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RESEARCH PAPER

Optimizing Stocking Density in Biofloc Culture of Juvenile Common Carp (*Cyprinus carpio*) Using Growth and Immune-biochemical Indices as Indicators

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Abstract

The optimizing stocking density for biofloc technology is essential for improving fish growth and profitability of aquaculture. The treatments consisted of different stocking density of 125, 250, 375, and 500 common carp (*Cyprinus carpio*) fish/m^3. Fish with an initial average weight of 17.10±0.3 g were fed three times per day in triplicates for 40 days with a commercial diet (25% crude protein), and the ratio of carbon to nitrogen 20 in each treatment. The water nitrogen substances enhanced by increased stocking density ($P<0.05$). The final growth (-19.75%), whole-body protein (-2.84%), lipid content (-7.27%) significantly decreased, and food conversion ratio (+67.29%) increased in the treatment of 500 fish/m^3 compared to the treatment of 250 fish/m^3 ($P<0.05$). Serum glucose (-11.09%), triglycerides (-11.24%), high-density lipoprotein (-26.16%), and low-density lipoprotein (-31.98%) levels significantly reduced, but serum cortisol concentrations (+13.08%) and, liver enzymes increased in the treatment of 500 fish/m^3 compared to the treatment of 250 fish/m^3 ($P<0.05$). Total protein (-9.01%), globulin (-12.80%), ACH50 (-4.97%), and lysozyme activity (-9.21%) significantly reduced in the treatment of 500 fish/m^3 compared to the treatment of 250 fish/m^3 ($P<0.05$). The best performance of fish was observed at stocking density up to 250 fish/m^3 in the biofloc system.

Introduction

Biofloc technology with the use of less water can be considered as an artificial feed-based intensive monoculture system for production of fish due to the increase in the cost of using water, the lack of water in recent years, and release of wastewater from traditional stocking system, which negatively affects the environment (Minaz & Kubilay, 2021). In addition, the culture of fish in biofloc system can reduces the need for formulated diets by affecting feeding rate (Najdegerami et al., 2016) and nutrients sparing (Samocha, 2019; Alimahmoudi & Mohammadiazarm, 2019), which leads to more profitability of aquaculture. Furthermore, the bioactive products of floc as a kind of probiotic had positive effects on growth and health status of fish through immune response, antioxidant defense, and changes in the blood parameters (Adineh et al., 2022). In this regards, hematological and blood biochemistry parameters have been powerful tools and becoming increasingly common in aquaculture studies (Esmaeili, 2021).

However, the stocking density directly affects many parameters like growth and survival, size variation, degree of comfortness, mortality, and water quality parameters (North et al., 2006; Hussain et al., 2014). Therefore, the benefits of the biofloc system could be hampered by unsuitable stocking density. Unbridled high stocking density had a harmful effect on fish performance. It reduces water quality and crowding, which lead to physiological changes, and as a result reduced growth performance and feed utilization

efficiency (Azim & Little, 2008; Crab et al., 2012; Minabi et al., 2020; Minaz & Kubilay, 2021).

On the other hand, the rapid growth and good survival of common carp (*Cyprinus carpio*) make it suitable for intensive culture in biofloc system (Najdegerami et al., 2016; Bakhshi et al., 2018; Adineh et al., 2019; Minabi et al., 2020; Tabarrok et al., 2020). Nevertheless, there is little information about optimal stocking density of common carp in biofloc system, and different stocking densities of common carp fish were used by several researches (Najdegerami et al., 2015; Bakhshi et al., 2018; Adineh et al., 2019; Minabi et al., 2020; Tabarrok et al., 2020; Adineh et al., 2022). Some studies reported negative effects of high densities on common carp under the biofloc system (Adineh et al., 2019; Adineh et al., 2022). Therefore, Minaz and Kubilay (2021) reported that optimal density causes an appropriate density of microbial flocs that is play important role in raising profit of fish production. Consequently, the productivity and profitability of the biofloc system can be influenced by stocking density. Therefore, we conducted this experiment for optimizing stocking density of juvenile common carp in biofloc system by evaluating, growth, feed utilization, proximate composition, some immune-biochemical blood indices, total heterotrophic bacteria count in water, and water quality parameters.

