± 0.03 log cfu/ml) for CN15, CN20 and CN25 tanks, respectively. The THC counts were varied between 1.1×10^6 – 1.28×10^7 cfu/ml (6.04 – 7.10 log cfu/ml), 1.18×10^6 – 1.84×10^7 cfu/ml (6.07 – 7.26 log cfu/ml) and 1.1×10^6 – 5.4×10^7 cfu/ml (6.04 – 7.74 log cfu/ml) for the CN15, CN20 and CN25 tanks, respectively. THC in control tanks were found significantly lowest most ($p < 0.05$) i.e., 6.2×10^4 cfu/ml (4.78 ± 0.02 log cfu/ml) within the range of 5.8×10^4 – 7.5×10^4 cfu/ml (4.76 – 4.91 log cfu/ml) indicated the abundance of autotrophic and chemoautotrophic nitrifying bacteria in control tanks (Table 4.24).

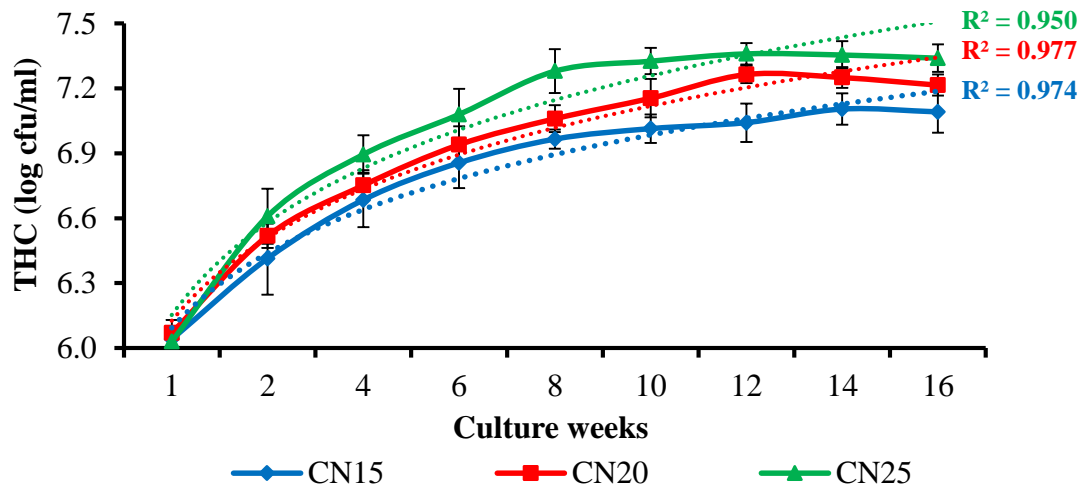
Table 4.24. Logarithmic values (mean±SD, n=3) of total heterotrophic bacteria recorded in control and biofloc tanks with different C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25), for rearing of Nile tilapia (*O. niloticus*) for 120 d

	CN15	CN20	CN25	Control
Total Heterotrophic Bacteria Count (THC) (log cfu/ml)	6.90 ^b ± 0.04 (6.04 – 7.10)	7.02 ^c ± 0.05 (6.07 – 7.26)	7.25 ^d ± 0.03 (6.04 – 7.74)	4.78 ^a ± 0.02 (4.76 – 4.91)

Values in the same row with different letters were significantly different (p< 0.05)

The heterotrophic bacterial growth patterns in BFT tanks in our study showed a typical bacterial growth curve as observed by Khanjani *et al.* (2021) and Minabi *et al.* (2020). The exponential phase of heterotrophic bacterial community in all biofloc tanks such as CN15, CN20 and CN25 groups were observed up to 8th week of culture which were remained almost constant as stationary phase with little changes till end. No declination phase of THC in biofloc tanks were observed during the culture period because of the regular input of fresh feed and external carbon sources (Fig.4.46).

Fig.4.46. Mean (±SD, n=3) total heterotrophic counts in control and biofloc tanks with different C:N ratios used for rearing of Nile tilapia (*O. niloticus*)



All the THC (log cfu/ml) growth curves for different C:N ratios were found to be fitted suitably in logarithmic trend line with R² value of 0.977, 0.950 and 0.974 for CN20, CN25 and CN15 tanks, respectively (Fig.4.46). Our findings in respect of higher heterotrophic bacterial count in BFT system with increasing carbon to nitrogen ratios were found to be similar with previous findings of Panigrahi *et al.* (2017) and Minabi *et al.* (2020). Higher C:N ratios favoured the growth of heterotrophic bacteria

and thus produced more flocs in the biofloc systems was also reported by (Xu *et al.*, 2016, 2022).

4.6.9. Pearson Correlation test ($p < 0.01$, 2-tailed) between different water quality parameters in control and biofloc tanks with different C:N ratios of 15:1, 20:1 and 25:1 for rearing of Nile tilapia (*O. niloticus*) for 120 d

The correlations between fortnights measurements of different water quality parameters such as temperature, pH, dissolve oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO_2), nitrate (NO_3), total alkalinity (TA), total suspended solids (TSS), biofloc volume (BV) and total heterotrophic counts (THC) were done by Pearson Correlation test at 99% significant level for each BFT system with different C:N ratios of 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25). In case of control tanks the correlations were measured for the water quality parameters such as temperature, pH, DO, TAN, NO_2 , NO_3 , total alkalinity only. The correlation matrix of water quality parameters in Table 4.25, Table 4.26 and Table 4.27 for three biofloc systems with different carbon to nitrogen ratios such as CN15, CN20 and CN25, respectively, showed almost similar model with minor deviations while considered those at 99% significant level ($p < 0.01$).

Dissolve oxygen (DO), pH and total alkalinity were found positively correlated ($p < 0.01$) with each other in all BFT systems except in CN20 where the relation between DO and total alkalinity was highly positive but insignificant ($p > 0.01$). Total alkalinity provided buffering capacity to prevent the abrupt changes of pH in water, hence the depletion of total alkalinity in all BFT tanks might affected the buffering capacity which resulted decreasing trend of pH in our study. Total alkalinity could be reduced as resulted of production of acidic compounds by fast growing heterotrophic bacteria, and also might be inorganic carbon utilization by the photosynthetic plankton community developed in the BFT system as the experimental setup was provided with 24 h light. Though there were no physical-chemical connections in between DO and pH, but in present study both were found downwards with proceeding of culture duration. Dissolve oxygen consumption by fish and floc community including heterotrophic bacteria were the major reason behind the reduction of DO in BFT system. Hargreaves (2012) stated that alkalinity, pH and DO concentration were the influencing factors for floc formation. The both pH and DO of all BFT tanks were found negatively correlated with both biofloc volume (BV) and

total heterotrophic count (THC) but not significant ($p>0.01$), which indicated the well maintained biofloc setup in this study (Table 4.25, 4.26 and 4.27).

The pH of all BFT system were found negatively correlated ($p<0.01$) with total suspended solids (TSS). The TSS were found to be an increasing trend in all BFT system due to higher heterotrophic bacterial abundance as TSS was used to describe floc level (Luo *et al.*, 2014). Similarly, dissolve oxygen (DO) were found negatively correlated with TSS in all BFT system but it was significant ($p<0.01$) for CN20 and CN25 only. As stated above the TSS was directly associated with abundance of heterotrophic bacterial population, hence the DO might be lowered due to higher oxygen consumption (Table 4.25, 4.26 and 4.27).

Total ammonia nitrogen (TAN) were found positively correlated ($p<0.01$) with nitrite (NO_2) in all BFT tanks as both TAN and NO_2 elevated and decreased simultaneously in all BFT systems in this study. Both TAN and NO_2 were found with an elevated level in initial period due to lower utilization of nitrogenous wastes by heterotrophic bacteria and the nitrification of TAN by nitrifying bacteria, respectively, which decreased drastically afterwards and remained within permissible limits till end (Table 4.25, 4.26 and 4.27).

Significant negative correlations ($p<0.01$) were existed with NO_2 against TSS, biofloc volume (BV) and total heterotrophic counts (THC) in all BFT tanks. Dominance of heterotrophic bacteria in the BFT system increased the biofloc volume, hence positive correlations ($p<0.01$) were found between THC and BV in all BFT tanks. Increased total suspended solids over time also resulted by higher BV and THC (Kamilya *et al.*, 2017), hence strong positive correlation ($p<0.01$) were noticed between THC and TSS in all BFT system. It was already described that higher THC could minimize both the TAN and NO_2 levels in BFT system. Similarly, strong negative correlation between TAN against TSS, BV and THC were also found in all BFT tanks (Table 4.25, 4.26 and 4.27).

In control tanks DO, pH and total alkalinity were found positively correlated ($p<0.01$) with each other and those were altogether negatively correlated ($p<0.01$) with NO_3 concentration. In any culture condition DO might decrease over time due to higher oxygen demand by cultured species. The buffering capacities were decreased due to increased nitrogenous waste that resulted lowering of pH in culture water. Nitrates accumulation was the most common phenomenon in presence of higher nitrifying and chemoautotrophic bacterial population (Table 4.28).

Table 4.25. Correlations between water quality parameters in biofloc system with C:N ratio of 15:1 (CN15) for rearing Nile tilapia (*O. niloticus*) for 120 d

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA	TSS	BV	THC
Temp	1									
pH	-.309	1								
DO	-.304	.946*	1							
TAN	.057	.568	.358	1						
NO ₂	.073	.596	.448	.935*	1					
NO ₃	-.721	.044	.082	-.526	-.646	1				
TA	-.360	.949*	.968*	.285	.343	.235	1			
TSS	.141	-.875*	-.739	-.877*	-.889*	.312	-.693	1		
BV	-.025	-.214	-.073	-.822	-.865*	.668	.067	.604	1	
THC	.018	-.689	-.515	-.955*	-.981*	.527	-.444	.941*	.817	1

Table 4.26. Correlations between water quality parameters in biofloc system with C:N ratio of 20:1 (CN20) for rearing Nile tilapia (*O. niloticus*) for 120 d

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA	TSS	BV	THC
Temp	1									
pH	-.152	1								
DO	-.187	.969*	1							
TAN	.121	.555	.489	1						
NO ₂	.181	.601	.560	.977*	1					
NO ₃	-.693	.287	.308	-.476	-.481	1				
TA	-.143	.835*	.827	.020	.101	.589	1			
TSS	-.036	-.912*	-.880*	-.832	-.870*	.089	-.558	1		
BV	.122	-.460	-.460	-.914*	-.865*	.358	.089	.727	1	
THC	.098	-.744	-.727	-.929*	-.933*	.241	-.265	.926*	.904*	1

Table 4.27. Correlations between water quality parameters in biofloc system with C:N ratio of 25:1 (CN25) for rearing of Nile tilapia (*O. niloticus*) for 120 d

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA	TSS	BV	THC
Temp	1									
pH	-.214	1								
DO	-.296	.991*	1							
TAN	.206	.525	.487	1						
NO ₂	.221	.563	.506	.980*	1					
NO ₃	-.683	.101	.203	-.588	-.642	1				
TA	-.227	.840*	.871*	.088	.097	.475	1			
TSS	-.056	-.899*	-.868*	-.829	-.848*	.257	-.606	1		
BV	.028	-.605	-.587	-.936*	-.932*	.472	-.180	.826	1	
THC	-.031	-.674	-.638	-.947*	-.967*	.498	-.235	.887*	.984*	1

*Correlation is significant at the 0.01 level (2-tailed).

Temp: Temperature, DO: Dissolve oxygen, TAN: Total ammonia nitrogen, TA: Total alkalinity, TSS: Total suspended solids, BV: Biofloc volume, THC: Total heterotrophic count. [Colour intensity towards green indicated positive correlation and red indicated negative correlation whereas, yellow indicated nil correlation.]

Table 4.28. Correlations between water quality parameters in control (without biofloc) for rearing of Nile tilapia (*O. niloticus*) for 120 d

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA
Temp	1						
pH	-.121	1					
DO	-.107	.976*	1				
TAN	.213	-.656	-.648	1			
NO ₂	-.265	-.624	-.556	.690	1		
NO ₃	.135	-.909*	-.812	.573	.675	1	
TA	-.145	.982*	.921*	-.665	-.677	-.968*	1

*Correlation is significant at the 0.01 level (2-tailed).

Temp: Temperature, DO: Dissolve oxygen, TAN: Total ammonia nitrogen, TA: Total alkalinity. [Colour intensity towards green indicated positive correlation and red indicated negative correlation whereas, yellow indicated nil correlation.]

4.6.10. Temperature measurements in control and biofloc system during culture of Singhi or Asian stinging catfish (*Heteropneustes fossilis*) for 180 d

In this study the culture of Singhi under biofloc system with different carbon to nitrogen ratio of 8:1 (CN8), 12:1 (CN12), 16:1 (CN16) along with control (without biofloc) were started on dated 23th September, 2021. The culture were continued for 180 d and ended on dated 21th March, 2022. The experiment was conducted in post monsoon, winter and spring season when the temperature became quite low which might not favoured biofloc system as the ideal temperature for heterotrophic bacteria was 25°C above (Azim and Little, 2008). Hence, thermostats (RSElectrical; China made) were used in this study to maintain the water temperature above 26°C throughout the experiment in individual cement tank hold 1200 l water for rearing of *Heteropneustes fossilis*. The water temperature range was recorded between 26.13–27.46°C for control and 26.14–27.34°C, 26.17–27.11°C and 26.21–27.33°C for BFT system of CN8, CN12 and CN16, respectively (Table 4.29).

The experimental setup was shaded with transparent asbestos sheet with facilities of diffuse sunlight though it and well natural air circulation. Each tank was provided with 100 watt bulb at 4’ top at night. The water temperatures were measured at 7 am daily and uniform deviations of temperature in all BFT treated and control tanks were observed throughout 180 d (Fig.4.47). No significant differences (p>0.05) of water temperature were observed among BFT treated and control tanks throughout the experiment, where the mean water temperature for 180 d were recorded as 26.62±0.06, 26.67±0.06, 26.64±0.05 and 29.69±0.05 for the control and BFT system of CN8, CN12 and CN16, respectively (Table 4.29). According to the Azim and Little

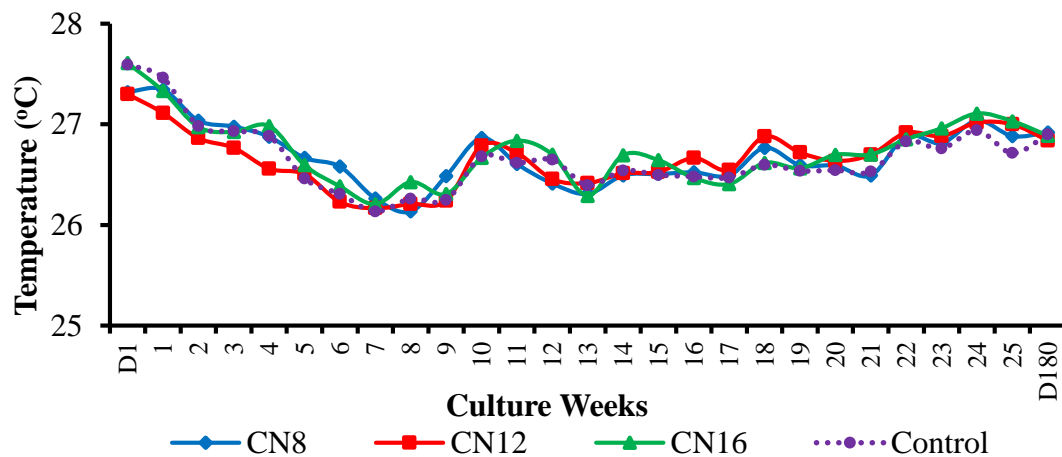
(2008) the optimal temperature to maintain floc stability were ranges from 26 to 32°C, hence temperature were maintained beyond 26°C for all the biofloc treated and control tanks in this study. The temperature was measured within the proper range of floc development (Hostins *et al.*, 2015).

