Effects of calcium carbonate nanoparticles on water quality, growth and metabolic activity of *Macrobrachium nipponense* in zero-water exchange biofloc system

R Fakhari[1], H Adineh[1*], H Jafaryan[1], M Harsij[1], M Sudagar[2]

[1]Department of Fisheries, Faculty of Agriculture Science and Natural Resources, Gonbad Kavous University, Gonbad, Iran

[2]Department of Aquaculture, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran

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Abstract

The purpose of the present research was to investigate the effects of adding calcium carbonate nanoparticles to the *Macrobrachium nipponense* diet in the biofloc system under zero exchange conditions. Oriental River prawn (inital weigh of 0.82 ± 0.07 g) were divided into four groups and fed four levels of calcium carbonate nanoparticles as following 0, 25, 50 and 100 mg kg^{-1} diet in biofloc system (CN0, CN25, CN50, and CN100) for 28 days. This study was applied complete randomized design with three replications. Water quality parameters were measured during the test period. Feed and growth parameters and some metabolic activities of hepatopancreas were measured. Physico-chemical water factors were in the appropriate range for this species. The concentrations of total ammonia nitrogen (TAN), nitrite, and nitrate were not significantly different between the experimental groups. The growth of prawns was significantly higher and feed conversion ratio was lower in CN25 and CN50 groups compared to the control group. The lowest AST and ALT activities were observed in CN25 and CN50 groups compared to the control. The prawns fed with experiment diets had significantly higher total protein, hemocyanin, glucose, and calcium compared to the control. Overall, the results showed diets containing Nano-calcium carbonate at levels 25-50 mg kg^{-1} in CN25 and CN50 groups could improve growth performance and metabolic activity of oriental river prawn in the biofloc system.

[*]**Correspondence** H. Adineh, Department of Fisheries, Faculty of Agriculture science and natural resources, Gonbad Kavous University, Gonbad, Iran (e-mail: adineh.h@gonbad.ac.ir).

Keywords: *Macrobrachium nipponense*, nanoparticles, physiology, biofloc technology

Introduction

Freshwater prawn farming plays an important role in the global aquaculture industry. Oriental river *Macrobrachium nipponense* is widely distributed in freshwater and low-salinity areas of rivers. Oriental river *M.*

nipponense is one of the important and commercial species of Palamonidae family of decapod crustacesns (Ma *et al.,* 2011), due to high resistance to temperature changes, good growth in natural conditions, and ease of reproduction. Currently, this species is an important target in Southeast Asian countries. This species abundantly found in fish ponds in the Northern provinces of Iran (Gilan, Mazandaran, and Golestan).

Biofloc technology (BFT) is a suitable and useful system based on the growth of microorganisms. Microorganisms play two major roles in maintaining water quality (by absorbing nitrogen compounds in the production of microbial protein) and nutrition (increasing production per unit area and reducing feed conversion ratio) in the aquaculture environment (De Schryver *et al.,* 2008; Avnimelech, 2009; Crab *et al.,* 2012). The main advantages of the culture system with biofloc technology are the use of nitrogenous wastes, the limited use of water and at least wastewater is released to the environment (Avnimelech, 2009; Emerenciano *et al.,* 2011), and so BFT is environmentally friendly. In addition, BFT systems enhance the growth parameters, feed performance, enzymatic activities, and immune and also antioxidant indexes of cultured shrimp (Xu *et al.,* 2013; Kumar *et al.,* 2017; Panigrahi *et al.,* 2020). In this regards, research has been reported on the effects of BFT on the growth and liver histology changes of Speckled shrimp (*Metapenaeus monoceros*) (Kaya *et al.,* 2019) and Pacific white shrimp *Litopenaeus vannamei* (Xu and Pan, 2012; Khanjani *et al.,* 2017), the effects of different types of feeds and salinity levels on Pacific white shrimp juveniles in a biofloc system (Khanjani *et al.,* 2020), and the effects of different feeding levels on Pacific white shrimp in zero water exchange system (Khanjani *et al.,* 2015).

