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Critical water quality parameters and certain non specific immune parameters of *Clarias gariepinus* juveniles raised in bioflocs system with cassava flour and rice bran as carbon sources

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Abstract
This study was conducted to examine effects of bioflocs culture on critical water parameters and certain non specific immune parameters in *C. gariepinus* juveniles. This experiment was conducted for 72 days, in 9 separate tanks with an aerated blower. Bioflocs culture had two different sources of carbon addition. This research consists of three treatments, carbon sources were added at CN ratio of 15:1. Results showed no significant difference in dissolved oxygen, pH, and temperature in all treatments. However, conductivity, Total dissolved solids and salinity showed significant difference across all treatment groups. Total ammonia nitrogen, nitrate and nitrite showed significant difference between control and bioflocs treatments. Blood parameters examined were serum lysozyme, myeloperoxidase activity, neutrophils, lymphocytes and monocytes percentage. Serum lysozyme and myeloperoxidase showed significant difference across treatments while neutrophils percentage showed significant difference between bioflocs culture and control experiment. Lymphocytes and monocytes showed no significant difference.

Keywords: biofloc, immune system, nitrite, dissolved oxygen, neutrophils

1. Introduction
Almost all the earth's natural resources have been harnessed, farmed, or cultured for man's various needs. These resources have benefited man in various ways which include provision of food, fuel, shelther, clothing and meeting the energy demands of man such as electricity, transportation e.t.c (FAO, 2010) [33]. However, as population increases, these resources decreased proportionally, in some particular cases these resources have depleted than they are being replenished while some cases have led to environmental pollution, loss of biodiversity, extinction of said resources and climate change e.t.c (FAO, 2014) [34]. Global fish production has grown steadily in the last five decades, with food fish supply increasing at an average annual rate of 3.2%, outpacing world population growth at 1.6%. World per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 20.3kg in 2017 (FAO, 2018) [29].
The aquaculture industry is a substantial global industry supplying a significant proportion of the aquatic food consumed and other aquatic products that are valuable sources of protein and essential nutrient components for global food security (FAO, 2018) [29]. To support the rapidly growing human population globally, food production industries such as aquaculture require an approach toward improving the output with minimal cost. Aquaculture is an activity in constant growth that requires maximizing resources and spaces. The rapid growth of global aquaculture is faced with environmental and economic imitations. Employment in the sector has grown faster than the world's population, the sector provides jobs to tens of millions and supports the livelihoods of hundreds of millions and fish continues to be one of the most traded food commodities worldwide (FAO, 2014) [32].
Amongst the challenges of aquaculture include competition for land and water resource, the introduction of exotic species around the globe, the over exploitation of ocean fish stocks to

obtain fishmeal and fish oil, the dispersion of pathogens, high level eutrophication, development of antibiotic resistance gene (FAO, 2010) [33]. Feed being a major input plays a major role in freshwater aquaculture, high protein content in the feed becomes unutilized and subjected to microbial decomposition which leads to the production of toxic inorganic nitrogenous compounds like ammonia, which is detrimental to the organism in the culture ponds (Avnimelech and Ritvo, 2003) [9].

In aquaculture, the accumulated waste must be removed continuously to maintain optimal growth conditions and the health of the cultured organism (Akinwole, 2005) [2]. The profitability of aquaculture depends on methods used in minimizing waste production or waste utilization as inputs to other production processes. Therefore, the management of solid wastes and dissolved substances is one of the most important aspects in the aquaculture industry today. In order to make aquaculture industry more successful, it is of utmost importance to develop technology that will increase economic and environmental sustainability (Kuhn *et al*., 2010) [41].

Biofloc technology is an emerging avenue in aquatic animal healthcare and nutrition not only horizontal but as well as vertical expansion (Daniel & Nageswari, 2017) [24]. This system involves the modification of physicochemical variables of the culture system to favour the proliferation of particular biotic communities have been adopted not only to improve the recirculation of nutrients (and the consequent detoxification of the system) but also to use the biomass of such biotic communities as direct food source for the cultured organisms. This system of aquaculture promises to solve some of the challenges and revolutionize aquaculture. In addition to maintaining good water quality, it provides an inexpensive source of feed and a higher efficiency of nutrient conservation. The water purification method using biofloc technology (BFT) was developed to make fish farming more cost effective and to increase nutrient utilization efficiency, reduce water use, provide additional feed, reduce effluent discharges and improve biosecurity (Avnimelech, 2006) [9]. The BFT has the advantage over all other techniques that it is relatively inexpensive, this makes it an economically viable approach for sustainable aquaculture.