Table 4.29. Mean (\pm SD, n=3) values of water quality parameters recorded in control and biofloc tanks with different C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16), for rearing of Singhi (*H. fossilis*) for 180 d

	CN8	CN12	CN16	Control
Temperature (°C) at 7.00 am	26.67 \pm 0.06 (26.14 – 27.34)	26.64 \pm 0.05 (26.17 – 27.11)	26.69 \pm 0.05 (26.21 – 27.33)	26.62 \pm 0.06 (26.13 – 27.46)
pH at 5.00 pm	7.78 ^a (7.44 – 8.10)	7.96 ^c (7.65 – 8.18)	7.86 ^b (7.54 – 8.19)	8.01 ^d (7.76 – 8.28)
Total Alkalinity (mg/l)	162.84 ^a \pm 0.42 (156.33–167.59)	167.28 ^{bc} \pm 2.28 (162.59–172.67)	164.34 ^{ab} \pm 1.93 (159.76–172.54)	169.66 ^c \pm 2.92 (165.68–173.30)
Dissolve Oxygen (mg/l) 7.00 am	6.47 ^{ab} \pm 0.05 (6.08 – 6.94)	6.52 ^b \pm 0.02 (6.02 – 7.01)	6.43 ^a \pm 0.05 (5.93 – 7.02)	6.70 ^c \pm 0.02 (6.25 – 7.03)
Total Ammonia Nitrogen (mg/l)	0.51 ^c \pm 0.02 (0.26 – 0.94)	0.21 ^b \pm 0.01 (0.09 – 0.75)	0.16 ^a \pm 0.00 (0.07 – 0.72)	0.64 ^d \pm 0.03 (0.35 – 0.91)
Nitrite (NO ₂) (mg/l)	0.14 ^c \pm 0.01 (0.08 – 0.25)	0.07 ^b \pm 0.01 (0.02 – 0.24)	0.05 ^a \pm 0.00 (0.02 – 0.22)	0.15 ^c \pm 0.01 (0.06 – 0.23)
Nitrate (NO ₃) (mg/l)	10.04 ^c \pm 0.25 (1.57 – 17.25)	5.87 ^b \pm 0.03 (1.11 – 9.20)	4.99 ^a \pm 0.09 (0.93 – 9.27)	13.15 ^d \pm 0.47 (1.10 – 23.01)
Total Suspended Solids (mg/l)	237.40 ^b \pm 5.21 (148.75–299.58)	270.52 ^c \pm 2.40 (155.40–346.17)	289.53 ^d \pm 3.31 (171.12–385.12)	20.85 ^a \pm 0.46 (11.46–31.52)
Biofloc volume (ml)	13.13 ^b \pm 0.46 (5.8 – 16.1)	16.98 ^c \pm 0.30 (10.1 – 19.6)	19.94 ^d \pm 0.56 (10.8 – 25.3)	1.09 ^a \pm 0.03 (0.2 – 1.7)

Values in the same row with different letters are significantly different (p<0.05).

Fig.4.47. Mean(n=3) temperature recorded in control and biofloc tanks with different C:N ratios used for rearing of Singhi (*H. fossilis*) for 180 d

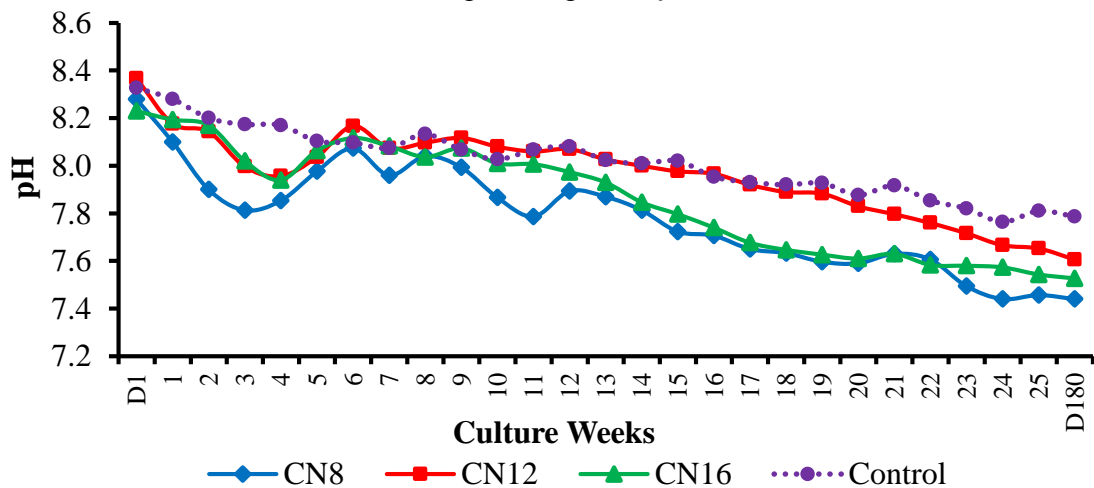


4.6.11. Measurements of pH in control and biofloc system during culture of Singhi or Asian stinging catfish (*Heteropneustes fossilis*) for 180 d

According to (Avnimelech, 2007) the pH should not less than 6.5 and must not greater than 9 in biofloc. A significantly lower trend ($p < 0.05$) of pH at 5.00 pm were recorded in all biofloc rearing tanks. Those lowering trends were significantly ($p < 0.05$) lowest most in CN8 groups followed by CN16 and CN12 groups. We observed the different declining patterns of pH for BFT system and control, as we noticed in our previous studies on Nile tilapia. The mean pH value of control (8.01 ± 0.02) within the range of 7.76–8.28 was significantly higher ($p < 0.05$) than all BFT system. The decreasing of pH in control was found downwards without any massive fluctuation, whereas, in BFT tanks an initial prominent drop of pH were observed in 3rd to 4th week which rose again up to 6th week (Fig.4.48). The mean value of pH during 180 d were noticed significantly different ($p < 0.05$) among BFT treated tanks with different C:N ratios. Those were found highest 7.96 followed by 7.86 and 7.78 in CN12, CN16 and CN8 groups, respectively (Table 4.29). The pH value of the biofloc tanks such as CN8, CN12 and CN16 were found in between 7.44–8.10, 7.65–8.18 and 7.54–8.19, respectively.

The decreasing pH level in BFT tanks were probably due to the incorporation of carbon sources to the biofloc medium which activated the heterotrophic bacteria to break down of organic matter (Zafar *et al.*, 2021). The pH values observed in our study were found optimal for culture of *H. fossilis* in BFT as described by Shamsuddin *et al.* (2022) and Zafar *et al.* (2021).

Fig.4.48. Weekly mean ($n=3$) pH recorded in control and biofloc tanks with different C:N ratios used for rearing of Singhi (*H. fossilis*) for 180 d

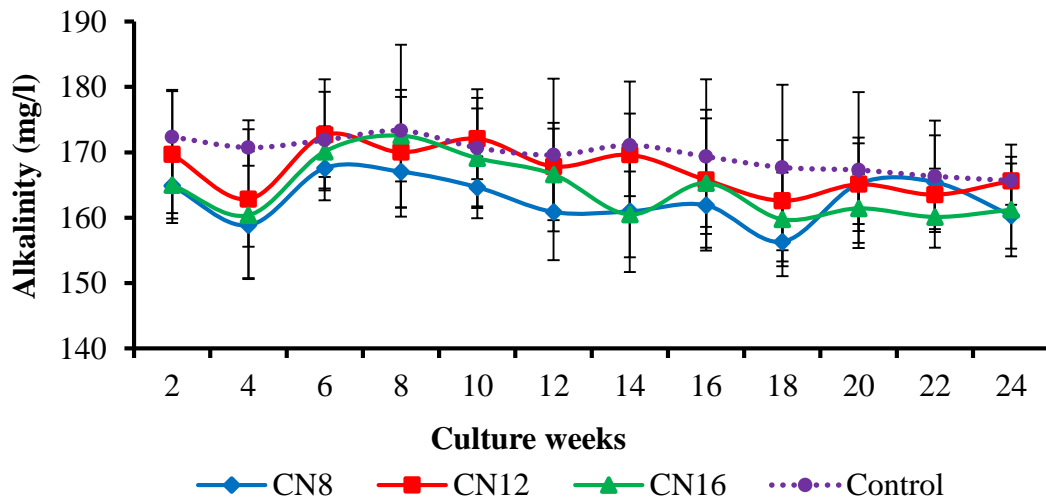


4.6.12. Measurements of total alkalinity in control and biofloc system during culture of Singhi or Asian stinging catfish (*H. fossilis*) for 180 d

The mean value total alkalinity of control Singhi groups (169.66 ± 2.92 mg/l) within a range of 165.68–173.30 mg/l were noticed significantly higher ($p < 0.05$) than BFT system of CN16 and CN8 groups but not from CN12 groups. The total alkalinity of CN12 (167.28 ± 2.28 mg/l) tanks within a range of 162.59–172.67 mg/l were found significantly higher ($p < 0.05$) than CN8 (162.84 ± 0.42 mg/l) tanks but not from CN16 (164.34 ± 1.93 mg/l) biofloc tanks. No significance differences ($p > 0.05$) of total alkalinity were observed between CN8 and CN16 biofloc tanks for rearing Singhi. Overall decreasing trends of fortnightly measured total alkalinity were observed throughout the 180 d culture for control and all biofloc treatment Singhi groups (Table 4.29).

The fluctuation of total alkalinity in BFT tanks (Fig.4.49) were found proportional to the changes of pH level in this study indicated the buffering capacity of the biofloc system highly influenced the pH level which were needed to be adjusted repeatedly by addition of $\text{Ca}(\text{HCO}_3)_2$ or NaHCO_3 (Azim and Little, 2008). Reduced level of both pH and alkalinity in the biofloc treatment groups compared to the control was due to the use of inorganic carbon by heterotrophic bacteria (Ebeling *et al.*, 2006). Our finding about reduced total alkalinity in BFT was supported by Panigrahi *et al.* (2017) and Menaga *et al.* (2019).

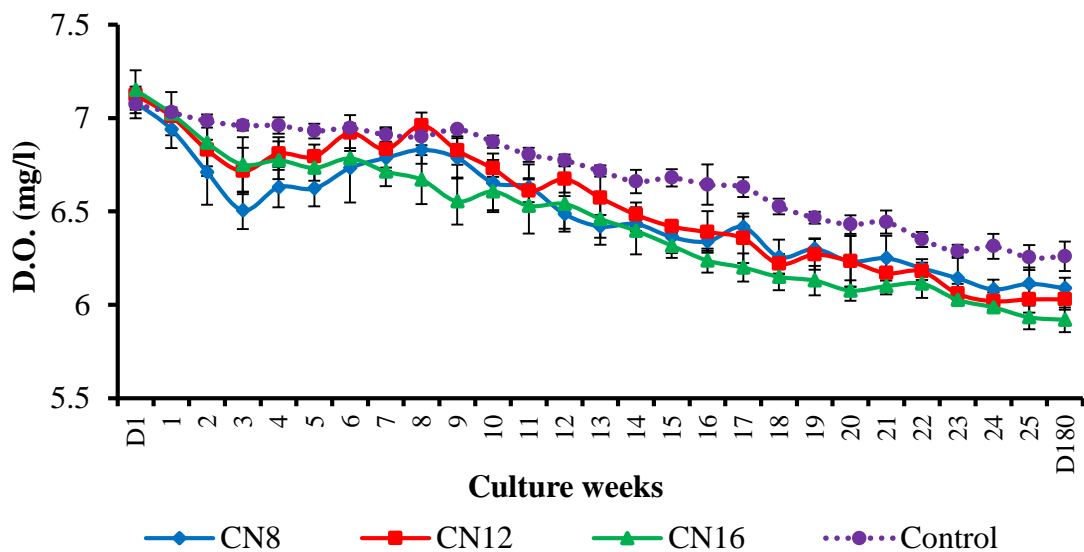
Fig.4.49. Mean (\pm SD, $n=3$) total alkalinity in control and biofloc tanks with different C:N ratios used for rearing of Singhi (*H. fossilis*) for 180 d



4.6.13. Measurements of dissolve oxygen in control and biofloc system during culture of Singhi or Asian stinging catfish (*H. fossilis*) for 180 d

In an intensive aquaculture system maintenance of adequate dissolved oxygen level was very crucial for decomposition of organic matters by the constant metabolic activity of microbes (Green and McEntire, 2017). The weekly measured mean dissolve oxygen (DO) levels of all biofloc treatment and control Singhi groups were found downwards throughout the experiment. The decreasing trends were quite consistent in control where in BFT groups initial drops of DO were observed (Fig. 4.50). The mean DO level of control (6.52 ± 0.02 mg/l) within a range of 6.25–7.03 mg/l was found significantly upper ($p < 0.05$) than all BFT treated Singhi groups. Among BFT groups the CN12 (6.52 ± 0.02 mg/l) and CN8 (6.47 ± 0.05 mg/l) were found insignificantly ($p > 0.05$) different, whereas, the mean DO level of BFT groups CN16 (6.43 ± 0.05 mg/l) was found significantly lower ($p < 0.05$) than CN12 groups but not than CN8 groups. The DO levels of all biofloc groups were recorded in between 6.08–6.94 mg/l, 6.02–7.01 mg/l and 5.93–7.02 mg/l for CN8, CN12 and CN16 groups respectively (Table 4.29). The lower DO level in CN16 biofloc groups were due to the activities of the comparatively larger heterotrophic community and also increased carbon dioxide concentration than BFT systems of CN8 and CN12 (Long *et al.*, 2015). However, desired DO levels for survival of *H. fossilis* were maintained in all experimental groups (Shamsuddin *et al.*, 2022 and Zafar *et al.*, 2021).