Divalent cations such as calcium (Ca^{2+}) are responsible for the formation of bioflocs by improvements in floc properties as measured by sludge volume index (SVI), capillary suction time (CST), specific resistance to filtration (SRF), cake solids, and floc strength (Sobeck and Higgins, 2002). Calcium is an essential element in the body that is required for bone formation, growth, cellular physiology, immune response, and blood coagulation (Reid *et al.,* 1993). Calcium carbonate is the most common form of calcium supplements (NRC, 1994). Calcium carbonate, a mineral chemical with the formula ($CaCo_3$), can also be produced synthetically. Calcium carbonate can affect crustacean biological activities such as reproduction, peeling, and growth by affecting water hardness (Mente, 2003; Houng *et al.,* 2010).

In recent years, interest in nanotechnology and the use of nanoparticles in commercial applications has increased. However, there is little information about the fate and behavior of engineered nanoparticles in the environment (Moges et al., 2020). The use of calcium carbonate nano-particles in the fish and crustacean diets has not been investigated and studies were for other animals. Also, no report has been published on the feasibility of *M. nipponense* culturing in the biofloc system.

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Therefore, this project was planned to improve the technical production of BFT and to provide a more sustainable system for shrimp culture. In our study, the effects of feeding *M. nipponense* with different levels of calcium carbonate nanoparticles in the biofloc environment were evaluated.

Materials and Methods

Prawns

Oriental river *Macrobrachium nipponense* were caught with a hand-held saucer around coastal waters of Golestan Dam located in Gonbad Kavous, Golestan Province, Iran. All prawn samples were immediately transferred to the aquaculture laboratory at Gonbad Kavous University. Prawns were acclimatized with laboratory conditions for 7 days. The prawns (inital weigh of 0.82 ± 0.07 g) were distributed into 12 aquaria (volume of 25 L) and reared for 28 days. The photoperiod was a 10 h:14 h light-dark cycle.

Biofloc Production

Biofloc production was performed in two fiberglass tanks with a water volume of 40 liters in the laboratory environment. In each tank, three air stones were created to make a circular flow of water for complete mixing. Biofloc material contained commercial feed, flour wheat, urea, and molasses. Previously produced microbial flocs were inoculated in the biofloc system at a rate of 10% of the total volume (Martins *et al.,* 2017). The carbon: nitrogen (C:N) ratio was maintained at 15:1 using the carbon source (molasses) described by Crab e*t al.* (2012). According to Craig and Helfrich (2002), 16% of protein is N. If the prawns are consumed 100 g of feed with 34.30% protein, the N amount consumed by the prawns is 5.48 g (Asaduzzaman *et al.,* 2008) and 4.11 g N ends up in water. On average 75% of the feed-N ends up in the water (Piedrahita, 2003). The C:N ratio was maintained at 15:1, so 61.65 g C per 100 g of feed needed for biofloc production. The amount of carbon source added will then depend on the C content of the carbon source. In case of molasses (containing 0.4 g C per g), 154.12 g of carbon source would be needed (Sierra-De La Rosa, 2009).

Experimental diets

The diet ingredients were prepared and uniformly mixed (Table 1). The basal diet was divided into four equal portions. Only water was added to prepare the control diet (for the control treatment). Calcium carbonate nanoparticles (CN) (average particle size 10-80 nm, morphology cubic or hexagonal, molecular weight 100.09) were obtained from Iranian Nanomaterial Pioneers Company, Iran. The other three portions were CN (for the CN-supplemented diets) 25, 50, and 100 mg kg^{-1}, respectively. Water was then added to produce stiff dough. Four experimental diets were prepared with varying levels of CN [0 (control), 25 (CN25), 50 (CN50) and 100 (CN100) mg kg^{-1}]. The dough was then passed through a meat grinder and the resultant strings were dried before being crushed in the appropriate size. The pellets were kept after drying in the refrigerator (4 ºC). Approximate analysis of protein, lipid, and ash was

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performed according to the AOAC method (1995) (Table 1). Through Kjeldahl method, crude protein content was analyzed in triplicate (Kjeltec 1030 Auto Analyzer, Tector, Sweden); using a Soxtec extraction unit, crude lipid determination was performed (model 1043 Extraction Unit; Tecator, Sweden); after burning the shrimp sample in a muffler oven at 550 ^{o}C for 12 h, the weight of the crude ash was determined by weighing the residue of the crude ash (Heraeus, Germany).

