E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2021; 9(3): 194-199

www.fisheriesjournal.com
Received: 15-03-2021
Accepted: 21-04-2021

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Intensive culture of Asian stinging cat fish *Heteropneustes fossilis* (Bloch, 1794) in the biofloc system: An attempt towards freshwater conservation

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DOI: https://doi.org/10.22271/fish.2021.v9.i3c.2480

Abstract
The experimental work focused on 90 days culture of *Heteropneustes fossilis* in the 1000lit biofloc (T1&T2) and non biofloc (C) system to observe growth and production with lowest possible water exchange. And also observe the isolation and enumeration of microorganism. The stocking density was 500fish/1000lit water in all tanks. Species reached 4.3 to 22.5gm, in T1, 24.0 in T2 tank and 18.5gm in C tank. The survival rate also high in T1&T2:88-95%and C tank it was80-90% and the FCR was1.5 in C and 0.6&0.8 in T1&T2 tanks respectively. The total weight gain and specific growth rate per day was in T1,T2 and C tanks 18.2, 20.0and 14.2gm&0.74,0.69and0.57respectively. Temperature ranges 26-32.5°C in all tanks. In biofloc tanks (T1&T2), pH has been found: 7.4-8.0, Dissolve Oxygen:4.8-5.9ppm, Free CO_2:4.2-4.5ppm, Alkalinity:295-300ppm, Ammonia:0.15-1.0ppm, Nitrate:0.00-10.5ppm, Nitrite:0.25-1.5ppm, TDS:1500-1900ppm. Whereas in C tank pH range: 6.5-7.6, Dissolve Oxygen:5.2-5.5ppm, Free CO_2:4.2-4.6ppm, Alkalinity:278-287ppm, Ammonia:0.07-1.2ppm, Nitrite:0.01-0.1ppm and TDS:1000ppm. Mean value of all types of bacterial colony forming unit /ml in experimental T1&T2 tanks was Anaerobic bacteria:11×10^6,Gram negative bacteria;9×10^4, *Bifidobacterium* sp 176×10^3, *Pseudomonas* sp:9.5×10^3, *Vibrio* sp:742×10^3, Nitrifying bacteria:1.8×10^3. Moreover, water exchange of 20% in T1 & T2 tank and 50% in C tank required in every week.

Keywords: Biofloc, freshwater, asian stinging catfish, bacterial colony, water exchange

1. Introduction
Water conservation is crucial for sustainable development. Due to the limited natural resources (water, land, etc.) and the impact on the environment, aquaculture industry facing challenge in sustainable development. (*Costa'Pierce et al*. 2012; *Verdegem.* 2013) [35]. The sustainable development of aquaculture industry should focus on utilize limited resources (water, space, energy and capital,) and have less impact on the environment, and generate high profitability and productivity, (*Asche et al., 2008*; FAO, 2017) [1]. Fisheries sector plays a very important role in the national economy; in the world India is positioned second in fish production, it occupying 5.43% comparing with global fish production, in which fishery sector share 1.0% of national GDP and annual growth rate of this sector is 6% (DAHD 2018-19). The aquaculture production is expected to climb from 40 MT (2008) to 82 MT by 2050 (FAO, 2010). *Heteropneustes fossilis is a* freshwater catfish species that provide cheap source of protein and has high economic value in our Indian market. Catfish has become one of the commodities that serves as food ingredients into the food menu of India. Fishery products continue to increase each year so there is need for innovation to increase the production (*Schneider et al.*, 2005) [29]. With the increase of aquaculture production, culture system is also intensified. The use of more inputs, like feed per unit area of land are requires to get more production in intensive aquaculture system (*Henriksson, et al.*, 2018) [21]. The fish is stocked at high density in intensive culture and growth rate can be maintained by the addition of feed. High amount of proteins present in commercially fish feed. *Avnimelech* (1994) [4] reported that, fish retain only 25% of fed and the rest is excreted and usually accumulated to the system as organic nitrogen, ammonia in faeces and feed residue. Hence, nutrients load is high in culture water as well as accumulation of toxic residues which come from feed wastage. Biofloc system is a system that prevents the accumulation of inorganic nitrogen (NH_4, NO_2) by maintaining the high C/N ratio.