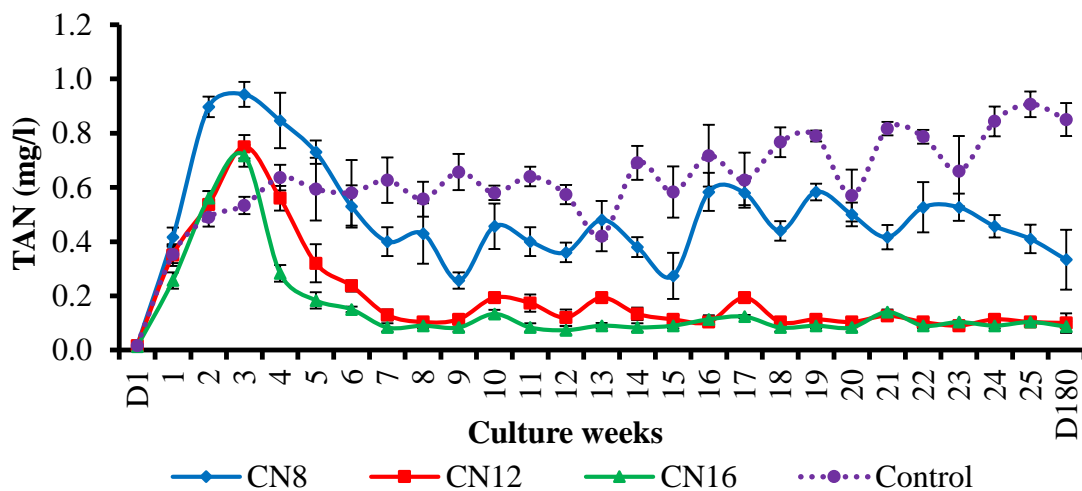
Fig.4.50. Weekly mean (\pm SD, n=3) dissolve oxygen in control and biofloc tanks with different C:N ratios used for rearing of Singhi (*H. fossilis*) for 180 d



4.6.14. Measurements of total ammonia nitrogen (TAN) and nitrite (NO₂) in control and biofloc system during culture of Singhi or Asian stinging catfish (*Heteropneustes fossilis*) for 180 d

The weekly measured mean total ammonia nitrogen (TAN) of control and all biofloc treatment Singhi groups were found significantly different ($p < 0.05$) with each other. The highest TAN was found in control (0.64 ± 0.03 mg/l) within a limit of 0.35–0.91 mg/l which was followed by BFT tanks of CN8 (0.51 ± 0.02 mg/l) within a range of 0.26–0.94 mg/l, CN12 (0.21 ± 0.01 mg/l) within a range of 0.09–0.75 mg/l, and, CN16 (0.16 ± 0.00 mg/l) had a range of 0.07–0.72 mg/l (Table 4.29). The TAN levels of both CN12 and CN16 tanks elevated suddenly up to 3rd weeks of culture to reach the maximum level and within next four weeks it reduced to below 0.2 mg/l, and continued at the almost same level till end, without any abrupt elevations. The TAN of CN8 was found with an initial elevation level up to 8th week but it was found inconsistent throughout 180 d of culture period. The TAN level of control groups were also found inconsistent throughout experiment but were recorded within the maximum permissible limit below 1 ppm (Fig.4.51).

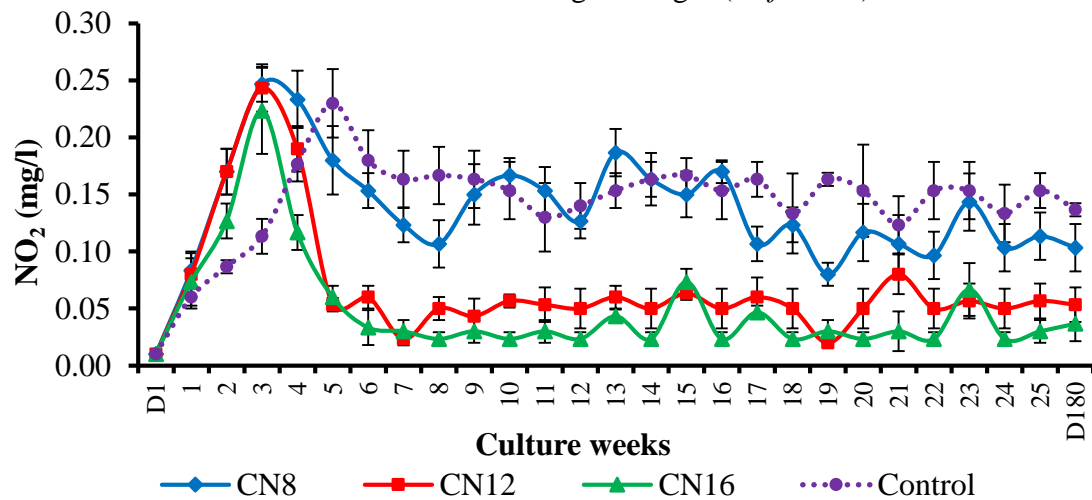
Fig.4.51. Weekly mean (\pm SD, $n=3$) total ammonia nitrogen in control and biofloc tanks with different C:N ratios for rearing of Singhi (*H. fossilis*) for 180 d



Alike TAN level, significant differences ($p < 0.05$) were found between control and all biofloc groups while considering the weekly measured mean nitrite (NO₂) concentration. It was recorded noticeably higher ($p < 0.05$) level of 0.15 ± 0.01 mg/l in control followed by 0.14 ± 0.01 mg/l, 0.07 ± 0.01 mg/l and 0.05 ± 0.00 mg/l in CN8, CN12 and CN16 tanks, respectively (Table 4.29). Sudden elevation at beginning followed by a consistent lower level of NO₂ concentration till end in CN12 and CN16

biofloc tanks were found analogous to the TAN curve just discussed above. The NO₂ concentrations of CN8 biofloc and control group showed unpredictable elevation overtime but below the maximum permissible limit throughout the experiment (Fig.4.52). The NO₂ concentrations in control and biofloc groups CN8, CN12 and CN16 were recorded in between 0.06–0.23 mg/l, 0.08–0.25 mg/l, 0.02–0.24 mg/l and 0.02–0.22 mg/l, respectively (Table 4.29).

Fig.4.52. Weekly mean (\pm SD, n=3) nitrite concentration in control and biofloc tanks with different C:N ratios for rearing of Singhi (*H. fossilis*) for 180 d



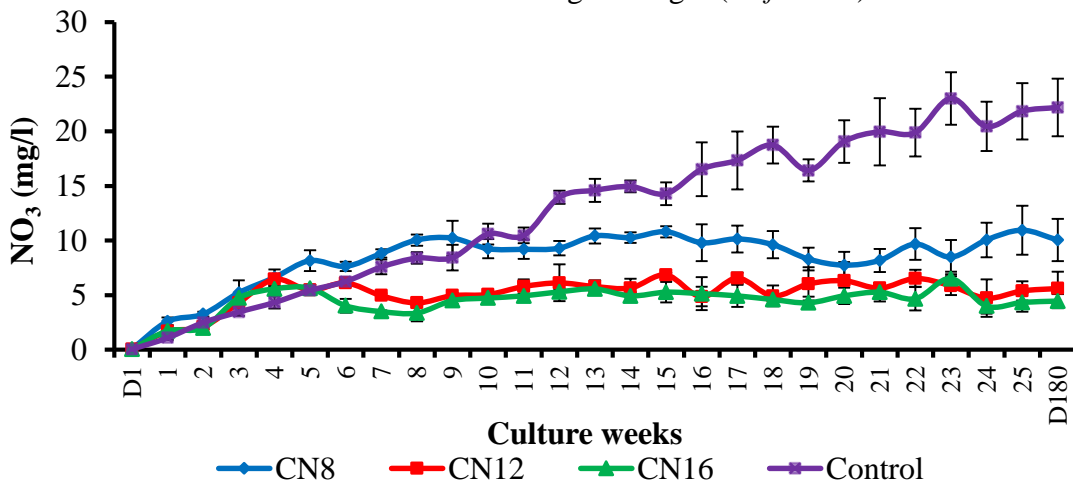
Low concentrations of total ammonia nitrogen and nitrite in biofloc groups with higher C:N ratios were due to the effective assimilation of those nitrogenous compounds by the microbial community in new cellular proteins (Burford *et al.*, 2004 and Ebeling *et al.*, 2006). Azim and Little (2008) showed that constant floc formation was needed to control the excess amount of ammonia in the biofloc system. Whereas higher and fluctuating TAN and nitrite level in CN8 biofloc groups probably due to the lower floc formation that might increase the ammonia level (Liu *et al.*, 2018a). Higher NO₂ level in CN8 tanks were due to the less dominance of heterotrophic bacteria over chemoautotrophic nitrifying bacteria, in compare to higher C:N ratios biofloc system. Due to the regular water exchange up to 15-20% in control tanks the TAN and NO₂ level were found within the desirable limits throughout the experiment. The weekly measured TAN and NO₂ concentration of BFT systems in this study also found quite similar with the observation of Shamsuddin *et al.* (2022) during culture of *H. fossilis* in biofloc system.

4.6.15. Measurements of nitrate (NO₃) in control and biofloc system during culture of Singhi or Asian stingray catfish (*H. fossilis*) for 180 d

The accumulation of nitrate (NO₃) in control and all biofloc groups were found significantly ($p < 0.05$) different with each other. Highest mean nitrate level during 180 d culture was recorded as 13.15 ± 0.47 mg/l within a range of 1.10–23.01 mg/l in control, followed by 8.59 ± 0.36 mg/l varied between 2.60–10.94 mg/l in CN8 tanks, 5.32 ± 0.04 mg/l varied between 1.68–6.53 mg/l in CN12 tanks and 4.40 ± 0.04 mg/l varied between 1.73 – 6.25 mg/l in CN16 biofloc groups (Table 4.29).

In this study the low nitrate level in BFT system with higher C:N ratios might be due to the lower availability of ammonia nitrogen for oxidation by nitrifying bacteria (Khanjani *et al.*, 2021). Accumulation of NO₃, but far below than optimum level indicated the lower but still existence of the nitrification process within the BFT system (Fig.4.53). Similar findings were also reported by Zafar *et al.* (2021) while culturing Singhi in biofloc system. However, the increasing NO₃ concentration in contrast to the decreasing NO₂ concentrations after normalization of initial TAN level in biofloc systems established the stabilized BFT system (Zafar *et al.*, 2021). In our studies the NO₃ levels of BFT tanks were found very low throughout the experiment, might be due to better utilization of inorganic nitrogen by heterotrophic bacteria (Liu *et al.* 2018a), however a slow but steady increase trend of nitrate level in control tanks might be resulted by **microbial respiration** (Chen *et al.*, 2018). In control tanks the regular exchange of 15-20% water was not found sufficient to reduce nitrate accumulation.

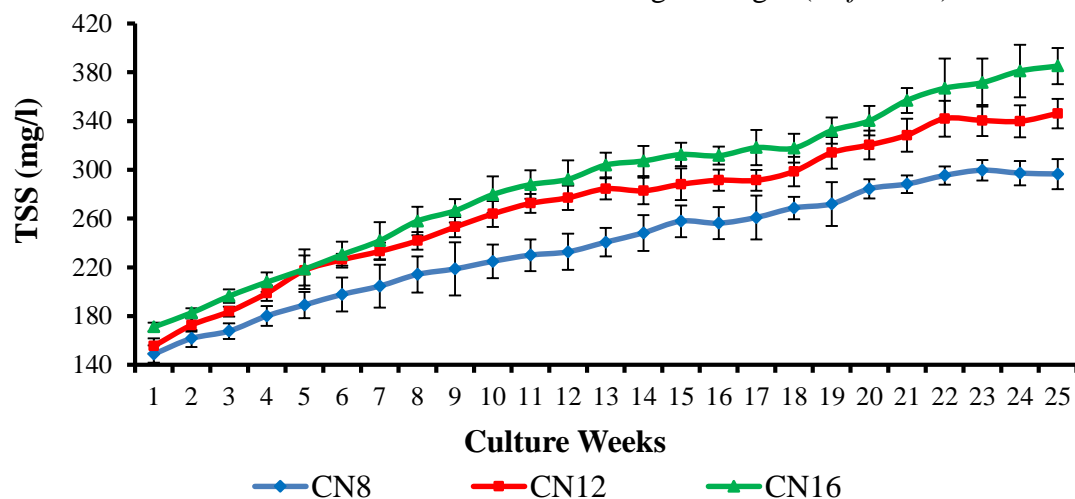
Fig.4.53. Weekly mean (\pm SD, n=3) nitrate concentration in control and biofloc tanks with different C:N ratios for rearing of Singhi (*H. fossilis*) for 180 d



4.6.16. Total suspended solids (TSS) in control and biofloc system during culture of Singhi or Asian stinging catfish (*Heteropneustes fossilis*) for 180 d

The overall mean total suspended solids (TSS) throughout culture period were found significantly higher ($p < 0.05$) with increasing C:N ratios. The highest TSS was recorded as 289.53 ± 3.31 mg/l in CN16 tanks followed by 270.52 ± 2.40 mg/l and 237.40 ± 5.21 mg/l in CN12 and CN8 tanks, respectively. The TSS level of all biofloc tanks showed an increasing trend till end of the experiment varied in between 148.75–299.58 mg/l, 155.40–346.17 mg/l and 171.12–385.12 mg/l for CN8, CN12 and CN16 BFT groups, respectively (Table 4.29 and Fig.4.54). Previous studies reported that TSS concentrations could gradually increase in the zero or low water exchange biofloc system with increased C:N ratios due to accumulation of high amount of organic substances and subsequently increased the growth rate of heterotrophic bacteria as well as other microbial biomass (Minabi *et al.*, 2020, Elayaraja *et al.*, 2020 and Dilmi *et al.*, 2022). The total suspended solids (TSS) should be below 500 mg/l in biofloc system (Elayaraja *et al.*, 2020) as higher concentration of TSS could have harmful effects on water quality and species respiration performance (Minabi *et al.*, 2020). The value of TSS in our study was recorded within the optimal range for fish culture.

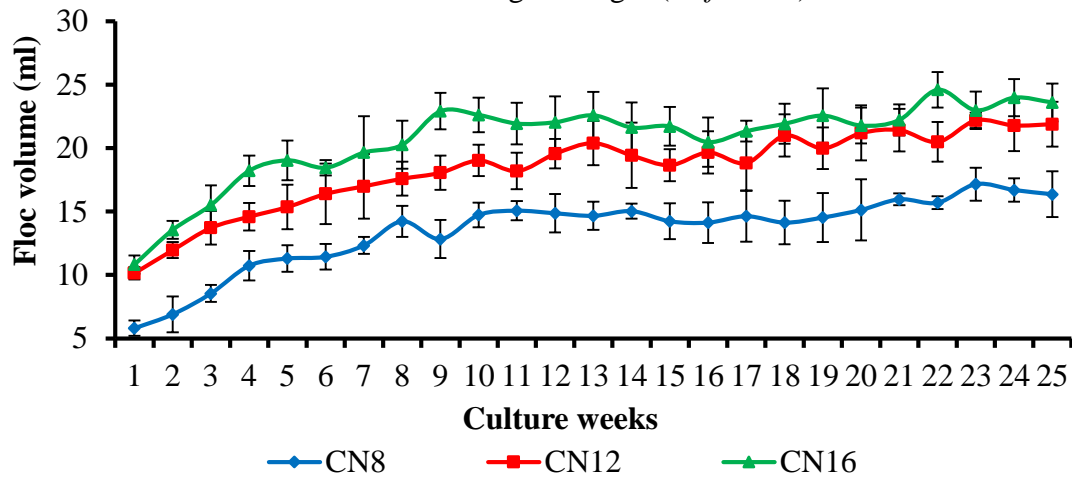
Fig.4.54. Weekly mean (\pm SD, $n=3$) total suspended solids in control and biofloc tanks with different C:N ratios for rearing of Singhi (*H. fossilis*) for 180 d



4.6.17. Measurements of biofloc volume in control and biofloc system during culture of Singhi or Asian stinging catfish (*H. fossilis*) for 180 d

Higher significant ($p < 0.05$) biofloc volume were found with higher carbon to nitrogen ratio BFT tanks. The mean of weakly measured biofloc volume (BV) of BFT tanks were found significantly higher ($p < 0.05$) with increased carbon to nitrogen ratios. Biofloc volume of CN16 biofloc tanks were recorded highest as 20.66 ± 0.41 ml that varied between 10.8–24.6 ml and it was found quite consistent in between 20–25 ml after 7th week of culture. Biofloc volume of CN12 and CN8 biofloc tanks were noted as 18.35 ± 0.24 ml and 13.49 ± 0.34 ml that varied between 10.1–21.9 ml and 5.8–16.7 ml, respectively (Table 4.29 and Fig.4.55).