Material and Methods

Experimental Groups and Feeding Methods

The experiment was performed at the fisheries laboratory of Khorramshahr University of Marine Science and Technology (Khuzestan, Iran), in compliance with university guidelines under the international and national ethics. Fish with an initial average weight of 17.10±0.3 g were placed into twelve cylinder-shaped polyethylene tanks (300 L volumes with 200 L filled water) at four stocking densities of 125, 250, 375, and 500 fish/m^3 with three replicates by minimum water exchanged. In addition, each tank was filled with fresh water tap and inoculated with 200 mg/L of microbial floc (Tabarrok et al. 2020) and aerated with four air stones. In following, fish fed a low protein commercial diet from 21 Beza company, Shiraz, Iran (25% crude protein, 6% crude lipid, 8% fiber, and 16.2 Mj Kg^{-1} gross energy) at 3% of body weight three times a day (09:00, 14:00, 18:00). In addition, Molasses (50% carbon amount) was used to adjust the carbon to nitrogen ratio of 20 in experimental groups. The photoperiod was 12 light: 12 darkness through the trial.

The water temperature determined with a thermometer, dissolved oxygen with DO Meter (WTW, Oxi 3210, Germany), and pH with pH meter (WTW Win lab, Germany). Additionally, total ammonia nitrogen (TAN), nitrite (NO_2-N), and nitrate (NO_3-N) of 100 mL of filtered water was determined using kits (AquaPAA Co, Khorramshahr, Iran). Moreover, 50 mL of water were passed through filter paper, and dried in an oven at 105°C for 1 to 3 h for calculation concentration of TSS (Azim and Little 2008). Furthermore, floc volume (FV) was assayed with an Imhoff cone (Avnimelech, 2015). Total heterotrophic bacteria count in water was determined by the method of APHA (2005). Therefore, the biofloc samples were serially diluted in sterilized physiological saline solution (0.9% NaCl). Thus, the Reasoner's 2A (R2A) agar plate was inoculated with 100 µL of the dilutions, and incubated for 37°C for 24 h. After that, total heterotrophic bacteria counts were determined as log $\times 10^6$ CFU/mL.

At the end of the experiment, feeding was stopped 24 h prior to weighing or blood sampling. All fish in each replicate tank were individually weighed at the end of the experiment after anesthetizing with 30 mg/L clove powder.

Growth and feed utilization were calculated by the following formula (Alimahmoudi & Mohammadiazarm, 2019).

Specific Growth Rate (SGR %/day) = [ln (final weight (g))-ln (initial weight (g))] /time of trial× 100.

Daily Feed Intake (DFI %) = feed consumption (g, dry) × 100/ [((initial weight (g) + final weight (g) + dead weight (g)/2) × time of trial)]

Daily Protein Intake (DPI %) = protein consumption (g, dry) × 100/ [((initial weight (g) + final weight (g) + dead weight (g)/2) × time of trial)]

Protein Efficiency Ratio (PER) = weight obtained (g)/ protein consumption (g)

Food Conversion Ratio (FCR) = feed consumption (g) / weight obtained (g)

Survival Rate = (number of fish at the end of trial / number of fish at the beginning of trial) × 100.

Biochemical Body Composition

Biochemical body composition of ten fish from each replicate tank were analyzed by standard methods (AOAC, 2000). The moisture content was assayed by an oven at 110°C and protein content determined by the Kjeldahl technique (Kjeltec TM2300, Foss, Sweden). In addition, the fat content was assayed by ether removal with a Soxhlet (Soxtec TM8000, Foss, Sweden), and ash was defined by burning in a muffle furnace at 550°C for six h.

Immune-biochemical Blood Parameters

Immune-biochemical parameters were measured in blood serum of ten fish from each replicate tank with non-heparinized syringes. Therefore, clotted blood was

centrifuged (3000 rpm for 10 min), and separated serum samples were kept at -80°C until further analysis.