Where we can alternatively fixing the waste in intensive culture by using Biofloc technology; Biofloc technology is most profitable and effective to reduce the waste of inorganic nitrogen. It stimulate the waste retention and in the aquaculture system it get converted to natural food for fishes which is known as biofloc; it works like a nutrients retention trap and reduces maintenance costs because it can be used as food supplement for the commercial cultured organisms, and by improving the food consumption rate it provides an added value (*Azim & Little,* 2008) [2]. Several studies have shown that the biofloc technology is used for reduces the production cost in intensive aquaculture and improving the water quality, productivity, biosecurity, feed efficiency. According to *Azim and Little* (2008) [7], for good quality water controlling capacity and producing extra source of protein, biofloc obtained attention in aquaculture. Because of nitrogenous waste up taking from the culture system and bacterial growth stimulation, waste of organic nitrogen and ammonia get converted into biomass of bacteria, if carbon and nitrogen are balanced in the solution and as a result production of microbiological protein.

Inducing the microbial community to uptake the ammonium (*De Schryver P., Verstraete W.*,2009) [15], fish serves as an additional of high value feed by harvesting of bioflocs and this technology also functions under minimum water usage and water exchange (*Suita et al.*, 2015) [32] So, the experiment was conducted to investigate the growth rates, water exchange, water quality parameter and microbial colony by using different agar media in biofloc (T1&T2) and non biofloc i.e. control (C) tank.

2. Material and Methods

2.1 Experimental setup

The study has been carried out for a periods of 90days at Amtalia, Rasulpur, Purba Medinipur. West Bengal, India, 21°50 '24 "N, 87°51' 4.4"E.

2.2 Experimental design

A randomized design ware used to carry out the experiment. Two tanks were filled with biofloc and one with non biofloc, 1000 lit watereach. The fish were cultured in a round size pool tarpaulin cage. Two Biofloc tank were T1, T2 and one non biofloc i.e. control tank C.

2.3 Fish stocking and maintenance

The cat fish are labyrinth fish so, it is possible to culture on biofloc system. Before releasing the catfish fingerlings the pool was filled with freshwater, and raw salt was added at the rate of 200gm/1000lit water and molasses was added 128gm for 100gm feed to maintain the C: N ratio *Heteropneustes fossilis*, mean body weight of fingerlings was 4.2 gm and standard length 8.0 cm were used for the experiment. At first they were acclimatized for 1 day and then released into tank. The fish were stocked at a rate of 500fishs/1000 lit water (*Basuki et al.,* 2018) [8]. In the total experiment period minimal water exchange (approximately 20%) was practiced in total culture periods for the T1&T2, where weekly 50% water exchange was followed for the C tank. A central aeration ring was used for solid suspension and maintained the oxygen levels. Aeration was provided by using a 0.5HP air blower throughout the experiment. Floating feed provided to fish on daily basis (crude protein of 30%) for a period of 90 days; each tank feed rate was 5%adjusted to total body mass of the fish *(Azim, &Little, 2008*) [7]. Daily two times feed rations were provide into two equal amounts in all the experimental tanks.

2.4 Fish growth measurements

Total body length of the fish was measured by using a normal scale and for measuring fish body weight electronic weighing balance machine ware used. At an interval of 15-days the growth of fish ware measured. From each tank randomly ten fish was collected and using digital balance body weight was measured; length was measured using a scale. Fish were collected for sampling before feeding and different growth parameters like Percentage weight gain (PWG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), survival rate were calculated as followed by *Sarker et al., (2016)* [28].

2.5 Water quality parameter analysis

We have studied the Physico-chemical parameters of water quality which affect cat fish culture and microorganisms aggregations. Selected study include Temperature, pH, DO and chemical parameters such as FreeCO_2, Alkalinity,Total ammonia nitrogen (TAN) and nitrate nitrogen (NO_3-N), nitrite. All were analyzed in laboratory and some data like temperature was collected onsite with digital thermometer and on every 15 days interval. The floc density was measured by the help of imhoff cone.