Fig.4.55. Weekly mean (\pm SD, n=3) biofloc volume in control and biofloc tanks with different C:N ratios for rearing of Singhi (*H. fossilis*) for 180 d



The increased floc volume in higher C:N ratios biofloc system was found due to higher microbial biomass synthesis resulted by regular addition of jaggery and fish feeds (Kamilya *et al.*, 2017). Elevated biofloc volume in BFT system with higher C:N ratios were also previously reported by Panigrahi *et al.* (2017), Mianbi *et al.* (2020) and Elayaraja *et al.* (2020). In this study the floc volume could not exceeds too much throughout the culture period probably due to the consumption of floc by *H. fossilis* to mitigate its feeding requirements within biofloc system.

4.6.18. Total heterotrophic count in control and biofloc system during culture of Singhi or Asian stinging catfish (*Heteropneustes fossilis*) for 180 d

The total heterotrophic count (THC) of biofloc system tanks were found directly proportional to the higher C:N ratios in this study. Significantly highest ($p < 0.05$) THC of 6.74 ± 0.06 log cfu/ml (6.48×10^6 cfu/ml) was recorded in CN16 tanks followed by THC of 6.56 ± 0.06 log cfu/ml (4.14×10^6 cfu/ml) and 6.34 ± 0.02 log

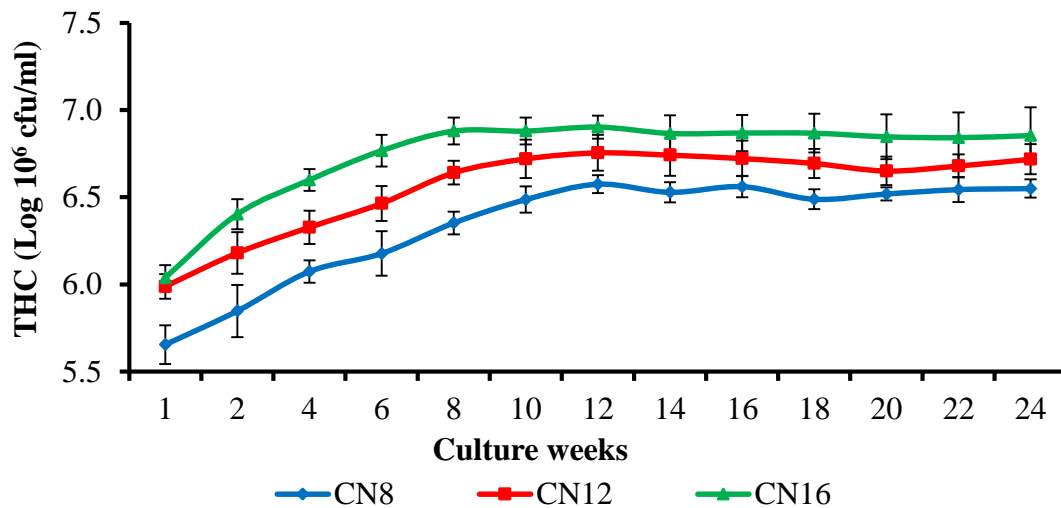
cfu/ml (2.58×10^6 cfu/ml) in CN12 and CN8 biofloc tanks, respectively. The THC levels of all BFT systems were varied between 5.65–6.58 log cfu/ml, 5.99–6.76 log cfu/ml and 6.04–6.90 log cfu/ml for CN8, CN12 and CN16 groups, respectively (Table 4.30). The THC of all BFT systems were found consistent from 10th week of culture (Fig.4.56). Higher heterotrophic bacterial count in BFT system with increasing carbon to nitrogen ratios while culturing Singhi in this study were found to be related with previous findings of Panigrahi *et al.* (2017) and Minabi *et al.* (2020). Elevated C:N ratios promoted the growth of heterotrophic bacteria as well as production of more bioflocs in the was also reported by Xu *et al.* (2016 and 2022).

Table 4.30. Logarithmic values (mean±SD, n=3) of total heterotrophic bacteria found in control and biofloc tanks with different C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1, for rearing of Singhi (*H. fossilis*) for 180 d

	CN8	CN12	CN16	Control
Total Heterotrophic Bacteria Count (THC) (log cfu/ml)	6.34 ^b ± 0.02 (5.65 – 6.58)	6.56 ^c ± 0.04 (5.99 – 6.76)	6.74 ^d ± 0.06 (6.04 – 6.90)	4.81 ^a ± 0.02 (4.76 – 4.91)

Values in the same row with different letters were significantly different (p<0.05)

Fig.4.56. Weekly mean (±SD, n=3) total heterotrophic count in control and biofloc with different C:N ratios for rearing of Singhi (*H. fossilis*) for 180 d



4.6.19. Pearson Correlation test (p<0.01, 2-tailed) between different water quality parameters in control and biofloc tanks with different C:N ratios of 8:1, 12:1 and 16:1 for rearing of Singhi (*H. fossilis*) for 180 d

The correlation between different water quality parameters such as temperature, pH, dissolve oxygen (DO), total ammonia nitrogen (TAN), nitrite (NO₂),

nitrate (NO₃), total alkalinity (TA), total suspended solids (TSS), biofloc volume (BV) and total heterotrophic counts (THC) for all three biofloc systems with different carbon to nitrogen ratios such as CN8, CN12 and CN16 showed the following patterns mentioned in Table 4.31, Table 4.32 and Table 4.33, respectively while considered those at 99% significant level ($p < 0.01$).

The pH of all BFT tanks except CN8 were found positively correlated at significant level ($p < 0.01$) with total alkalinity. As total alkalinity provided buffering capacity to the culture medium, its depletion in CN12 and CN16 BFT tanks might affected the buffering capacity, resulted a declining pH in this study. Whereas, in CN8 BFT tanks that relation was positive but not significant ($p > 0.01$), might be due to other negative factors like fluctuation of TAN and NO₂ on pH (Table 4.31).

The dissolve oxygen (DO) and pH of all BFT tanks with different C:N ratios showed significant ($p < 0.01$) positive correlation with each other and together they showed significant ($p < 0.01$) negative correlation with total suspended solids (TSS) of all BFT tanks. Although no physical-chemical connections were existed between DO and pH, but in the present study both were found downwards overtime. Dissolve oxygen utilization by fish and floc and production of their metabolic wastes were the chief reasons for the reduction of DO and pH, respectively in BFT system with higher TSS concentration. Azim *et al.* (2008) demonstrated that pH of the BFT system was inversely related with heterotrophic bacterial population which was also shown in this correlation matrix but not significant ($p > 0.01$). The DO of CN12 and CN16 tanks were found negatively correlated ($p < 0.01$) with biofloc volume (BV) due to higher oxygen consumption (Table 4.31, 4.32 and 4.33).

In CN12 and CN16 BFT system the total ammonia nitrogen (TAN) and nitrite (NO₂) showed significant ($p < 0.01$) positive correlation with each other and together they showed significant ($p < 0.01$) negative correlation with TSS, biofloc volume (BV) and total heterotrophic count (THC). Both TAN and NO₂ varied concurrently in CN12 and CN16 BFT systems in this study. Elevation of TAN and NO₂ at beginning due to nitrogenous wastes by heterotrophic bacteria and the nitrification of TAN by nitrifying bacteria, respectively, were decreased severely later and remained at low level till end. Immobilization of nitrogenous compound like TAN and NO₂ increased overtime with dominance of heterotrophic bacteria which eventually elevated the BV and TSS level in BFT system (Azim and Little, 2008) (Table 4.32 and 4.33).

Whereas, in CN8 BFT system the aforesaid degree of correlation were same alike higher C:N ratios but it was found negative significant ($p < 0.01$) correlation between TAN and BV, TAN and THC, and TAN and NO_3 only. Nitrate accumulation were significantly higher ($p < 0.05$) in CN8 group in compare to CN12 and CN16 groups could have been associated with higher contribution of nitrifying bacteria in CN8 (Azim and Little, 2008) (Table 4.31).

The NO_3 concentration of CN8 BFT tanks were found positively ($p < 0.01$) correlate with BV and THC. The NO_3 could have been accumulated due to incorporation of feed and microbial respiration in lower dominance of heterotrophic bacterial condition (Chen *et al.*, 2018). Gaona *et al.* (2016) also described the NO_3 level might increase overtime with TSS level in biofloc system which found positively correlate with NO_3 in CN8 tanks (Table 4.31).

The THC of all BFT system were showed considerably ($p < 0.01$) positive correlation with TSS and BV of all BFT tanks, were found similar with the findings of Kamilya *et al.* (2017) (Table 4.31, 4.32 and 4.33).

In control tanks for rearing of *H. fossilis*, significant ($p < 0.01$) positive correlation were existed in between pH, DO and total alkalinity. The pH, DO and total alkalinity altogether showed significant ($p < 0.01$) negative correlation with TAN and NO_3 concentration. The TAN and NO_3 showed significant ($p < 0.01$) positive correlation due to existence of well nitrification process in control tanks (Table 4.34).

Table 4.31. Correlations between water quality parameters in biofloc system with C:N ratio of 8:1 (CN8) for rearing Singhi (*H. fossilis*) for 180 d

	Temp	pH	DO	TAN	NO_2	NO_3	TA	TSS	BV	THC
Temp	1									
pH	-.476	1								
DO	-.323	.957*	1							
TAN	.533	.141	.307	1						
NO_2	.182	.400	.482	.639	1					
NO_3	-.438	-.292	-.417	-.856*	-.484	1				
TA	-.251	.457	.445	.029	-.198	-.197	1			
TSS	.025	-.824*	-.900*	-.614	-.693	.693	-.208	1		
BV	-.266	-.531	-.627	-.839*	-.604	.912*	-.151	.864*	1	
THC	-.308	-.519	-.649	-.845*	-.546	.888*	-.217	.853*	.955*	1

*Correlation is significant at the 0.01 level (2-tailed).

Temp: Temperature, DO: Dissolve oxygen, TAN: Total ammonia nitrogen, TA: Total alkalinity, TSS: Total suspended solids, BV: Biofloc volume, THC: Total heterotrophic count. [Colour intensity towards green indicated positive correlation and red indicated negative correlation whereas, yellow indicated nil correlation.]

Table 4.32. Correlations between water quality parameters in biofloc system with C:N ratio of 12:1 (CN12) for rearing Singhi (*H. fossilis*) for 180 d.

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA	TSS	BV	THC
Temp	1									
pH	-.657	1								
DO	-.705	.915*	1							
TAN	.011	.370	.532	1						
NO ₂	.076	.256	.443	.981*	1					
NO ₃	-.251	-.302	-.214	-.306	-.297	1				
TA	-.493	.743*	.649	.041	-.119	-.297	1			
TSS	.418	-.797*	-.878*	-.829*	-.764*	.425	-.429	1		
BV	.274	-.673	-.781*	-.895*	-.840*	.457	-.343	.957*	1	
THC	.062	-.394	-.530	-.923*	-.888*	.402	-.094	.820*	.916*	1

*Correlation is significant at the 0.01 level (2-tailed).

Table 4.33. Correlations between water quality parameters in biofloc system with C:N ratio of 16:1 (CN16) for rearing Singhi (*H. fossilis*) for 180 d

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA	TSS	BV	THC
Temp	1									
pH	-.266	1								
DO	-.167	.971*	1							
TAN	.411	.554	.615	1						
NO ₂	.503	.473	.610	.918*	1					
NO ₃	-.101	-.430	-.353	-.628	-.362	1				
TA	-.614	.709*	.592	.027	-.133	-.353	1			
TSS	.115	-.898*	-.953*	-.740*	-.750*	.389	-.444	1		
BV	.002	-.729*	-.764*	-.871*	-.810*	.566	-.265	.899*	1	
THC	-.464	-.483	-.581	-.975*	-.958*	.552	.085	.724*	.857*	1

*Correlation is significant at the 0.01 level (2-tailed).

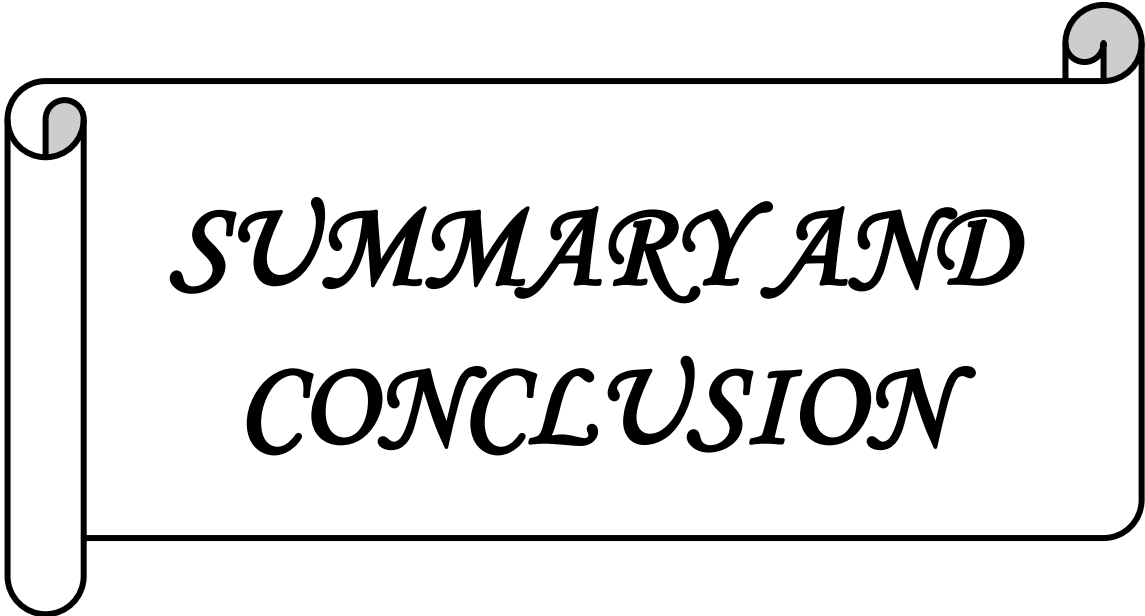
Table 4.34. Correlations between water quality parameters in control (without biofloc) for rearing Singhi (*H. fossilis*) for 180 d

	Temp	pH	DO	TAN	NO ₂	NO ₃	TA
Temp	1						
pH	-.142	1					
DO	-.205	.965*	1				
TAN	.242	-.789*	-.768*	1			
NO ₂	-.580	.121	.187	.013	1		
NO ₃	.011	-.937*	-.949*	.733*	-.103	1	
TA	-.397	.912*	.926*	-.750*	.215	-.862*	1

*Correlation is significant at the 0.01 level (2-tailed).