African catfish, *Clarias gariepinus*, of the Clariid genus is known as the special freshwater fish in Nigeria and the West African sub-region of high economic value and delicacy. Because of its rapid growth rate, high production, and good disease resistance capability, *C. gariepinus* has become an important aquaculture species in Nigeria. There have been several investigations on the applications of biofloc technology to *C. gariepinus* culture, and these have mostly been positive (Ekasari *et al*., 2016; Romano *et al*., 2018) [27, 49]. This study was designed to evaluate the effect of bioflocs technology using rice bran and cassava flour as carbohydrate sources on critical water quality parameters and certain non specific immune parameters of *C. gariepinus* juveniles

2. Materials and Methods

2.1 Description of Experiment Site

This experiment was conducted at the Teaching and Research Farm of Department of Fisheries and Aquaculture Technology, Federal University of Technology Akure, Ondo State, Nigeria.

2.2 Sample Collection

2.2.1 Fish for the Experiment

Ten thousand individuals of *Clarias gariepinus* juveniles of average weight of 9.0 ± 0.23g were obtained from a reliable hatchery. The fish were transported live using open headed container to the department of Fisheries and Aquaculture Research Farm of Federal university of Technology Akure. The fishes were acclimatized for 14 days prior commencement of experiment.

2.2.2 Carbon sources for bioflocs

Carbohydrates sources used for the experiment were Rice bran and cassava flour. Rice bran was sourced and procured from an animal feed mill with good biosecurity practices, while cassava flour was procured from a cassava processing plant. Both carbohydrate sources were transported to the department of Fisheries and Aquaculture Research Farm.

2.3 Experimental Design

Raw rice bran was obtained from a livestock feed ingredient store in Akure, Nigeria, then hammer milled to powdery form (425µm), while cassava flour was procured from a cassava processing plant. Biofloc system was prepared by adding 2mg of ammonium chloride (NH4Cl) to 1litre of water for 7 days as a source of nitrogen preceding the experiment. The rice bran and cassava flour were incubated with commercial *Bacillus megaterium* (15L of water obtained from tilapia culture system) with aeration for pre treatments to initiate biofloc production. *Clarias gariepinus* juveniles were randomly and equally distributed into 9 rectangular outdoor culture tanks ($2m^3$ each) in three experimental groups in triplicate following a completely randomised design. A thousand individuals of *Clarias gariepinus* each were placed in each tank. This experiment was conducted for a period of 72 days, fishes were fed twice daily (morning and evening) with a commercial fish feed (45% CP).

2.4 Experimental Procedure

Nine units of outdoor rectangular concrete tanks (2m3 each) at the Department of Fisheries and Aquaculture Technology, Federal University of Technology, Akure Nigeria, were assigned for this experiment. Before the experiment, tanks were prepared (cleaned, dried) and filled with fresh water. Fishes were acclimated for 14 days, bioflocs was initiated 7 days prior the commencement of the trial. After acclimatization, three different culture systems were applied i.e. control (without carbon addition), biofloc (with Rice bran as carbon source) and biofloc (with cassava flour as carbon source) systems. The acclimated *Clarias gariepinus* were randomly distributed into 9 tanks. Fishes were fed twice daily at 5% body weight (08.00 and 17.00hours) with a locally available commercial fish feed (40% crude protein). The organic carbon source in the biofloc system was added daily at a dose depending on the estimated total feed nitrogen added to the culture tank on the previous day and the expected C/N ratio in the water as described by Avnimelech (1999) and De Schryver *et al*. (2008). A pre-weighed carbon source was mixed in a glass beaker with the water collected from the corresponding culture tanks and was added and poured directly into the water column after first feeding (Avnimelech, 1999). Aeration was supplied by an air blower installed at 9 lines (5l/min per line).

2.5 Water Quality Parameters

Temperature, pH, and dissolved oxygen (DO) concentration were measured daily by using thermometer, pH meter, and DO meter respectively. Specific conductivity, salinity, and

total dissolved solids (TDS) were measured using the EXTECH instrument (ExStik ii model) according to the standard method for the examination of water and wastewater. However, total ammonia nitrogen (TAN) was measured weekly in line with the standard procedure described in the standard for water and wastewater quality analyses (APHA, 1998). Analyses of the nitrite (NO2-N) and nitrate (NO3-N) concentrations were performed every 5 days as described by Strickland & Parsons (1972) [51].