2.6 Plate culture methods for bacterial colony calculation

For microbial analysis the biofloc water was collected in imhoff cone and water was stored in sterile glass bottles during the culture period. The sample were preserved in icebox and processed within few hours. In order to know the microbial load and microbial diversity in biofloc tanks serial dilution of the respective water samples were made and plated on the following culture media.

Media used

1. Pseudomonas agar- Selective media used for the *Pseudomonas* sp.
2. Vibrio agar- Selective media used for the *Vibrio* sp.
3. Mac-Conkey agar- For Gram negative enteric bacilli selective media used.
4. Bifidobacterium agar- Selective media used for the *Bifidobacterium* sp.
5. Anaerobic agar- Selective media used for anaerobic bacteria.
6. Nutrient agar- Selective media used for the cultivation of microbes supporting growth of a wide range of non-fastidious organism.

The quantities of the dominant culturable microflora were enumerated through dilution plating technique on the basis of colony forming (cfu) in selective media by following the standard protocol as stated in the Himedia Manual (www.himedialabs.com). Prepared media were autoclaved at 121º C at 15 psi for 15 min. Then the media were cooled (-42 ºC) and poured into the sterile petridishes after autoclaving, under aseptic condition. After solidification, 0.1 ml of respective dilution was spread on the medium. For cultivation of aerobic bacteria, the inoculated plates were incubated at 37 ºC for 24 hours in BOD incubator. Anaerobic bacteria were cultivated at 37 ºC for 24 hour in a CO_2 incubator (5% CO_2).

2.7 Data analysis
The bacterial counts results were processed by log10 transformation. The co relation co efficient values were calculated by analysis tool pack Microsoft Excel. Only significant relationship was discussed.

3. Results
3.1 Preparation biofloc
In biofloc system carbon source requires for microbial community development. The microbial protein is high protein and certain micronutrients contribute to fish growth and recycling of nutrients into fish biomass (*Avnimelech.*, 1999) [3]. Heterotrophic bacteria biomass production is dependent on carbon level (*Schneider et al*., 2006) [30]. Molasses has been used for the carbon source to promote the microorganisms development (*Avnimelech,* 1999) [3]. The observation of biofloc water under the light microscope, the microorganisms like algae, rotifers, nematodes and other water dwelling organisms was contained high number. The presence of microorganisms was much higher in the biofloc culture (T1&T2) water tank, which was formed by the addition of 128gm molasses for every 100gm feed (*Avnimelech,* 2009) [5] to maintain C:N ratio 20:1. Biofloc contains bacteria, and other micro organisms, protozoa and zooplankton. On aggregates zooplankton like copepods and rotifers ware grazed. *Azim and Little*, (2008) [7] observed that, Rotifers, Protozoa, and Oligocheta generally present in biofloc system. For cultured organisms the presence of microorganisms and particle matters in the culture water serve as potential food source of food. The floc is a complex form of organic matter, physical substrate and a large range of microorganisms such as phytoplankton, free and attached bacteria, aggregates of particulate organic matter and grazers, such as copepods, ciliates and flagellates protozoa and rotifers.

3.2 Survival rate growth performance and bacterial colony in biofloc tanks
In every 4th week interval the survival rate of two biofloc tank (T1 & T2) was high than the non biofloloc(C) tank (Table 1). And also growth performance indicators like Final Average Body Weight (ABW), Final Average Body Length (ABL), Percent weight Gain (PWG%), Specific Growth Rate (SGR)%/day, FCR of *H. fossilis* was highest in two biofloc tanks compare to non biofloc tank, of 90 days culture period. where Initial Average Body Weight (ABW), Initial Average Body Length (ABL), was same in all tanks (Table 2). The bacterial colony concentrations varied and ware very high in nutrient agar. *Pseudomonas* sp, Nitrifying bacteria, and other beneficial microorganism were grown dominant in the biofloc system water (Table 4).