Temp: Temperature, DO: Dissolve oxygen, TAN: Total ammonia nitrogen, TA: Total alkalinity. [Colour intensity towards green indicated positive correlation and red indicated negative correlation whereas, yellow indicated nil correlation]

CHAPTER -5



*SUMMARY AND
CONCLUSION*

5. SUMMARY AND CONCLUSION

5.1. Summary

A survey was undertaken during October-December, 2020 by the randomly selected 110 biofloc farmers from thirteen districts of West Bengal to assess the biofloc farming practices. Till then no such information were available on record regarding the biofloc culture practices adopted by the farmers in West Bengal. Perhaps this study was done as a pioneer in that regard. Majorities of the respondents were found young, educated, least experienced and small scale investors. Unemployment during the pandemic COVID-19 situation since March, 2020, was the driving force to adopt this technology by the young enthusiastic generation of state. *Oreochromis niloticus* (Nile tilapia) was found the most common followed by *Anabas cobojius* (Gangetic koi), *Heteropneustes fossilis* (Singhi) and *Clarias batrachus* (Magur), and *Pangasianodon hypophthalmus* (Pangus). Disease outbreaks and mass mortalities were found very common to all. Disease prevention strategies and therapeutic measurements were found quite unsatisfactory. Most respondents failed to achieve their expected profit due to disease occurrences and mass mortalities. Only, 13.6% farmers were found highly satisfied, whereas, 62.7% were found dissatisfied but continue the culture and 23.7% were found absolutely disappointed. Possibilities for expanding that technology in West Bengal was also in doubt as majorities apprehended the biofloc techniques might not be sustained in coming days.

In this study the effects of different carbon to nitrogen (C:N) ratios in jaggery based BFT on water quality, growth, feed performances, welfare, body composition of both *O. niloticus* and *H. fossilis* were investigated in a separate 120 d and 180 d trial, respectively. A completely randomized design (stocking density of 312.5 g/m³ in 4000 l water in circular tarpaulin tank) was used in triplicates, where a control group was reared in clear water without biofloc and three BFT Nile tilapia (0.5±0.05 g) groups were reared in different C:N ratios i.e., 15:1 (CN15), 20:1 (CN20) and 25:1 (CN25), respectively. Similarly, Singhi (5±0.05 g) were reared in control and BFT with C:N ratios of 8:1 (CN8), 12:1 (CN12) and 16:1 (CN16) in another completely randomized design (stocking densities of 2.33 kg/m³ in 1500 l water in rectangular cement tank). Jaggery containing 46.7% organic carbon was used as only additional carbon source in this study. The fishes were fed twice daily with commercial diet at 5-

2% of their body weight. Daily water exchanges up to 15-20 % were done in control tanks while the BFT system were maintained by zero water exchange except the evaporation losses.

Significant higher survival rates (%) were found in CN15 ($91.39\pm 0.86\%$) and CN20 ($90.43\pm 0.21\%$) BFT groups compare to the CN25 and control Nile tilapia groups. The final body weight (FBW), weight gain (WG), daily weight gain (DWG) and specific growth rate (SGR) (% d) of all BFT groups were found significantly higher than the control but no significant differences were found among the BFT Nile tilapia groups with different C:N ratios. However, among BFT Nile tilapia groups, a non significant highest FBW, WG, SGR and DWG were recorded in CN15 groups i.e., 160.35 ± 2.16 g, 159.82 ± 2.16 g, $4.76\pm 0.01\%$ d and 1.33 ± 0.02 g. The highest final biomass (FBM) were obtained 366.31 ± 3.54 kg from the CN15 groups which was found significantly ($p<0.05$) higher than CN25 (344.58 ± 13.16 kg) but not than CN20 groups (358.73 ± 5.48 kg). The feed conversion ratio (FCR), feed efficiency ratio (FER) and protein efficiency ratio (PER) for all BFT groups were found significantly superior than control Nile tilapia group, but not significantly varied among BFT groups. Lowest FCR (1.10 ± 0.03) and highest FER (0.91 ± 0.02) and PER (3.22 ± 0.08) were recorded for the CN25 groups followed by the CN20 and CN15 groups. Somatic indices of control and CN25 Nile tilapia showed significantly higher gastroscopic index (GSI%) and hepatosomatic index (HSI%) than CN15 and CN20 BFT Nile tilapia groups. Conditioning factors (CF) were found insignificantly different among BFT and control Nile tilapia groups.

Singhi or *Heteropneustes fossilis* reared in the BFT for 180 d, showed better growth performances than control. Highest significant survival rates (%) were found in CN12 ($96.43\pm 0.43\%$) and CN8 ($96.81\pm 0.58\%$). Among BFT groups FWG and DWG as 52.12 ± 1.53 g and 0.29 ± 0.01 g, respectively in CN12 group were found significantly higher than CN8 and CN16 groups. The FBW (57.65 ± 1.53 g) and FBM (38.92 ± 1.21 kg) of CN12 were significantly higher than CN16 but not than CN8. No significant differences of specific growth rates (SGR%) were found among BFT treated Singhi groups while they were found significantly higher than control fish groups. Highest SGR% was recorded as 1.30 ± 0.01 in CN12 groups. All BFT treated Singhi groups showed significantly well feed utilization than control, but FCR and PER varied insignificantly among BFT groups, where lowest FCR and highest PER

were observed as 2.61 ± 0.06 and 1.35 ± 0.02 , respectively in CN12, followed by CN8 and CN16. No significant differences of somatic indices were found among BFT treated and control Singhi groups.

In this study no considerable differences of crude protein and ash contents of Nile tilapia carcass were noticed between control and all BFT Nile tilapia groups. Though, the lipid content (% DW) of CN20 ($25.12\pm 2.66\%$) was found significantly higher ($p<0.05$) than control but not significantly higher than CN15 and CN25 biofloc groups. Whereas, no considerable differences of lipid and ash contents of Singhi carcass were noticed between control and all BFT treated groups, but, the crude protein content (% DW) of CN12 ($66.20\pm 2.84\%$) was found significantly higher than control but not significantly higher than CN8 and CN16 biofloc groups. The proximate composition of the biofloc collected on 60th d from the BFT system showed increasing trend of crude protein and ash content with increased C:N ratios during culture of both Nile tilapia and Singhi fish. Lower lipid content was found in floc developed in all BFT systems with different C:N ratios.

In this study the serum biomarkers for stress like glucose and total protein; liver biomarkers like alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP); ionic balance biomarkers like calcium and chloride; haematological parameters like total erythrocytes count (TEC), total leucocytes count, (TLC) haemoglobin and haematocrit; innate immune parameters like respiratory burst activity (RBA), myeloperoxidase assay (MPO) and serum lysozyme assay; and antioxidant assays like superoxide dismutase (SOD) and catalase (CAT) were measured from the blood and serum samples collected on 60th followed by 120th d, and on 90th followed by 180th d for Nile tilapia and Singhi groups, respectively. Besides the serum ionic balance, all the aforesaid parameters showed significantly superior results in BFT treated Nile tilapia compare to control on both 60th and 120th d, except the haematological parameters which were found significantly higher 120th d only. Among BFT treated Nile tilapia the CN15 and CN20 showed significantly better results than CN25 in every aspect on 120th d but not 60th d. In spite of higher biomass along with significantly higher serum glucose, ALT, AST and ALP level on 120th d than 60th d in all treatment groups, the BFT treated Nile tilapia groups showed higher haematological, non specific and antioxidant properties on 120th d compare to 60th d, whereas, it was found either reverse or unchanged in control Nile tilapia group.

Serum glucose, ALT, AST and ALP of control *Heteropneustes fossilis* group were found significantly higher than BFT Singhi groups on both 90th and 180th d, whereas, serum total protein, haematological properties was found significantly lower than BFT groups on 180th d only. The innate immune and antioxidant properties were also found higher in BFT treated Singhi groups than control. Within BFT groups, the CN12 showed significantly higher hematological, innate immune, antioxidant properties and total serum protein, compare to CN8 and CN16 Singhi groups on 180th d but not on 90th d. The said welfare parameters were measured significantly superior on 180th d compare to 90th d in CN12 fish group only.

The liver histology of Nile tilapia showed significant increase of vacuoles in cytoplasm, degeneration of cytoplasm and cellular hypertrophy in control group followed by CN25 groups. Significantly lower aforesaid changes were observed in both CN15 and CN20 Nile tilapia groups. Histological studies of Nile tilapia intestine also showed significantly higher swollen lamina propria, mucinous degeneration, loss of absorptive vacuole, degeneration of epithelium and necrotized area, in control fish followed by CN25 group.

While observing the liver histology of Singhi, significant higher amount of vacuoles in cytoplasm, degeneration of cytoplasm and cellular hypertrophy were found in control group followed by CN16 groups. Histological studies of Singhi intestine showed significantly increase of damage in lamina propria, degeneration of epithelium and loss of brush border structure in control fish followed by CN16 BFT group. Significantly lower levels of aforesaid changes were observed in CN8 and CN12 Singhi groups.

Except temperature all the water quality parameters of control and BFT tanks were varied significantly during the culture of both Nile tilapia and Singhi for 120 d and 180 d, respectively. The water qualities parameters like higher dissolve oxygen (DO), pH and total alkalinity were observed in control tanks compare to BFT treated tanks for rearing of both. The DO, pH and alkalinity were found positively correlated with a downward trends with culture duration and lower levels in BFT tanks with increasing C:N ratios for both culture. Significant lower level of TAN, NO₂ and NO₃ were recorded in all biofloc tanks than control. Among BFT treated groups significant higher inorganic nitrogen immobilization were observed in BFT tanks with higher C:N ratios. However, the TAN, NO₂ and NO₃ were maintained within the permissible

limits in all biofloc tanks for rearing of Nile tilapia and Singhi for 120 d and 180 d, respectively. The C:N ratio of 8:1 were not found suitable to immobilize the inorganic nitrogen effectively due to fluctuation of TAN and NO₂ during 180 d of rearing of Singhi.

Significant increasing trend of total suspended solids (TSS), floc volume (FV) and total heterotrophic bacterial load with higher C:N ratios were observed throughout the rearing period for both Nile tilapia and Singhi. The highest mean values and maximum values of TSS (376.88 ± 8.16 mg/l and 581.40 mg/l), BV (29.94 ± 0.56 ml and 39.57 ml), THC (7.25 ± 0.03 log cfu/ml and 7.74 log cfu/ml) were recorded in CN25 tanks, were found significantly higher than CN20 and CN15 tanks for Nile tilapia culture. Similarly the mean and highest values of TSS (289.53 ± 3.31 mg/l and 385.12 mg/l), BV (19.94 ± 0.56 ml and 25.3 ml), THC (6.74 ± 0.06 log cfu/ml and 6.90 log cfu/ml) were recorded utmost in CN16 tanks for Singhi culture. The TSS, FV and THC in all biofloc tanks were found positively correlated with each other and together they were negatively correlated with TAN, NO₂, DO, pH and total alkalinity levels in all BFT tanks.

5.2 Conclusion

Biofloc procedure could leave the target crop at risk of disease challenges and benefit issues if systems fail to manage suspended solid level in tank water; hence farmers were still need to understand the discrete microbial dynamics in well manner. Apart from monitoring ideal water quality parameters, preparation of bacterial inoculums by good quality probiotics and carbon sources should be considered with equal importance. Selection of suitable species, correct feeding and maintenance of proper carbon nitrogen ratio to prevent nitrogen accumulation in tank should be the top most priority in biofloc system, because this critical gap made production management difficult and might force the farmers remain unprepared to address any emerging issues during culture period. The culture system required a start-up period and hence the newcomers were suggested to initiate the culture in small scale for standardizing it and were also advised to be more conscious during winter as the system might not sustain equally throughout the year. More emphasis should be put on disease prevention strategies rather than disease treatment, because no appropriate disease treatment protocol for biofloc culture system was available till date. Provision

of rehab tank for quarantine, observation and treatment should be mandatory. Farmers were also advised to make proper documentation of their findings for future strategies. Nile tilapia was found to be the most suitable species for biofloc system in this survey, but the farmers were advised for Gift tilapia or Monosex tilapia to overcome the problem of non-uniform growth. Singhi and Magur also can be considered for high market price, but farmers should be more conscious for its longer culture duration. Culture of Gangetic koi and Pangas in biofloc tank were still needed to be standardized. Too much dependence on Youtube video and social media for every emerging problem often might misguide the farmers. More intervention of Government sector in that endeavour was urgently needed in terms of conducting awareness programme, training for proper guidance, bringing farmers under subsidized scheme and insurance fold. The problems of small and marginal biofloc framers could be better addressed by organizing them through institutional efforts.

The higher feeding performances of BFT treated Nile tilapia in this study indicated that biofloc system could reduce supplementary feed utilization due to the development of microbial flocs, recycling of the remaining feeds and wastes and the feed reutilization of the nutrients by fish which resulted in the enhancement of growth and feeding performance. Whereas, for rearing of Singhi in biofloc the higher C:N ratio more than 12:1 could not be effective for its growth and feed utilization performances. At the same time our finding also suggested that higher C:N ratio of more than 20:1 and 12:1 could weaken the growth of Nile tilapia and Singhi in biofloc system, respectively. In those cases the microorganisms might not be benefited for growth of cultured fishes because of the negative effect of the microorganisms on controlling water quality.

BFT system with different C:N ratios might not produce nutritionally rich Nile tilapia in terms of muscle protein content but for Singhi it could have been possible to some extent. Probably duration of culture and type of carbon sources could have some effects on it which needs further investigation. Though biofloc contained few essential amino acids, fatty acids and minerals, vitamins and other nutritional elements as reported by various studies but lower lipid content in floc developed in all BFT system with different C:N ratios in this study supported the requirements of supplementary feeds with appropriate lipid content.

Summary and Conclusion

Increasing amount of both protein and ash content, higher volume of floc and heterotrophic bacterial load in BFT system with increasing C:N ratio might not be effective for fish growth always. The floc developed in the BFT system with higher C:N ratios contained high ash content might lower the digestibility of other ingredients in the diet. Apart from that the excessive heterotrophic bacterial load in BFT system with higher C:N ratios could effectively immobilize the toxic inorganic nitrogenous components of water but simultaneously it might increase the biological oxygen demand in a short time led declining the growth of fish in biofloc system.

The BFT system could effectively reduce the physiological stress, improve haematological, innate immune and anti-oxidant properties of Nile tilapia and Singhi more effectively in lower C:N ratios viz. 15:1 and 12:1, respectively than higher C:N ratios. Those might be linked with other environmental factors like high TSS level, high biological oxygen demand, higher floc and heterotrophic bacterial load in higher carbon content biofloc system, which created stress to the aquatic organisms. Higher level of floc volume, heterotrophic bacterial load or higher nutrient flow in BFT system with higher C:N ratios could hamper the liver hepatocytes and intestine histology of cultured Nile tilapia and Singhi, that could be reflected on final production. Overall, the C:N ratios of 15:1 and 12:1 appeared to provide best balance between water quality, growth and feeding performances, fish health, histology of digestive organs and biochemical compositions of *Oreochromis niloticus* and *Heteropneustes fossilis*, respectively. No doubt Nile tilapia or *Oreochromis niloticus* was found as a suitable species for rearing in biofloc system in this study, but from practical point of view, due to slow growth rates Singhi or *Heteropneustes fossilis* could not be considered as a suitable species for such an intensive aquaculture approach like biofloc technology.

CHAPTER -6



*FUTURE SCOPE
OF RESEARCH*

6. FUTURE SCOPE OF RESEARCH

There are ample scopes for conducting various researches on rearing fresh water fish in biofloc system by maintaining different carbon to nitrogen (C:N) ratios with help different carbon sources. Few of those have been highlighted below.