The serum cortisol concentration was determined by radioimmunoassay (RIA) based on the method of Ghaedi et al. (2015). Furthermore, glucose, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, and albumin assayed with diagnostic kits (Pars Azmoon, Karaj, Iran) by auto-analyzer (Mindray BS-200, Shenzhen Mindray Bio-Medical Electronics Co., Ltd, Shenzhen, China). In addition, albumin values were reduced from total protein to measure the amount of globulin.

The serum complement hemolysis (ACH50, unit/mL) was assayed based on the manner of Sunyer and Tort (1995) with alterations by Yeh et al. (2008). Therefore, the activity of ACH50 was reported as a unit/mL for the samples. Lysozyme activity was measured by turbidimetric method (Demers and Bayne 1997) with *Micrococcus lysodeikticus* (Sigma chemicals).

Statistical Analysis

Data from different experimental treatments were examined by one-way ANOVA, followed by Duncan's post hoc test at the significant level of P<0.05. In addition, Kolmogorov-Smirnov and Levene's tests were used for the normality and the homogeneity of variance of data in SPSS version 26 (Chicago, Illinois, USA). The statistics were shown as mean plus standard error (SE).

Results

The obtained data showed that water quality parameters including TAN, NO_2-N, and NO_3-N concentrations were considerably higher in the group with stocking density of 500 fish/m^3 than the other groups (Table 1; P<0.05). The differences in PH, oxygen, and temperature values were not significantly different among experimental groups (P>0.05). The floc volume (FV) and TSS concentration showed a major enhancing trend by increased stocking densities (P<0.05). Therefore, the FV and TSS concentration were significantly higher at stocking densities of 375 and 500 fish/m^3 than the stocking densities of 125 and 250 fish/m^3 (P<0.05). In addition, total heterotrophic bacteria count of water showed a gradual increasing trend with enhanced stocking densities (Figure 1; P<0.05).

The growth and feeding parameters contained FW, SGR (%/day), DFI%, DPI%, and PER meaningfully reduced in the group with stocking density of 500 fish/m^3 compared to other groups (Table 2; P<0.05). In addition, the groups with stocking densities of 375 and 500 fish/m^3 showed a significant increase in FCR compared

Table 1. Water quality parameters in biofloc system during rearing juvenile common carp.

	Density125	Density 250	Density 375	Density 500
TAN(mg/L)	0.27±0.03^{a}	0.36±0.07^{a}	0.39±0.06^{a}	0.63±0.01^{b}
NO2 (mg/L)	1.38±0.11^{a}	1.46±0.08^{a}	1.49±0.19^{a}	1.80±0.10^{b}
NO3 (mg/L)	126.78±0.66^{a}	129.80±1.84^{ab}	133.22±1.05^{b}	145.00±2.51^{c}
PH	7.90±0.03 $^{n.s}$	7.86±0.05	7.83±0.07	7.57±0.07
O_2 (mg/ L)	7.26±0.43$^{n.s}$	7.24±0.17	7.32±0.16	7.12±0.24
Temperature (C^{o})	24.95±0.13$^{n.s}$	25.11±0.15	25.10±0.16	24.90±0.07
FV (ml/L)	47.50±0.74^{a}	49.65±0.65^{a}	84.76±1.35^{b}	97.79±1.47^{b}
TSS (mg/L)	486.67±4.47^{a}	497.66±5.00^{a}	858.33±6.17^{b}	974.33±3.18^{b}

A different lowercase in the same row denotes statistically significant differences (mean of three replicate tanks ± SE, P<0.05). ns, not significant differences (P>0.05).