2.6 Blood and Serum Collection
Blood and serum samples were collected for immunological assays. Fishes were anesthetized with clove oil at 50 µl per litre of water before collecting blood samples. Blood was drawn from the caudal vein of fish using 1.0 ml hypodermal syringe and 24 gauge needles, and rinsed with 2.7% EDTA solution before use. The collected blood were immediately transferred to the test tube coated with thin layer of EDTA (as an anticoagulant) and shaken properly in order to prevent clotting.

Serum collection was done without the use of anticoagulant and separated from blood by keeping the tubes in slanting position for close to 2h and centrifuged at 1370 g for 15 min at 4 °C, followed by collection of straw coloured serum with micropipette and stored at 20 °C for further analysis.

2.7 Blood and Serum Assays
2.7.1 Myeloperoxidase activity
Total myeloperoxidase content present in serum was measured according to Quade and Roth, 1997. About 15 µl of serum was diluted with 135 µl of Hank Balanced Salt Solution (HBSS) without Ca2+ or Mg2+ in 96 well plates. 25 µl of 20 mM 3,3-5,5 tetra methyl benzidine hydrochloride and 25 µl of 5 mM H_2O_2 (both substrates of MPO) were added. The colour change reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid (H2SO4). Plate was centrifuged at 400 g for 10 min, and 150 µl of the supernatants, present in each well, were transferred to new 96 well plates. The OD was recorded at 450 nm in a micro plate reader.

2.7.2 Serum lysozyme activity
Serum lysozyme activity was measured using spectrophotometric assay by (Anderson and Siwicki, 1995) [47]. 3ml of *Micrococcus luteus* or a gram variable bacteria suspension in phosphate buffer was put in a cuvette, to which 50 µl of diluted serum sample will be added. The content of cuvette was properly mixed for 15s and measured using a spectrophotometer at 450 nm. The reading of lysis of the bacteria was taken immediately. Lysozyme activity is expressed as U/min.

Other Haematological values were measured by following standard methods as described by Terry *et al*. (2000) [52].

2.8 Statistical Analysis
Data collected were analyzed by one factor analysis using Statistical Package for Social Science (SPSS 23) and significant difference in the mean was compared using the New Duncan Multiple Range Test (NDMRT).

3. Results
3.1 Water Quality Parameters of Various Treatments
Table 1 shows the range of water quality parameters of all treatments measured during the experimental period. Temperature and DO in water of all treatments were in optimal condition for fish culture. Also range of pH in all treatments during the course of the experiment showed a relatively stable pH while Total dissolved solids is higher in both bioflocs treatments

The water temperature of the different treatments ranged from 24.2 °C to 25.6 °C, however, no significant difference existed between the treatments. Water quality parameters at the end of the experimental period revealed considerable variation. The values for dissolved oxygen ranged between 7.87- 12.10 mgL−1 during the experimental period. The control (7.8mgL−1) had significantly higher DO than BC (5.40mgL−1) and BR (4.90mgL−1). The mean pH ranged from 7.74 to 8.37. The total dissolved solids (TDS) fluctuated from 283.57 to 871.57mgL−1 in the experimental groups. The TDS in rice bran biofloc and cassava flour biofloc were significantly higher than the control. The concentration of total ammonia nitrogen (TAN) within the experimental groups varied significantly (p<0.05) with the highest mean values of 0.32mgL−1 in the control, 0.17mgL−1 in BR, and the lowest mean value of 0.11mgL−1 in BC, respectively (Table 2). A similar trend was observed in nitrite values among the Treatment groups. The control group had the highest value of 0.39mgL−1 followed by BR (0.23mgL−1) and the lowest in BC (0.29mgL−1). The values recorded for nitrate among the treatments showed that BR had the highest (25.2mgL−1) and the least (16.18mgL−1) in the control. The alkalinity values also show the same trend with BR having the highest (125.9mL−1) and control having the least value (113.7mgL−1).