- More study on understanding the microbial mechanisms involved in the process of flocculation i.e., quorum sensing and controlling effect on pathogenic microbes in BFT system with different C:N ratios.
- Metagenomics studies for analysing the genetic and species level information of microorganisms developed in BFT system with different C:N ratios by various carbon sources.
- Histopathological alterations in gills and digestive organs of fish at different culture stages in BFT system with different C:N ratios.
- More research on immune gene expression studies in BFT reared fish at different culture stages, with different C:N ratios.
- Incorporation of feed additives to improve appearance for enhancing the market acceptability of BFT reared fish.
- Culturing other commercially important freshwater fishes in BFT system with different C:N ratios.

CHAPTER -7



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7. BIBLIOGRAPHY

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CHAPTER -8



APPENDICES

8. APPENDICES

Annexure-I

Interview Schedule for Biofloc Farmers

1. Farmers' category:

- (a) Age (Years):
- (b) Educational qualification:
- (c) Experience (Years):
- (d) Training source:

2. Culture practices:

- (a) Cultured fish species:
- (b) Stocking Size (nos./kg):
- (c) Stocking Density (nos./1000 l):
- (d) Tank materials:
Indoor/outdoor:
Culture season:

3. Water quality monitoring practices:

Water quality checking before stocking:

Measuring Temperature, TDS, pH, DO, NO₂, TAN, NO₃:

Measuring Alkalinity, Hardness, H₂S, CO₂, Salinity:

Continues aeration:

: Yes/No

Using Imhoff cone:

Raw salts and dolomite application:

Partial water exchange in emergency:

Thermostat and Generator provision:

4. Heterotrophic bacteria management:

- (a) Bacterial inoculums preparation:
- (b) Desired floc volume (ml):
- (c) Desired C:N ratio:
- (d) Commercial Probiotics:
Estimation of floc volume (ml):
Estimation of C:N ratio:
Estimation of Bacterial load CFU/ml:
Using molasses or others as carbon source:
Additional carbon source used:

: Yes/No

5. Feed managements:

- (a) Supplementary feed:
Commercially formulated feed:
Watching feed intake:
Using check tray:
Checking stored feed:
Calculating FCR:
- (b) Feed preferences by cultured species among
Heterotrophic bacterial floc and supplementary feed:

: Yes/No

6. Disease preventive management:

KMnO₄ bath before liberation of fish:

Watching feeding at initial stage:

Removal of uneaten feed:

Frequent checking of TAN, NO₂, pH, DO, TDS, floc, temperature:

Documentation of test reports:

: Yes/No

Removal of dead fishes:

Regular observation for any external lesions:

Weekly checking of fish growth:

Incorporation of vitamin/ minerals with feed:

Hospital tank:

7. Diseases and mortality:

(a) Type of fish diseases and symptoms:

(b) Age group mostly affected by disease:

(c) Most disease occurrence month from commencement of culture to end:

(d) Most disease occurrence season:

(e) Type of mass mortality:

8. Therapeutic measures:

(a) Confidence : **High**: Sufficient to treat fish without taking help from others.
for therapy **Medium**: Can treat fish by taking help from others.

Low: Unable to treat fish even after getting advice from others.

(b) Taking therapeutic measures:

Antibiotics; Medicines or chemicals:

Satisfied with diseases control:

: Yes/No

Consult with friends, private or government sector:

Depend on Youtube or social media group:

9. Production: In Ton/ 10000 litre

10. Economics:

(a) Project cost : In Lacs

(b) Profit/ Loss : **High Profit** (>10% of total project cost including capital cost in first crop & >50 % of culture cost in subsequent crop).

Medium Profit (5-10% of total project cost including capital cost in first crop & 25-50% of culture cost in subsequent crop).

Low Profit (<5% of total project cost including capital cost in first crop & <25% of culture cost in subsequent crop).

No Profit (Less than project cost in first and subsequent crop).

11. Overall experience and prospect:

(a) Satisfied with biofloc technique and regarding continuation in future:

(b) More investment:

Suggest others:

: Yes/No

Looking for additional livelihood:

Involve other family member:

A study on ground reality of freshwater fish farming in biofloc tank in West Bengal, India

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Introduction of land-based biofloc technology has brought huge changes in concept of fish farming. The practice of biofloc farming is gaining popularity amidst COVID-19 situation. For the present study on biofloc tank farming in West Bengal, 110 biofloc farmers who had harvested at least one crop, were selected through simple random sampling method, from a list of 390 biofloc farmers from 13 districts of West Bengal. Data were collected from the selected respondents during the last quarter of 2020, according to a structured interview schedule on every minute aspect on culture strategies, technical issues, profit gain and prospect. *Oreochromis niloticus* was found to be the most cultured species followed by *Anabas cobojus*, Catfishes *Heteropneustes fossilis* and *Clarias batrachus*, *Pangasianodon hypophthalmus* and *Labeo bata*. Majorities of the respondents were found young, educated, least experienced and small scale investors. Disease outbreaks and mass mortalities were found very common to all. Disease prevention strategies and therapeutic measurements were found quite unsatisfactory. Most respondents failed to achieve their expected profit. Only 13.6% farmers were found to be highly satisfied, whereas, 62.7% were dissatisfied but continue the culture and 23.7% were highly disappointed. Synchronizing C:N ratio with microbial dynamics by the farmers was found to be the major gap in technical knowhow.

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Key words Biofloc culture, Freshwater fish, West Bengal

Introduction

Expansion and intensification of aquaculture production is one of the top priorities among aquaculturists to meet the demand of animal protein for increasing global population which has reached 7.9 billion in 2021. Producing more aquatic crops without considerably expanding the usage of natural water and land resources as well as developing environment-friendly sustainable aquaculture systems to provide reasonable returns for economic support and social sustainability, the biofloc technology have gained attention during last few years (Avnimelech, 2009; Ahmad *et al.*, 2017). Now-a-days biofloc technology is widely used for culturing numerous shrimp and fish species by microbial immobilization of ammonium with limited water exchange facilities (Ekasari *et al.*, 2015). Biofloc technology is based on the maintenance of elevated levels of suspended microbial flocs by providing continuous aeration and additional carbohydrates to permit organic material decomposition aerobically (Avnimelech, 2006). Getting alternative occupational area during COVID-19 pandemic has become a major challenge at present. The National Fisheries Development Board, Hyderabad has implemented some key projects including biofloc technology for environmental sustainability, better management practices. Government of India has approved the "Pradhan Mantri Matsya Sampada Yojana" with an estimated investment of 20,050 crores for coming five years with intention to bringing '2nd Blue Revolution'. Biofloc technology also has got importance among this National dream venture to mitigate the critical gaps in fish production in our country. West Bengal Government also has put special emphasis on biofloc technology with the intention of

employment generation amidst the COVID-19 pandemic situation. Azim and Little (2008) reported that the biofloc technology can increase the fish production by around 45% over the traditional aquaculture systems. In reality, majorities of the natural water resources are being utilized by traditional and non-professional aqua culturists. A person not having own water area needs huge investment for starting fish culture in pond, whereas setting up of a small biofloc unit on land with minimum investment is quite easy. Lot of social media groups are proactive to promote biofloc technology by presenting how this intensified culture technique can be easily performed by ordinary man. A lot of private paid trainers throughout the state are also equally promoting it by their own efforts. As a result, common people without knowledge of fisheries science have entered into this field and are facing difficulties. Keeping this in mind, a survey was conducted to assess the present scenario of freshwater fish culture in biofloc tanks in West Bengal. The survey was conducted not only to unravel the emerging problems and lacunae associated with this technique, but also to find out the possible remedies to mitigate all those critical gaps.

Materials and methods

A survey was undertaken during October-December, 2020 to assess the farming practices of biofloc technology in tanks by the randomly selected 110 biofloc farmers from thirteen districts i.e., Purba Midnapur, Paschim Midnapur, North-24 Parganas, South-24 Parganas, Howrah, Hooghly, Purba Bardhaman, Bankura, Nadia, Murshidabad, Malda, Dakshin Dinajpur and Uttar Dinajpur of West Bengal. The districts were selected considering the preponderance of freshwater fish culture in biofloc tanks as information received from the progressive farmers, fish seed suppliers, fishery experts and also State fishery officials. A list of 30 farmers from each district, who were performing biofloc tank culture in September, 2020 and had harvested at least one crop earlier, was prepared with inputs from aforesaid sources. A total of 130 farmers (10 from each of the 13 districts) were selected by simple random sampling method without replacement technique and were contacted; however, a total of 110 farmers of those volunteered to share information on their biofloc farming practices, constituted the sample size of this study. The interview schedule as depicted below was used to gather data from those 110 farmers. All the collected information

were accumulated, percentage calculated and presented in graphical forms by MS Excel 2007 to understand the current scenario of biofloc tank farming of freshwater fish in West Bengal. The structured interview schedule had questionnaire on following aspects:

Farmers' category

(a) Age (Years), (b) Educational qualification, (c) Experience of biofloc culture (Years), (d) Training source

Culture practices

(a) Cultured fish species, (b) Stocking size (gm/fish), (c) Stocking Density (nos./1000L) (d) Tank materials; Overhead shed; Culture season

Water quality monitoring practices

Water quality checking before stocking; Measuring TDS, pH, DO, NO₂-N, Temperature, TAN, NO₃-N, Alkalinity, Hardness, H₂S, Free CO₂, Salinity; Continuous aeration; Imhoff cone; Raw salts, molasses and dolomite application; Partial water exchange in emergency; Thermostat, Generator provision. : Yes/No

Heterotrophic bacteria management

(a) Bacterial inoculum preparation, (b) Desired floc volume (ml), (c) Desired C:N ratio (d) Commercial Probiotics used, Estimation of floc volume (ml), C:N ratio, Bacterial load CFU/ml; Using molasses or others as carbon source : Yes/No

Feed management

(a) Supplementary feed used; Commercially formulated feed; Monitoring feed intake; Using check tray; Checking stored feed; Calculating FCR : Yes/No
(b) Feed preferences by cultured species among heterotrophic bacterial floc and supplementary feed

Preventive health management

Potassium permanganate (KMnO₄) bath before stocking of fish; Noticing feeding at initial stage; Removal of uneaten feed; Frequent checking of free Ammonia, NO₂-N, pH, DO, TDS, floc, temperature; Documentation

of test reports; Removal of dead fishes; Regular observation for any external lesions; Weekly monitoring of fish growth; Incorporation of vitamin/ minerals with feed; Hospital tank : Yes/No

Diseases and mortality

(a) Type of fish diseases and symptoms, (b) Age groups most affected by disease, (c) Most disease occurrence month from commencement of culture to end, (d) Most disease occurrence season, (e) Type of mass mortality

Therapeutic measures

(a) Confidence for therapy : High: Sufficient to treat fish without taking help from others,
Medium: Can treat fish by taking help from others,
Low: Unable to treat fish even after getting advice from others.
(b) Taking therapeutic measures; Antibiotics; Medicines or chemicals; Satisfied with diseases control; Consult with friends, private or government sector; Depend on Youtube or social media group : Yes/No

Production

Ton/ 10,000 litre water volume

Economics

(a) Project cost : In Rs.
(b) Profit/ Loss : High Profit (>10% of total project cost including capital cost in first crop & >50 % of culture cost in subsequent crop)/
Medium Profit (5-10% of total project cost including capital cost in first crop & 25-50% of culture cost in subsequent crop)/
Low Profit (<5% of total project cost including capital cost in first crop & <25% of culture cost in subsequent crop)/
No Profit (Less than project cost in first and subsequent crop).

Satisfaction and prospect

(a) Satisfaction with biofloc technique and about continuation
(b) More investment; Suggest others; Looking for additional livelihood; Involve other family member : Yes/No

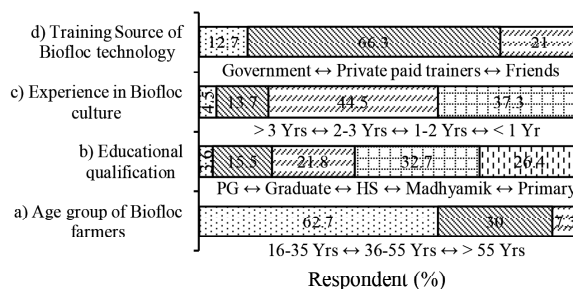


Fig. 1. Age, education, experience and training of biofloc fish farmers

Results and discussion

Farmers' category

Majority of the biofloc farmers were found to be young with good educational background (Fig. 1), however most of them had very little experience in biofloc culture (Fig. 1). Unemployment during the pandemic COVID-19 situation since March 2020 was the driving force to adopt this technology by the young enthusiastic generation of the state. Most were trained by different private paid trainers but training by Government organizations was negligible.

Culture practices

Majority of the farmers were found culturing more than one species but not multispecies in one tank. Nile tilapia, *Oreochromis niloticus*, was found to be most popular species and cultured by 89 farmers, followed by *Anabas cotojius* (Koi) (55 farmers), Catfishes like *Heteropneustes fossilis* (Singhi) and *Clarias batrachus* (Magur) (33 farmers), and *Pangasianodon hypophthalmus* or commonly known as Pangas by 17 farmers. Unexpectedly, 6 respondents were found to be culturing *Labeo bata* which is not considered as a recommended species for biofloc (Fig. 2a). Few farmers had initiated culture of *Ompok bimaculatus* (butter catfish/ Pabda) and *Mystus* sps. (Tengra) but were unable to harvest due to sudden mortality, while few were found still struggling with it. As complete data were not obtained on pabda and tengra from the respondents, these two fish species were not considered in this study. Among the cultured species, the most preferred stoking sizes were 0.5-1 gm/fish (Fig. 2b). Low stoking densities were maintained for bata, pangas, singhi and magur, whereas tilapia and koi farmers were found to maintain

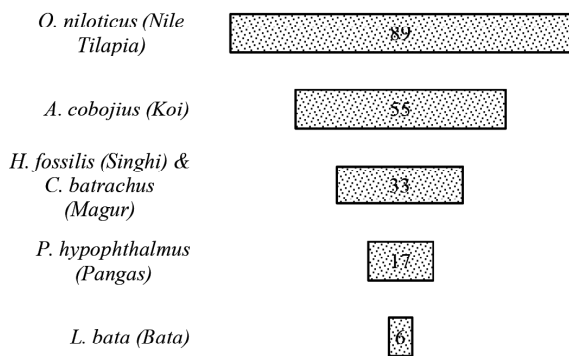


Fig. 2a. Fish species cultured by 110 respondents (values are in numbers)

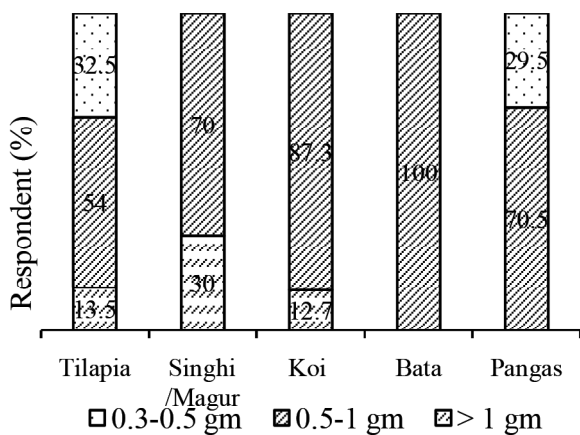


Fig. 2b. Stocking Size (gm/fish) of different species in biofloc culture

higher stocking densities (Fig. 2c). Higher stocking densities usually impact water quality and impart main stress to the aquatic life (de Oliveira *et al.* 2012). Tarpaulin tank supported by iron mesh was found most popular over cement and fibre glass or plastic tub (Fig. 2d). Maximum culture setup was found in indoor facility (Fig. 2d). Due to disease predominance and difficulties in maintaining heterotrophic bacteria at low temperature, seasonal culture (excluding December-February) was found most common.