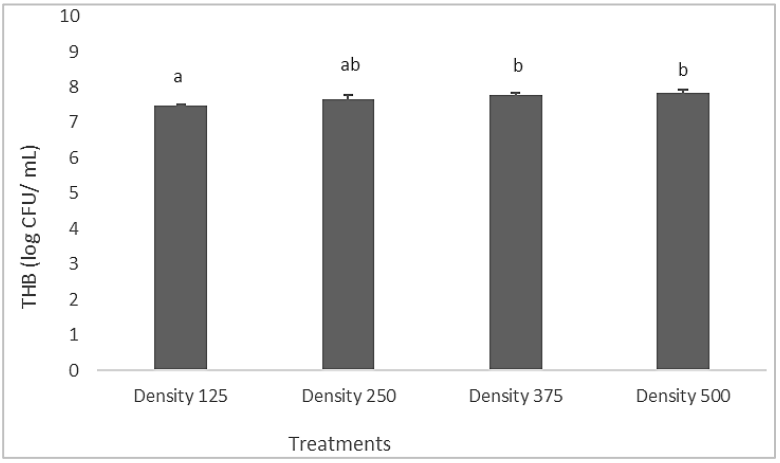

Figure 1. Total heterotrophic bacteria count (log CFU/ mL) of water at the end of experiment. A different lowercase in each column denotes statistically significant differences (mean of three replicate tanks ± SE, P<0.05).

to the groups with stocking densities of 125-250 fish/m^3 (P<0.05). The survival rate was not considerably affected in experimental groups (P>0.05).

Whole protein content of fish at stocking density of 500 fish/m^3 was significantly lower than the other groups (P<0.05). Furthermore, the lipid content of fish at stocking density of 500 fish/m^3 significantly decreased, but the moisture content of fish increased compared to the groups with stocking densities of 125 and 250 fish/m^3 (P<0.05). Nevertheless, ash content of fish was not significantly different among all groups (Table 3; P>0.05).

The data of serum biochemical and immunological indices are presented in Table 4. Therefore, the amounts of glucose, HDL, and LDL were significantly reduced in the group with stocking density of 500 fish/m^3 compared to the other groups (P>0.05). The amount of triglyceride was significantly lower in the groups with stocking density of 375 and 500 fish/m^3 than the groups of 125 and 250 fish/m^3 (P<0.05). On the other hand, serum cortisol and ALT activity were significantly higher in the group of 500 fish/m^3 than the groups of 125 and 250 fish/m^3 (P<0.05). The AST activity was significantly lower in the groups with stocking density of 375 and 500 fish/m^3 than the groups of 125 and 250 fish/m^3 (P<0.05). Furthermore, the ALP activity were considerably higher in the group with stocking density of 500 fish/m^3 than the group with stocking density of 125 fish/m^3 (P<0.05).

In addition, based on the results of immunological parameters the group with stocking density of 500 fish/m^3 showed considerably lower total serum protein than the groups with densities of 125 and 250 fish/m^3 (P<0.05). On the other hand, there was no significant difference in albumin values among different groups (P>0.05). The significant decreases in the amount of globulin, ACH50, and lysozyme activities were observed in the groups with stocking densities of 375 and 500 fish/m^3 compared to the stocking densities of 125 and 250 fish/m^3 (P<0.05).

Table 2. Growth and feed utilization of reared juvenile common carp in biofloc system at the end of experiment.

	Density125	Density 250	Density 375	Density 500
FW (g)	28.23 ± 1.52^{b}	28.14 ± 2.20^{b}	25.37 ± 0.25^{ab}	22.58 ± 0.82^{a}
SGR (%/day)	1.26 ± 0.07^{b}	1.25 ± 0.1^{b}	1.00 ± 0.01^{b}	0.70 ± 0.06^{a}
DFI (%)	3.37 ± 0.04^{b}	3.36 ± 0.05^{b}	3.23 ± 0.00^{b}	3.08 ± 0.03^{a}
DPI (%)	0.84 ± 0.01^{b}	0.84 ± 0.01^{b}	0.80 ± 0.00^{b}	0.77 ± 0.01^{a}
PER (g/fish)	1.46 ± 0.08^{b}	1.43 ± 0.08^{b}	1.20 ± 0.00^{b}	0.90 ± 0.10^{a}
FCR	2.63 ± 0.13^{a}	2.66 ± 0.20^{a}	3.26 ± 0.03^{b}	4.45 ± 0.35^{c}
Survival (%)	100	100	100	100

A different lowercase in the same row denotes statistically significant differences (mean of three replicate tanks ± SE, P<0.05).

Table 3. Whole body composition of reared juvenile common carp in biofloc treatments at the end of experiment.