Table 1: Water Quality Parameters of various treatments

	pH	Temperature	Conductivity	TDS	DO
Control	7.74-8.37	24.26-25.4	364.43-517.29	283.57-367.72	7.81-12.04
BR	7.99-8.09	24.21-25.31	400.71-989.26	320.14-688.71	5.40 - 8.11
BC	7.79-8.02	24.24-25.63	559.57-1314.14	389.29-871.57	4.91 - 7.74

Table 2: The average values of total ammonia nitrogen, nitrite, nitrate, and alkalinity

	TAN (mgL−1)	Nitrite (mgL−1)	Nitrate (mgL−1)	Alkanility (mgL−1)
Control	0.32 0.01^a	0.39 0.02^a	16.18 1.31^a	113.7 1.62^a
BR	0.11 0.02^b	0.23 0.02^b	23.03 0.71^a	118.1 2.29^a
BC	0.17 0.01^b	0.29 0.01^b	25.2 1.03^b	25.2 1.03^b

Means ±SD on the same row with different superscripts are statistically significant (p<0.05). TAN: total ammonia nitrogen

3.2 Immunological Assays / Non Specific Immune Parameters
Data obtained from various immunological parameters assessed is shown in Table 3, most non-specific immune parameters showed significant difference across treatment groups. Serum lysozyme activity (U/min) was found to be significantly different across treatment groups, when compared with control serum lysozyme values increased

significantly. Also Myeloperoxidase activity (MPO) (OD at 450 nm) showed significant difference across treatments, MPO showed higher value for control compared with bioflocs culture. Neutrophils percentage showed significant difference between bioflocs culture and also between bioflocs and control. Lymphocytes percentage shows no significant difference across treatment groups while neutrophils percentage shows significant difference between biofloc treatments. However both parameters have higher percentages across both bioflocs culture. Monocytes was significantly higher in bioflocs culture compared with control experiment but shows no significant difference across treatments

Table 3: Non specific immune parameters of *C. gariepinus* raised in bioflocs system using cassava flour and rice bran as carbon source

Parameters	BC	BR	C
Neutrophils (%)	63.00 ± 1.73^{b}	60.00 ± 4.16^{a}	61.67 ± 1.45^{ab}
Lymphocytes (%)	35.33 ± 2.60^{a}	39.33 ± 4.81^{a}	38.33 ± 1.45^{a}
Serum Lysozyme (U/min)	0.96 ± 0.01^{c}	0.89 ± 0.02^{b}	0.61 ± 0.01^{a}
Myeloperoxidase activity (OD at 450 nm)	0.91 ± 0.01^{c}	0.83 ± 0.05^{b}	0.61 ± 0.01^{a}
Monocytes	1.66 ± 0.57^{a}	1.50 ± 1.00^{a}	0.66 ± 0.57^{a}

a, b = Means on the same row with different superscripts are statistically ($p<0.05$) significant
BC= Biofloc Cassava, BR= Biofloc Rice Bran, C= Control

4. Discussion

Temperature and Dissolved oxygen observed throughout this experimental period was in an optimal range. However the optimal level of dissolved oxygen in experiment can be due to the addition of aeration all through the course of the experiment. pH values were also stable and within limits for culturable species during the course of the experiment. DeSchryver *et al*. (2008) [12] suggested that nitrogen uptake by heterotrophic process that likely to dominate BFT system consumes alkalinity half than nitrification (3.57 g alkalinity/ 7.5g NH4+-N). As alkalinity concentration relates to the buffering capacity of water, thus it could be suggested that in BFT system, the effect of the high concentration of CO2 resulted from fish and microbial respiration has an effect on water pH. The presence of NO2-N and NO3-N in both control and BFT treatments indicates the occurrence of nitrification processes in both culture systems.

The potential benefits of BFT may be multi layered which may include maintaining water quality, which would minimize stress as well as increase biosecurity and potentially reducing prevalence of diseases. Another factor may also include the consumption of bioactive compounds in the bioflocs that leads to an enhanced nutritional status and or immunity of the fishes (Xu & Pan 2013; Ekasari *et al*. 2014; Ahmad *et al*. 2016) [55, 27, 1] This study demonstrated that bioflocs enhanced water quality for performance and certain immunological parameters during the course of experiment. The survival rate of bioflocs treatment was higher ($p<0.05$) than the control (non biofloc treatment). This result indicated that bioflocs was able to increase the survival rate which may be as a result of improved water quality.