3.4 Water quality parameters
All the physico-chemical parameter like temperature, dissolve oxygen, pH , free carbon dioxide, alkalinity, ammonia, nitrate, nitrite etc. were found in acceptable range throughout culture operation (Table 3) and they agreed well with the findings of *Boyd* (1979) [9]. The hardness, calcium(Ca^{2+}), magnesium (Mg^{2+}) potassium (K^{+}) iron (Fe^{2+}) ware almost same in all tanks those ware 300ppm, 0.0ppm, 60ppm ,60ppm and 0.05ppm. TDS ranges was 1500-1900ppm in two biofloc tanks (T1&T2) where non biofloc tank (C) was 1000ppm.

4. Discussion
After catfish fingerling was stocked, there was change in water quality parameter. Catfish fingerling has a good chance to survive as long as well maintain the water quality. However, throughout the experiment sometime water quality parameter suddenly decreases and leads to the death of the fish. The survival rate changes in every month or every four weeks. The mean value of T1 showed significantly (P<0.05) the highest survival while in C showed the lowest (Table: 1).
In this experiment, for the growth of *H. fossilis* supplementary feeds are supplied. Growth in terms of weight, length, SGR and weight gain of individual *H. fossilis* were statically significantly higher values (P<0.05) in T1 & T2 system than C system. The fastest growth was observed in T1&T2 system than C. At the beginning we have started the experiment with mean body weight of fish was 4.3gm. It also shows the positive effects of feeding quality and water quality on fish growth, but the significant level was different in each tank. Among the three systems significantly (P<0.05) the highest mean final weight was recorded in T2, the weight increases T1 18.2±0.64gm T2 20±0.99gm and C 14.2±0.63gm (Figure:1). During the experimental period, higher survival rate of *H. fossilis* influencing factors are tank preparation, healthy fingerlings, and stocking, feed quality, physico-chemical parameters, and good management practices, *Choudhury et al.* (1978) [12]. Chiu *et al*. (1989) [11] state that lowering the FCR value, digestibility plays an important role by efficient food utilization. Digestibility factor depends on frequency of feeding, daily feeding rate and type of food used. There was gradually decrease in FCR in T1 and T2 system than C system. The final FCR was in T1 tank 0.6 T2 tank 0.8 and C 1.5. The FCR value of T1 was found to be statistically significant (P<0.05) which indicated that lower amount of feed was needed to produce one unit fish biomass and highest was found in C. For different treatment the FCR values ware acceptable and indicated better utilization of food, which is identical to observation of *Reddy and Katro* (1979) [27]. Growth performances results (Table:2) showed that fish growth performances was enhanced in biofloc system. For different culture species *Azim and Little and Luo et al*. (2014) [25] also reported the identical findings. In comparison between biofloc to non biofloc higher performances in growth of biofloc system may be due to the intake of high amount of feed. In culture system microorganism and particles presence provide an extra source of food to the cultured species (*Crab et al.*, 2012) [14] and (*Schneider et al.*, 2005) [29]. Microorganisms play an important role in feed intake of *H. fossilis* and thereby species growth performance increased. Hence, logically it is assumed that additional intake of feed with nutritional value feed can enhance the fish growth performances. At the end of the experiment, the SGR (% per day) were T1:0.74, T2:0.69, and C: 0.57 respectively. The result of the experiment revealed that significantly (P<0.05) the highest SGR value (0.74) was recorded in T1 while lowest (0.57) was obtained in C. Similar types of observation recorded different species and different stocking densities by *Soedibya et al.* (2018) [31].
The pH value found to be in the range 7.6-8.0, 7.4-7.8 and 6.5-7.6 in T1, T2 & C tanks respectively, it indicates slightly alkaline water. According to *Swingle* (1969) [33], the pH range between 6.5 to 9.0 is suitable for catfish culture, Azad *et al.* (2004) [6] recorded pH ranging from 6.18 to 9.16 in polyculture ponds .The pH value was at its peak in tank T1 in the 7th weak due to high concentration of ammonia. (*Wurts, William*, 2003) [36]. The Temperature range lies between 26-32°C, beside catfish growth (Table:3), temperature of the water also affecting formation of floc (*De Schryver& Verstraete*,2009) [15]. However, there was no significant