	Density125	Density 250	Density 375	Density 500
Protein (% DM)	70.63 ± 0.32^{b}	70.68 ± 0.24^{b}	70.56 ± 0.73^{b}	68.67 ± 0.41^{a}
Lipid (% DM)	14.04 ± 0.25^{bc}	14.30 ± 0.27^{c}	13.65 ± 0.29^{ab}	13.26 ± 0.19^{a}
Ash (% DM)	$9.32\pm0.07^{n.s}$	9.22±0.04	9.35±0.08	9.42±0.06
Moisture (%)	74.59 ± 0.20^{a}	75.86 ± 0.09^{b}	76.36 ± 0.31^{bc}	77.07 ± 0.30^{c}

A different lowercase in the same row denotes statistically significant differences (mean of three replicate tanks ± SE, P<0.05). DM; Dry matter, ns, not significant differences (P>0.05).

Table 4. Serum biochemical and immunological indices of reared juvenile common carp in biofloc system at the end of experiment.

	Density125	Density 250	Density 375	Density 500
Glucose (mg/dL)	145.66 ± 2.02^{b}	142.46 ± 5.60^{b}	138.63 ± 2.00^{b}	126.66 ± 2.03^{a}
Triglyceride (mg/dL)	314.17 ± 7.44^{b}	312.61 ± 3.69^{b}	282.95 ± 6.46^{a}	277.45 ± 5.01^{a}
HDL (mg/dL)	29.00 ± 0.57^{c}	28.66 ± 0.88^{c}	26.66 ± 0.33^{b}	21.16 ± 0.44^{a}
LDL (mg/dL)	32.25 ± 0.14^{d}	32.83 ± 1.30^{c}	28.00 ± 0.57^{b}	22.33 ± 0.66^{a}
Cortisol (ng/dL)	43.94 ± 1.28^{a}	44.87 ± 0.90^{a}	48.23 ± 1.60^{ab}	50.74 ± 2.26^{b}
ALT (U/L)	8.83 ± 1.01^{a}	9.00 ± 1.15^{a}	11.66 ± 2.02^{ab}	14.00 ± 0.57^{b}
AST (U/ L)	211.00 ± 13.00^{a}	248.00 ± 10.00^{ab}	268.00 ± 14.00^{b}	278.00 ± 13.00^{b}
ALP (U/ L)	104.66 ± 8.95^{a}	123.16 ± 7.07^{ab}	123.88 ± 5.91^{ab}	151.00 ± 9.05^{b}
Total protein (g/ dL)	3.58 ± 0.05^{b}	3.55 ± 0.11^{b}	3.32 ± 0.06^{ab}	3.23 ± 0.08^{a}
Albumin (g /dL)	$1.15\pm0.05^{n.s}$	1.13±0.11	1.14±0.03	1.12±0.08
Globulin (g/ dL)	2.43 ± 0.04^{b}	2.42 ± 0.09^{b}	2.18 ± 0.05^{a}	2.11 ± 0.03^{a}
ACH50 (U/ mL)	104.38 ± 0.26^{b}	104.62 ± 0.16^{b}	100.67 ± 0.42^{a}	99.42 ± 0.73^{a}
Lysozyme (U/ mL)	35.63 ± 0.33^{b}	36.23 ± 0.24^{b}	33.29 ± 0.32^{a}	32.89 ± 0.37^{a}

A different lowercase in the same row denotes statistically significant differences (mean of three replicate tanks ± SE, P<0.05).

Discussion

The finding of the current experiment confirmed the beneficial effects of biofloc system with minimum water exchange on water quality and performance of juvenile common carp. Therefore, the bacteria biomass by absorbing nitrogen substances led to keep water quality parameters at stocking density of 125 up to 250 fish/m^3. Nevertheless, water quality deterioration was observed at stocking density of 500 fish/m^3.