Balancing the concentration of ammonium in the biofloc culture system by adding a carbon source is possible because the heterotrophic bacteria in biofloc can absorb ammonia 40 times faster than nitrifying bacteria found in non-biofloc system. The relationship between adding carbon via carbohydrates in biofloc, reducing ammonium, and producing microbial proteins has been reported already by Avnimelech (1999) [7]. This relationship depends on the microbial conversion coefficient, the C/N ratio in the microbial biomass, and the carbon content of the added material. Avnimelech (2005) [9]. demonstrated that the addition of carbohydrate lessens the need for dietary protein concentration and also decreases the TAN level in the system. The average concentration of TAN observed was higher in the control group (Table2). However, the mean value of a lower concentration of NO3-N was observed in the Biofloc treated group. This low level probably relates to NO3-N uptake by microbes in the treatments (Hargreaves, 2006) [37]. In general, the TAN concentrations found in this study were still in optimum ranges for *C. gariepinus* production (Taslihan *et al*., 2003) [51]. Rostika (2014) [49]. recommended total dissolved solids (TDS) levels up to 1000mgL−1 to be appropriate for the culture of *C. gariepinus*, but beyond this level, it may lead to stress. The values recorded in this study in the biofloc and control groups were less than the threshold for *C. gariepinus* culture. The lower pH values in the biofloc tanks may be appropriated to high respiration rates by the large quantities of micro organisms, which might consequently increase CO_2 concentrations. A similar trend was observed by Wasielesky *et al.*, (2006) [55]. Also, the decrease in pH during the chemolithotrophic process as reported by Chen *et al.*, (2006) [19] leads to the release of CO2 and H^+ into the culture medium. Dissolved oxygen observed during the experimental period was within the range required for culturing *C.gariepinus* production, which is an indication of the positive effect on plankton nutritional quality (Azim *et al.* 2008) [11].

Azim and Little (2008) [11] stated that, the presence of optimum concentration microbial cell in biofloc was able to increase fish health status. Bacterial cells in the biofloc accumulate the poly-β-hydroxybutyrate (PHB) with alleged role in microbial pathogens inhibition of fish culture. The PHB content in biofloc consumed by fish was able to increase its immune system so that the fish can be more resistant to environmental interference during treatment.

Blood tissue is a reflection of the physical and chemical changes occurring in organisms that gives detailed information on the general metabolism and physiological status of fish in different groups of age and habitat (Ahmad *et al.*, 2017) [1]. Blood analysis is an essential component for successful fish farming, management and diseases investigation. Also the blood condition of the fish is essential with regards to the health, food, economic and post-harvest needs of fish farmers and consumers.

Lysozyme activity functions as primary defence of non specific humoral immunity, its capability to disrupt cell walls of pathogens makes it a natural antagonist to harmful organisms which include bacteria and viruses. Neutrophils are considered the source of lysozyme, lysozyme has been found predominantly in fish serum and mucus (Ellis 2001) [28]. An increased level of lysozyme has been found to be a natural protective mechanism (Basha *et al*., 2013) [12] and it helps destroy gram positive bacteria. In this study the serum

lysozyme showed significant difference with biofloc culture with cassava flour as carbon source having the highest value. It can thus be inferred that *C. gariepinus* juveniles serum lysosome activity was enhanced in biofloc culture.

MPO is a peculiar and specific hemeprotein released by neutrophils, secreted and functional during activation of neutrophils and produces hypohalous acids to carry out their antimicrobial activity. MPO activity also showed significant difference, the actions of MPO may also contribute to the initiation and pathogenesis of inflammatory related disease (Basha *et al*., 2013) [12]

Neutrophils are an important component of host defence against many bacterial, viral and fungal infections, it produces cytokines to recruit immune cells to infected areas of the fish. The evaluation of neutrophil function is valuable for assessment of the health status, in this study, neutrophils percentage was found to show significant difference between the bioflocs culture and also between the non bioflocs culture. Although percentages were high in all treatment groups with the highest value in bioflocs culture using cassava flour as carbon source.

Monocytes are capable of differentiating between pathogenic and non pathogenic mycobacteria, it produces cytokines the primary cells involved in phagocytosis. Monocytes showed no significant difference, however it was higher in both biofloc culture compared with the non bioflocs culture. Lymphocytes percentage also shows no significant difference.

5. Conclusion

Both carbohydrate sources used in this research (Cassava flour and rice bran) has proven to be of immense benefit in the culture medium and for the culture organism (*C. gariepinus*). Both carbohydrate sources utilised for biofloc treatments improved innate immunity as well as maintained good water quality for the cultured *C. gariepinus* than the control treatment.

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