(*P*>0.05) variation among the tanks. In addition, temperature affect the activity of microbes in the floc, for example the primary structure of floc are composed extracellular polysaccharide formed by microbes was higher at 25°C (*Krishna &Van Loosdrecht* 1999) [24] and for *H. fossils* culture DO found to be optimum and that was 5.0-5.9ppm. In the T1&T2 tanks Temperature and pH significant (*P*<0.05) affected the percentage of weight gain (PWG%). According to *Boyd* (1982) the alkalinity to be more than 20 mg/lit in fertilized ponds. The production increases with the increase in total alkalinity. The variations of total alkalinity in all tanks were within the productive range for aquaculture **(***Wahab et al.*,1995; Kohinoor *et al*.,1998), few factors of environmental affect growth, feed efficacy and feed consumption of fish (*Brett.*,1979). The permissible DO limit for *H. fossils* culture is 4 ppm and in study the value was found to be ranging between 5.0-5.9 ppm in all tanks. Higher value of of DO concentration was found in all experiment tanks due to continuously aeration in the tanks which were equipped. For water mixing and avoiding biofloc deposition aeration needed, food remains and feces could increase the ammonia concentration (*Hargreaves.*, 2013) [20].

The ammonia is a primary byproduct of protein metabolism, the level of Ammonia, nitrate and nitrite in the T1&T2 system was significant (*P*<0.05) to the PWG. The mean values of ammonia-nitrogen (NH4-N) contents in the present study were significantly (*P*<0.05) highest in C (0.9±0.2) followed by T2 (0.6±0.12) and T1 (0.6±0.09 ppm) (Table:3). According to *Ekasar*i (2009) [17] due to utilization of inorganic and organic compound of nitrogen by heterotrophic bacteria in the culture system the quality of water remain balanced for floc in the system. In the biofloc culture system nitrification process occur due to presence of high concentration NO_3-N. While concentration of NO_2-N in two T1&T2 treatments tanks seems to be relatively stable. The concentration nitrate nitrogen ware 0-10.5 ppm, nitrite nitrogen 0-1.5ppm and ammonia was 0.1-1.0ppm throughout the culture period. The concentration of nitarate gradually increases with time in biofloc system while in non biofloc system nitrate concentrations remain constant. This observation was similar to the observation of *Azim and Little*, (2008) [7]. According to the *Ebeling et et al*., (2006) [16], biologically nitrogen can be removed by algae through photoautotrophic pathway, chemo-autotrophic oxidation pathway by and immobilization through heterotrophic bacteria from the aquaculture system.

The number of total bacterial colony was high in two biofloc tanks (T1&T2) in nutrient agar media because all types of bacteria can grow in nutrient agar media. For the cultivation of microorganisms of less fastidious Nutrient Agar are used. The nitrifying bacteria, anaerobic bacteria, gram negative bacteria, pseudomonas were dominant in biofloc tanks (Table:4). The nitrifying bacteria helped in nitrification process and all other bacteria in biofloc tanks were also helpful for food and ammonia recycling. Similar types of work observed by *Putra et al.*,2017 [26], stated that probiotics can decompose organic materials, suppress the growth of pathogenic, culture media quality improved and maintain balance the microbial load and have positive health and growth effect on fish.