Water quality monitoring practices

Water quality is an important aspect of biofloc management and respondents were found to be aware about the ideal water quality parameters. They knew that deviation from the optimum range might adversely affect the crop. Monitoring of major water quality parameters like pH, DO, NO₂-N, TAN, TDS and floc

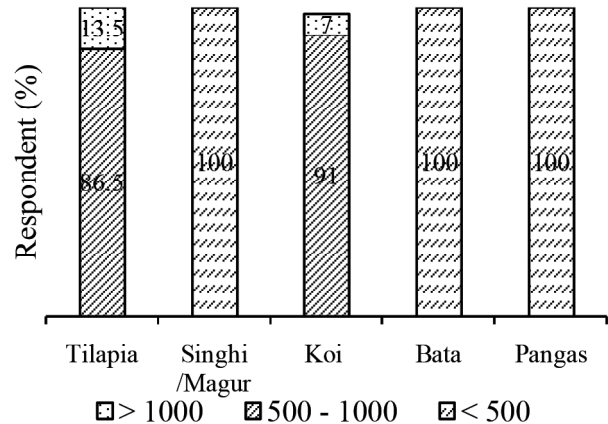


Fig. 2c. Stocking density (Nos./1000 L water volume) of different species

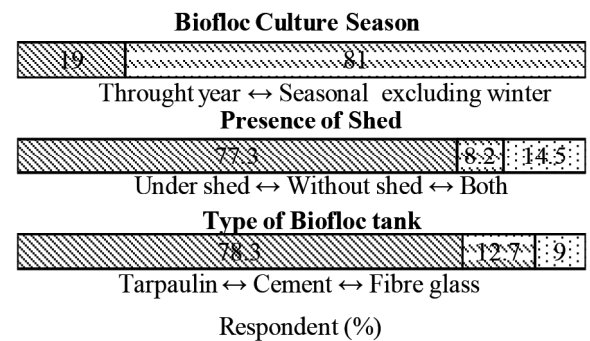


Fig. 2d. Tank type, overhead and seasonality of biofloc culture

volume measurement by Imhoff Cone was found to be a common practice (Fig. 3). But monitoring of others parameters like NO₃-N, temperature, salinity, alkalinity, hardness, H₂S and free CO₂ was not common. In general, aquaculture system water exchange is important to maintain water quality from deterioration. Hence, minimum or zero-water exchange culture techniques increase nitrogen levels in water (Randall and Tsui, 2002). Most respondents used to perform partial water exchange during emergency. Provisions for emergency power supply and thermostats were found limited (Fig. 3). Farmers not having inverter/ generator facilities often faced culture failure during long power cut-off, mainly in rural areas. The worst experience in this regard was faced by many farmers after cyclonic storm Amphan on 16 May, 2020 which affected electricity supply badly in West Bengal.

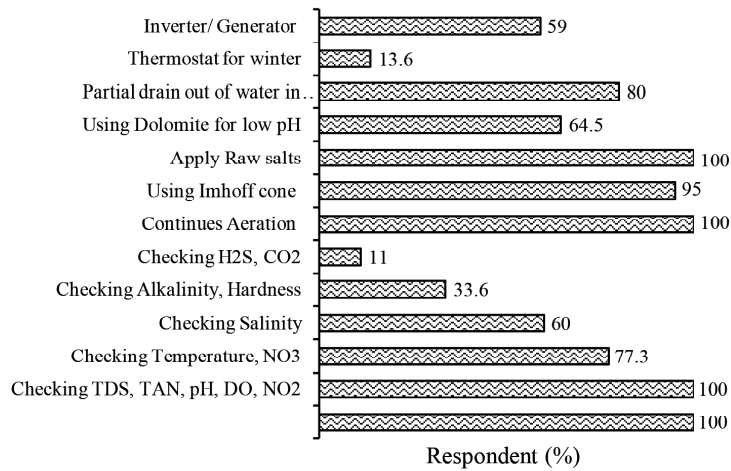


Fig. 3. Water quality monitoring practices adopted by the biofloc farmers

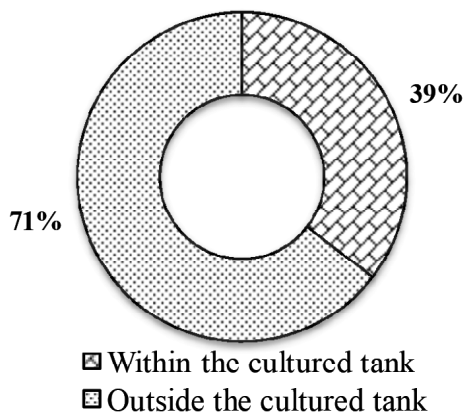


Fig. 4a. Place of bacterial floc preparation adopted by farmers

Heterotrophic bacteria management

Preparation of heterotrophic bacterial floc is the most crucial in biofloc culture. Majority of the farmers were found to prepare bacterial inoculum outside the tank, *i.e.*, by mixing carbon source and probiotics in small bucket with continuous aeration, which was called as "FCO" (Fermented Carbon Organic) by them (Fig. 4a), while others prepared it directly into the culture tank with continuous aeration prior to the fish stocking. Maintaining optimum floc levels was also considered as a critical issue because high fluctuation of pH and alkalinity, high concentrations of inorganic nitrogen might have chronic effects on fish health (Azim and Little, 2008). Most commonly used floc volume by farmers for culturing Tilapia and Koi was 10-15 ml and, for Singhi, Magur and Bata it was 5-10 ml and, for Pangas it was 15-20 ml.

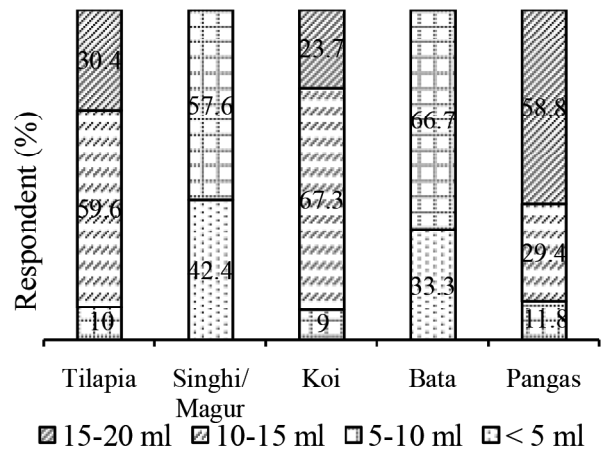


Fig. 4b. Ideal floc volume or desired Imhoff Cone measurement (ml)

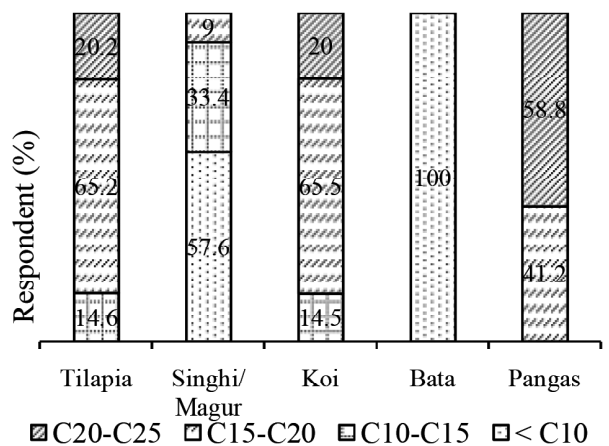


Fig. 4c. C:N to be achieved by biofloc farmers for different cultured species

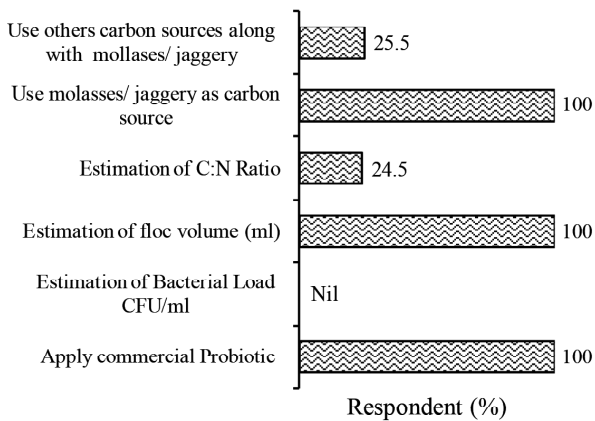


Fig. 4d. Heterotrophic bacteria management practices by biofloc farmers

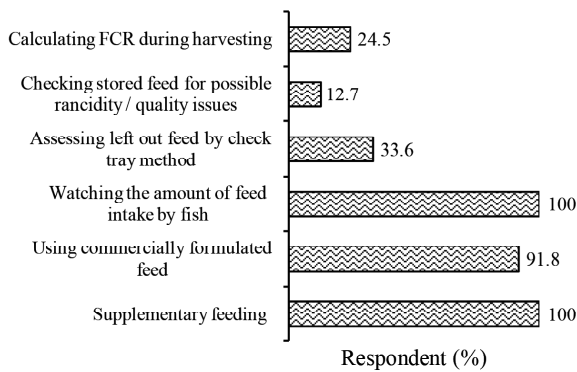


Fig. 5a. Fish feed managements by the biofloc farmers

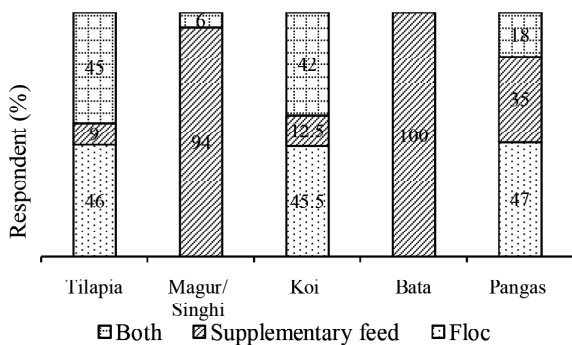


Fig. 5b. Feed preferences for different species in biofloc culture

it was 15-20 ml (Fig. 4b); this variation was due to differences in feeding habits of different fish species. Biofloc formation in the water column of tank is correlated to the dissolved ammoniacal nitrogen from feed as well as fish excretions by heterotrophic bacteria (Wang *et al.*, 2016), so the optimum range of TAN must be

controlled within suggested ranges. Only limited farmers had clear idea about the estimation of carbon:nitrogen (C:N) ratio and they tried to maintain it (Fig. 4d). Despite lack of proper knowledge, all the farmers were found familiar with the term 'carbon and nitrogen ratio' and expressed their views on preferred C:N ratio (Fig. 4c) and, majority of the farmers were found to have conception that higher C:N ratio was always directly proportional to the floc volume. Controlled C:N ratio by adding organic carbon is the prime management measures for biofloc tank, but availability of inexpensive and easily available carbon source is also important to run this system (Zaki *et al.*, 2020). All farmers used molasses or jaggery in case of non-availability of molasses, as carbon sources, but their assumptions on organic carbon (%) in their given carbon sources were found different across the state. Commercial probiotics were used by all but they neither had provisions to estimate bacterial load (CFU/ml) in their tank, nor did they even try to estimate it through outsourcing (Fig. 4d). Though it was practically impossible for them, but unfortunately, most farmers blindly believed that observation of water colour and floc volume was enough to realize the existence of sufficient heterotrophic bacteria in tank water.

Feed management

All the farmers had used supplementary feed but major lacuna in feeding practice was associated with limited practices of assessing left out feed by check tray or feeding tray methods, checking stored feed for possible rancidity as well as quality and, calculating FCR during harvesting (Fig. 5a). Consideration of carbon content in uneaten supplementary feed was completely ignored by the majority. Regarding feed preferences, merely all singhi, magur and bata growers selected supplementary feeds unanimously, whereas, tilapia, koi and pangas farmers indicated floc as well as supplementary feeds both (Fig. 5b).

Preventive health management

The health status of aquatic organisms largely depends on the farming system (M'balaka *et al.*, 2012). A lot of demerits were found in respect of taking preventive measures for disease concerned. Non-removal of uneaten feed led to subsequent water quality deterioration which was very common. In spite of

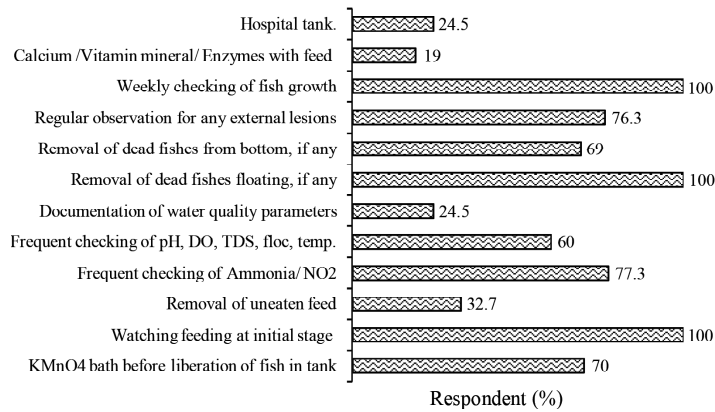


Fig. 6. Preventive health management practices by farmers

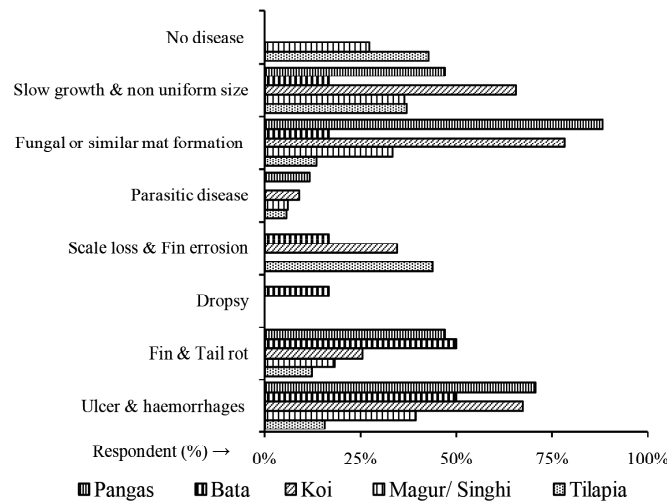


Fig. 7a. Fish diseases and symptoms observed by biofloc farmers for different cultured species

checking important water quality parameters, few of them documented their observations in a systematic manner which might be an important tool for taking preparatory measures to combat any diseases and for case studies by fishery experts. Farmers used to check fish growth weekly. Incorporation of calcium, vitamin-mineral mixtures or any digestive enzyme along with feed to enhance the fish health status was practised rarely (Fig. 6). Rehab tank for quarantine and observation of any suspected fishes was performed by only one fourth of respondents (Fig. 6).