Table 1. Ingredients and proximate compositions (%) of the experimental diets

Ingredient	%	Composition	%
Fish meal	15	Crude protein	34.30
Meat meal	10	Crude fat	8.5
Soybean meal	19	Moisture	14
Wheat meal	35	Crude ash	8.9
Fish oil	2		
Soybean oil	2		
Corn meal	10		
Starch	1		
Lysine	0.5		
Methionine	0.5		
Vitamin premix	2		
Mineral premix	3		

Water analysis

Temperature, dissolved oxygen, pH, and salinity were measured with a multiparameter meter (Hack, Model 2000). Water quality parameters including total alkalinity, total ammonia nitrogen (TAN), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), and total phosphorus (TP) were analyzed following the standard methods for water parameters analysis on days 7, 14, 21, and 28 of the experiment (APHA, 1998). Every 7 days, the biofloc volume (BFV) was determined using the Imhoff cone, where the biofloc volume was recorded after 30 minutes of deposition from 1000 ml of water (Avnimelech, 2009).

Feed and growth parameters

At the end of the test, the feed and growth parameters were calculated:

Weight gain = (final wight (g) – initial weight (g)) × 100/ initial weight (1)

Specific growth rate = (Ln final weight (g) – Ln initial weight (g) / test days (2)

Feed conversion efficiency = (weight gain / feed consumed) × 100 (3)

Condition factor = (final wight (g) / final length $(cm)^3$) × 100 (4)

Metabolic activity

Feeding was stopped 12 hours before sampling. All prawns were counted and weighed after collection. In each tank, hepatopancreas from ten prwan were separated to measure metabolic activity. The total protein, glucose, calcium, cholestrol, AST, ALT, and hemocyanin were determined using commercially available kits. Total protein was determined based on Shi *et al.* (2006). According to the method of Kunst *et al.* (1983), glucose concentration was determined by a commercial kit (Pars-Azmun Co., Tehran, Iran) with colorimetric assay at 546 nm using a

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spectrophotometer. Ca^{2+} levels were measured using an absorption spectrophotometer (Li and Cheng, 2012). Cholesterol levels were measured using a commercial kit (Pars-Azmun Co., Tehran, Iran) with the enzymatic-calorimetric method. Homocyanin was determined according to the method of Adachi *et al.* (2001) using a spectrophotometer with a wavelength of 340 nm.

Statistical

Data were presented as mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Before analysis, normality and homogeneity of variance were checked with the Shapiro-Wilk and Levene's tests, respectively. The differences were measured in statistical with SPSS at $p < 0.05$ level.

Results

The results of water quality parameters in the experimental groups during 28 days trial are shown in Table 2. Phosphate in CN25 group was higher than CN50 and CN100 groups. Also, BFV in CN100 group was higher than CN25 group. The water variables ranges were into the acceptable levels for shrimp culture.