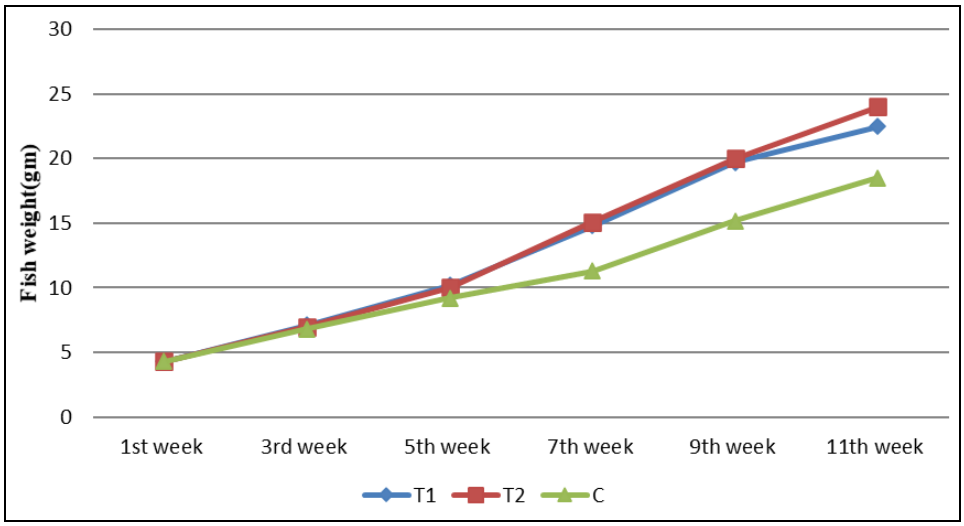

Fig 1: Every fifteen days interval fish growth

Table 1: Survival rate (SR) per month or every 4th weak

Tanks	Initial (%)	Week 4(%)	Week8 (%)	Week 12(%)
T1	100	95	90	88
T2	100	96	89	87
C	100	90	85	80

Table 2: Growth performances in three tanks

Parameters	**T1**	**T2**	**C**
Initial ABW gm	4.3	4.3	4.3
Final ABW gm	22.5	24.0	18.5
Initial ABL cm	8	8	8
Final ABL cm	16	16	13
PWG (%)	423.25	458.14	330.23
SGR(%/Day)	0.74	0.69	0.57

DOC(Days)	90	90	90
FCR	0.6	0.8	1.5

ABW, average body weight; ABL, average body length; PWG, percent weight gain; SGR, specific growth rate; DOC, days of culture; FCR, food conversion ratio;

Table 3: The mean value, SE and ranges of water quality parameters of all tanks during total culture period

Tanks	Temp(°C)	pH	Dissolved Oxygen(ppm)	Free carbon dioxide(ppm)	alkalinity (ppm)	Free Ammonia (ppm)	Nitrate (ppm)	Nitrite (ppm)
T1	29.7±0.99 (26-32.5)	7.8±0.05 (7.6-8.0)	5.3±0.14 (5.0-5.9)	4.4±0.03 (4.3-4.5)	298±0.3 (297-300)	0.6±0.09 (0.23-0.83)	8.5±1.7 (0.0-10.5)	1.14±0.1 (0.25-1.5)
T2	29.6±0.94 (26-32)	7.7±0.08 (7.4-8.0)	5.3±0.15 (4.8-5.8)	4.3±0.04 (4.2-4.5)	297±0.6 (295-300)	0.6±0.12 (0.15-1.0)	8.4±1.6(0.00-10.2)	1.13±0.2 (0.22-1.5)
C	29.6±0.94 (26-32)	7.1±0.16 (6.5-7.6)	5.2±0.07 (5.2-5.5)	4.4±0.06 (4.2-4.6)	286.2±2.2 (278-287)	0.9±0.2 (0.14-1.5)	-----	0.1(0.02-0.14)

Table 4: The mean values of bacterial colony of two biofloc tanks

Media Name	Cfu/ml of Water Sample
Nutrient Agar	11×10^6
Anaerobic bacteria	9×10^4
Gram negative bacteria	176×10^3
Bifidobacterium sp.	9.5×10^3
Pseudomonas sp.	742×10^3
Vibrio sp.	1.8×10^3
Nitrifying bacteria	573×10^4

5. Conclusion

The use of biofloc technology proved to be an effective for more fish production, high survival rate and higher growth rate than non biofloc intensive culture. Biofloc is an alternative food source for fish. Biofloc can be recommended for fish farmers for more production with less or minimum water exchange. The freshwater may be conserved by using such techniques.

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