Diseases and mortality

Occurrences of disease and fish mortality are major problems in all type of culture practices including biofloc

tank culture system. In present study, only 42.7% Tilapia farmers never faced any disease, followed by 27.3% Singhi/Magur farmers (Fig. 7a). But, disease occurrences were very abundant in Pangas and Koi farming. Presence of ulcer and haemorrhages, fin rots and tail rot, generally of bacterial aetiology, were found to be very common. Occurrence of dropsy was confirmed by Bata farmers only. Parasitic infections were negligible. Scale loss, possibly due to poor water quality, and fin erosion either due to infection or might be the attack by co-species during shortage of feed, were reported as one of the vital problems for Tilapia and Koi. The major disease in Pangas, Koi, Singhi, Magur was mat like fungal growth. In koi, first a hazy film appeared on skin or fin which rose into white to pink bumps and was reported untreatable. This symptom was quite similar to

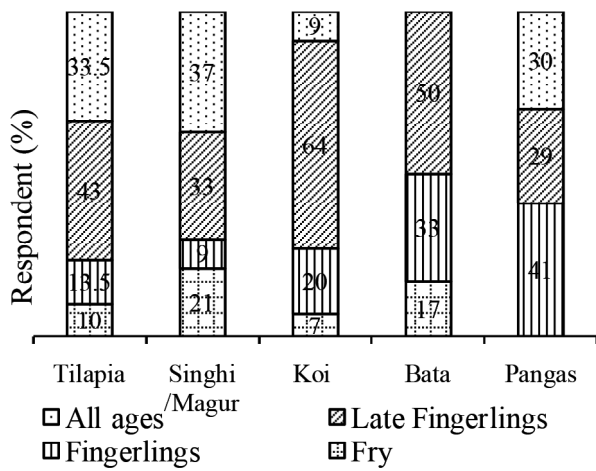


Fig. 7b. Fish age group mostly affected by disease outbreaks

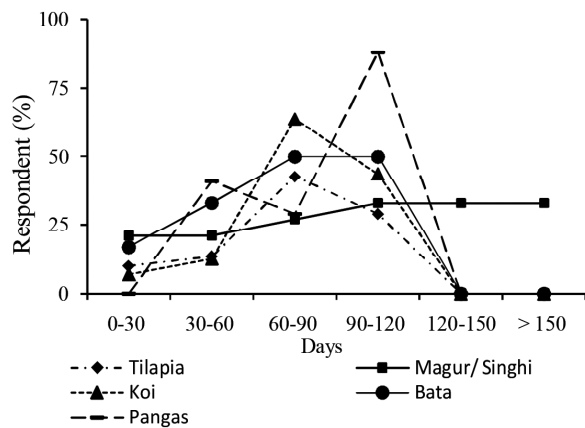


Fig. 7c. Most disease occurrence period from commencement of culture

Lymphocystis viral infection and needs detailed investigation. Slow growth as well as non-uniform size was also reported for all cultured fishes (Fig. 7a). Late fingerling stage was reported as the most disease affected group for Tilapia, Singhi, Magur, Koi and Bata, whereas, fingerling was found the mostly affected group for Pangas. Fry size was reported as the least disease affected group (Fig. 7b). During the whole culture period maximum disease outbreaks occurred during 3rd and 4th month for Tilapia, Koi and Bata whereas, for Pangas it was 4th month (Fig. 7c). Disease outbreaks occurred every month in Singhi/ Magur during 6 month culture (Fig. 7c). Most farmers skipped culture during the winter season except Singhi/Magur farmers. So, highest

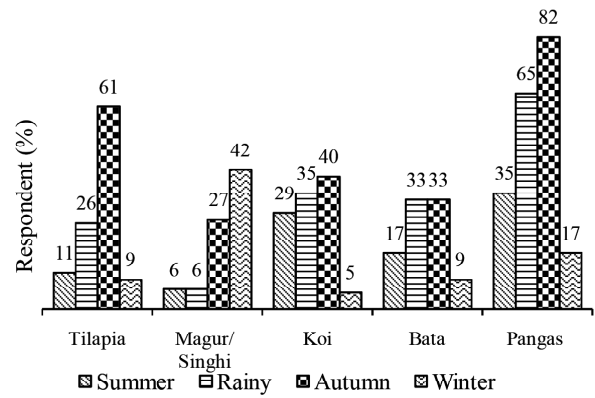


Fig. 7d. Seasonal occurrence of diseases for different cultured species

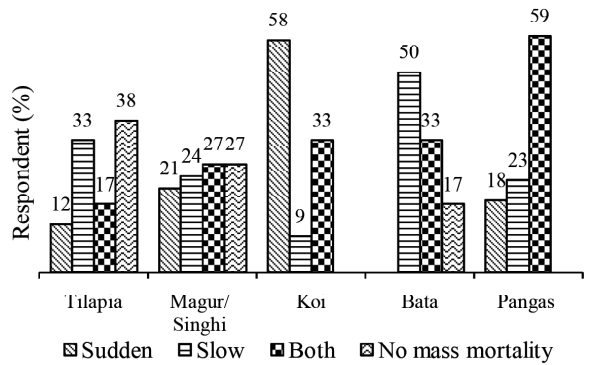


Fig.7e. Type of mass mortality for different cultured species

disease prevalence occurred during autumn, followed by rainy and summer season for all fishes, whereas, for Singhi/Magur it was found to be the winter (Fig. 7d). Fluctuation of temperature and stress were the major triggering factors for disease occurrences. Mass mortalities of cultured fishes were also reported. Sudden mass mortalities were found most common in koi and slow mass mortalities in Bata. Both sudden and slow mortalities were found highest in Pangas. Farmers who never faced mass mortalities were mostly Tilapia growers (Fig. 7e). It is needless to say that fish mortality was a major concern among all the farmers.

Therapeutic measures

Disease management and therapeutic measures adopted by the respondents were found suboptimal to very poor as only few farmers were confident to treat their fishes (Fig. 8a). Treatment with antibiotics and medicines or chemicals were found to be limited, as most of the

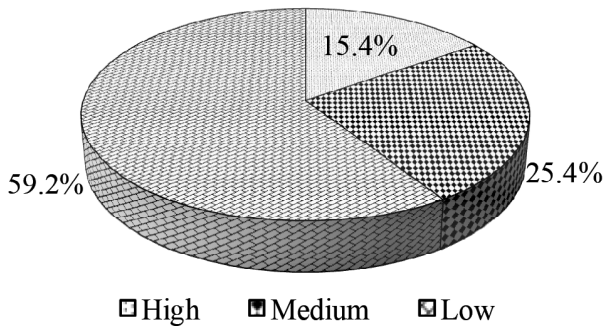


Fig. 8a. Confidence on disease therapy by biofloc farmers

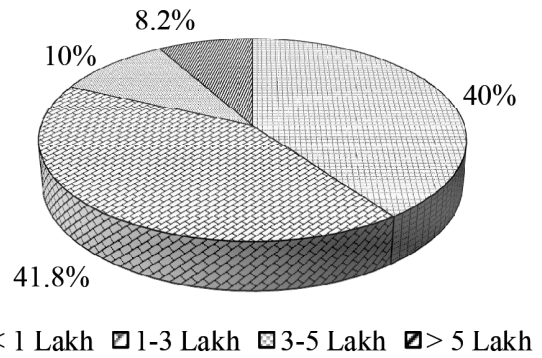


Fig. 10a. Project investment by the farmers in Rs. (lakh)

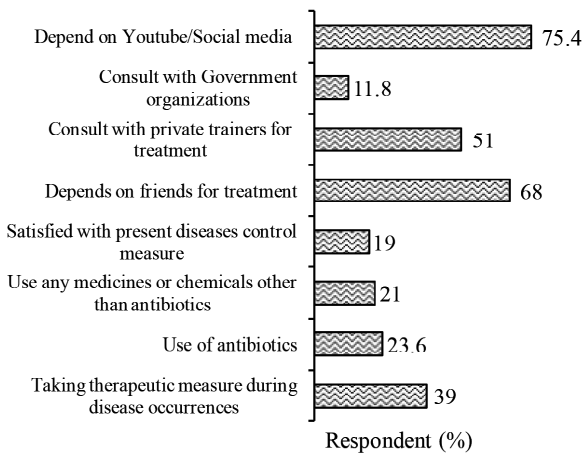


Fig. 8b. Therapeutic measures against diseases practised by biofloc farmers

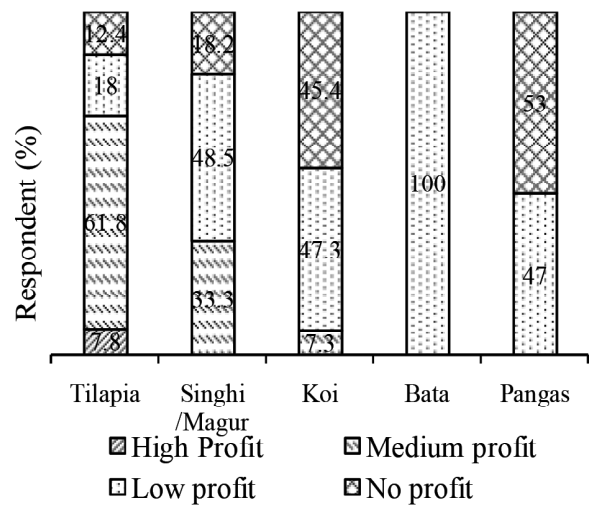


Fig. 10b. Profit gain by the Biofloc farmers for different cultured species

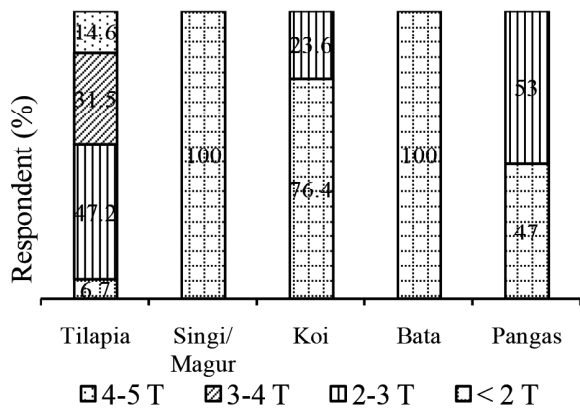


Fig. 9. Production of different cultured species in biofloc tank (Ton/10000L)

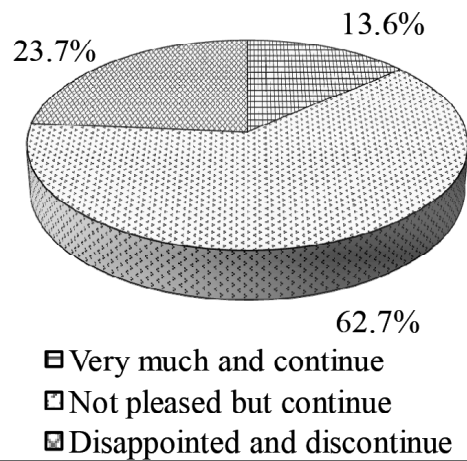


Fig. 11a. Farmers satisfied with biofloc technology

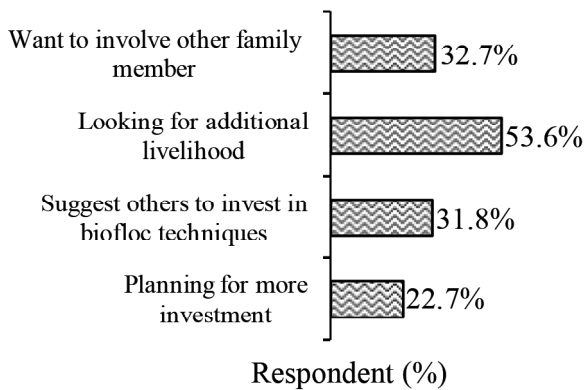


Fig. 11b. Prospect of biofloc culture

farmers had failed earlier or had no idea of disease identification and treatment (Fig. 8b). Therapeutic intervention was effective in catfishes only. Most of the farmers were found influenced by Youtube videos, social media groups in Facebook, Whatsapp and also depended on their friends, private trainers for disease control (Fig. 8b).

Production

Biofloc technology has gained importance, especially among unemployed youths, because they believed that highly profitable intensive culture system can be manipulated easily, but in reality most of the fish seeds did not survive till the end of the culture, majorly due to adverse water quality, cannibalism and diseases. An overall scenario of fish production clearly indicated that harvesting above 3 and 4 ton/ 10,000 L water volume was achieved by tilapia farmers only (Fig. 9).

Economics

Majorities of respondent were found small scale investors (Fig. 10a). The profit gain was categorised in four groups as mentioned in the questionnaire no. 10b and presented graphically in Fig. 10b. High profit was reported by 7.8% Tilapia farmers only, while majority belonged to medium profit groups. Despite high production, the profit from Tilapia was not satisfactory because of low market price. Singhi/Magur farmers were also unable to reach their target due to slow growth, non-uniform size and disease occurrences. All the Bata farmers belonged to the low profit group due to low

production in contrast to investment cost. Profits earned by Koi and Pangas farmers in 4 months were found very unsatisfactory, as majority faced financial loss due to excessive mortalities (Fig. 10b). Apart from disease and mortality, the problem of Koi was associated with low acceptability by consumers for unpleasant colour, whereas, for Pangas it was low market price.

Farmers' satisfaction and prospect of biofloc culture

Only 13.6% respondents were found satisfied with this tank biofloc culture techniques and decided to go forward without vacillate. Majority of the respondent were not found happy with this upcoming aquaculture technique yet they did not give up. Whereas, 23.7% farmers were found disappointed and decided to stop the culture thenceforth (Fig. 11a). Possibility of higher investment, recommendation to other farmers to adopt biofloc culture and involving family members were also found limited among the respondents as they apprehended the techniques might not be sustained in coming days (Fig. 11b).

Conclusions

Selection of suitable species, maintaining ideal water quality conditions, use of good quality probiotics, correct feeding and maintenance of proper C:N ratio to prevent nitrogen accumulation in tank should be the top most priorities in biofloc system; any critical gap makes production management difficult and may force the farmers to address emerging issues during culture. The culture system requires a start-up period and hence the newcomers are suggested to initiate the culture in small scale for standardizing it and gaining experience. They are also advised to be more cautious during winter. More emphasis should be put on disease prevention strategies than treatment since no appropriate disease treatment protocol for biofloc culture system is available till date. Provision of rehab tank for quarantine, observation and treatment should be mandatory. Farmers are also advised to make proper documentation of their findings for adoption of future strategies. Nile tilapia was found to be the most suitable species for biofloc system in this survey; the farmers may try culture of Gift or Monosex tilapia to overcome the problem of non-uniform growth. Singhi and Magur also can be considered for high market price, but farmers should be

more conscious about longer culture duration. Culture of koi and pangas in biofloc tanks are still needed to be standardized. Too much dependence on Youtube video and social media for every emerging problem often may misguide the farmers. More intervention of Government sector in this endeavour is urgently needed in terms of conducting awareness programme, training for knowledge and capacity building, bringing farmers under subsidized schemes and insurance fold. The problems of small and marginal biofloc framers can be better addressed by organizing them through institutional efforts.

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Curriculum Vitae

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Immunology: Immunodiffusion, Immunoprecipitation, ELISA, Western Blot etc.

Molecular Biology: DNA and RNA extraction, PCR, Agarose gel electrophoresis, etc.

Research Papers:

Mali, P., Ghosh, S., Dash, G., Abraham, T.J., Chowdhury, S. and Das, S.K. (2021). A study on ground reality of freshwater fish farming in biofloc tank in West Bengal, India. *Journal of Inland Fisheries Society of India*, **53 (3&4)**: 132-142.

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