On the other hand, the floc density and TSS concentrations were raised at stocking density of 375 up to 500 fish/m^3. Avnimelech (2012) reported that a suitable amount of FV is up to 50 ml/L in rearing units for cultured fish, which was observed at stocking densities of 125 up to 250 fish/m^3 in this experiment. It was stated that increased floc density more than optimal value of 50 ml/L from fish fecal and waste feed induce to system crash and water quality deterioration at high stocking density (Azim and Little, 2008; Crab et al., 2012; Minabi et al., 2020; Minaz and Kubilay 2021) in similarity with our result. In addition, Avnimelech (2012) confirmed that TSS concentrations could increase up to 1000 mg/L in fish ponds, but Azim & Little (2008) reported suitable TSS standards going from 400 to 600 mg/L. Therefore, in our study, the TSS values were up to 500 mg/L at stocking densities of 125 up to 250 fish/m^3.

The growth performance and feed utilization of fish reduced at density of 500 fish/m^3 that accompanied with reduced protein and lipid content of fish body. Previous studies also reported reduced growth performance of fish at high stocking density in biofloc system (Lima et al., 2018; Liu et al., 2018; Adineh et al., 2019). Although, it was reported that floc as a rich protein-lipid has positive effect on fish growth and body composition (Samocha, 2019; Alimahmoudi and Mohammadiazarm, 2019), but increased floc density can result in water quality deterioration with negative effect on fish performance at high stocking density (Minaz and Kubilay, 2021). Furthermore, it was described that high stocking density through chronic crowding stress has adverse results for growth, feed utilization, and welfare of aquaculture organisms (Suarez et al., 2015; Naderi et al., 2019; Liu et al., 2018; Adineh et al., 2019).

In this regards, serum cortisol concentration increased, and the amounts of glucose, triglyceride, HDL, and LDL decreased at high stocking densities. In similarity with our results, lower HDL, LDL, and triglyceride levels has been obtained in reared rainbow trout, *Oncorhynchus mykiss* at high stocking density compared to the low stocking density due to crowding stress (Suarez et al., 2015). In addition, Adineh et al. (2019) reported the reducing glucose content of reared common carp in biofloc system due to crowding stress. It was reported that crowding stress caused upper cortisol concentration in cultured aquatic organisms (Barcellus et al., 1999; Suarez et al., 2015). Therefore, the reduction in glucose and lipid content of fish serum was occurred due to utilization of glycogen and lipid stores for reducing the effect of stress situation (Mommsen et al., 1999). Furthermore, the liver enzymes activity of reared fish elevated at high density in this study that could be caused by crowding stress and water quality deterioration. Therefore, it was stated that crowding stress has adverse effects on liver function through a rise in liver enzymes activity of common carp fish (Adineh et al., 2019; Liu et al., 2018; Naderi et al., 2018).

Furthermore, the increased serum cortisol concentration of reared fish at high density due to crowding stress and poor water quality caused weak immune status. In this regards, it was stated that increased cortisol concentration by crowding stress negatively affects immune reactions of cultured aquatic species (Belo et al., 2005; Santos et al., 2010; Suarez et al., 2015; Adineh et al., 2019) through immune-related genes (Lin et al., 2018; Yarahmadi et al., 2016). Therefore, fish displayed a reduced amount of total protein, globulin, ACH50, and lysozyme activities at the stocking density of 375-500 fish/m^3. Similar to our results, reduced ACH50, lysozyme activities, total protein, and globulin were detected in cultured common carp during crowding stress situations in the biofloc system (Yin et al., 1995; Adineh et al., 2019; Adineh et al., 2022).

Conclusion

Finally, stocking juvenile common carp at density of 125 up to 250 fish/m^3 in biofloc system caused optimal floc density with improved growth performance, feed utilization, immune-biochemical parameters, and water quality. However, higher stocking densities above 375 fish/m^3 induced negative effects on health status and performance of fish through chronic crowding stress and water quality deterioration.

Ethical Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health guide (NIH Publications No. 8023, revised 1978). The protocol was approved by the Committee on the Ethics of Animal Experiments of Khorramshahr University of Marine Science and Technology (IR.KMSU.REC.1394.003).

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Author Contribution

Shoheila Nazarpour performed the experiment and lab analysis. Hamid Mohammadiazarm was the supervisor that planned the experiments, analyzed the data, and prepared the manuscript.

Conflict of Interest

The authers declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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