Table 2. Water quality parameters of prawns fed nano-calcium carbonate in biofloc environment for 28 days

	Control	CN25	CN50	CN100
Temperature (°C)	26.06± 0.23	26.23± 0.05	25.86± 0.66	25.56± 0.49
DO (mg L^{-1})	4.62± 0.73	3.94± 0.32	4.29± 0.40	4.19± 0.67
pH	7.48± 0.06	7.41± 0.24	7.47± 0.14	7.50± 0.08
Salinity (g L^{-1})	0.42± 0.02	0.43± 0.02	0.41± 0.01	0.44± 0.02
EC (µs cm^{-1})	896.00± 50.48	884.00± 24.12	881.40± 27.51	917.40± 48.84
TDS (mg L^{-1})	426.80± 26.33	428.25± 16.41	415.40± 15.93	439.40± 25.11
Alkalinity (mg L^{-1} CaCO3)	371.31± 7.57	340.12± 15.18	366.65± 29.55	365.18± 13.33
Phosphate (mg L^{-1})	0.16± 0.03^{ab}	0.21± 0.02^{a}	0.11± 0.03^{b}	0.12± 0.03^{b}
BFV (mg L^{-1})	7.45± 0.86^{ab}	6.80± 0.54^{b}	7.13± 0.72^{ab}	8.41± 0.62^{a}
TAN (mg L^{-1})	0.094± 0.037	0.081± 0.022	0.089± 0.023	0.088± 0.024
NO_3 (mg L^{-1})	0.56± 0.09	0.48± 0.07	0.37± 0.05	0.51± 0.10
NO_2 (mg L^{-1})	0.05± 0.004	0.04± 0.005	0.04± 0.003	0.05± 0.005

Data are presented as mean ± SD. Data were analyzed through one-way ANOVA besides Duncan comparisons. Data with different superscript letters (a, b and c) in the same row mean significant differences among experimental groups ($p < 0.05$).

The fish growth performance and feed efficiency are presented in Table 3. The final weight, weight gain, and specific growth rate were significantly ($p<0.05$) improved in fish fed diets containing 25 and 50 mg CN kg^{-1} diet as compared to the control group in the biofloc system. Statistical analysis showed that there was no significant difference in the condition factor of fish in experimental groups. However, feed conversion ratio (FCR) was decreased significantly in prawns fed CN25 and CN50 supplemented diets than the control group ($p<0.05$). The survival rate was ranged from 71.66 % (control) to 91.41% (CN50) with a statistically significant difference ($p<0.05$).

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Table 3. Production parameters of *M. nipponense* fed different levels of nano-calcium carbonate in biofloc environment for 28 days

	Control	CN25	CN50	CN100
IW (g)	0.80± 0.07	0.80± 0.08	0.79± 0.07	0.91± 0.06
FW (g)	1.22± 0.14^{b}	1.58± 0.23^{a}	1.49± 0.20^{a}	1.37± 0.23^{ab}
WG (g)	0.42± 0.12^{b}	0.77± 0.19^{a}	0.70± 0.14^{a}	0.56± 0.20^{ab}
SGR (% day^{-1})	1.39± 0.37^{b}	2.23± 0.40^{a}	2.11± 0.24^{a}	1.72± 0.59^{ab}
CF (%)	1.17± 0.64	1.28± 0.44	1.51± 0.34	1.61± 0.57
FCR	2.83± 0.86^{a}	1.50± 0.41^{b}	1.61± 0.33^{b}	2.17± 0.69^{ab}
FCE	38.11± 11.75^{c}	70.75± 18.07^{a}	64.09± 13.37^{ab}	49.74± 14.83^{bc}
SR (%)	71.66± 9.05^{b}	83.35± 9.46^{ab}	91.41± 7.52^{a}	76.67± 14.83^{ab}

Data are presented as mean ± SD. Data were analyzed through one-way ANOVA besides Duncan comparisons. Data with different superscript letters (a, b and c) in the same row mean significant differences among experimental groups ($p < 0.05$). IW, initial weight; FW, final weight; SGR, specific growth rate, CF, condition factor; FCR, feed conversion ratio; FCE, feed conversion efficiency; SR, survival rate.

The measured metabolic activity is shown in Table 4. Hepatopancreas total protein, hemocyanin, and glucose of prawns fed CN were significantly ($p<0.05$) higher than the control group. AST and ALT activities was decreased significantly in prawns fed nano-calcium carbonate (CN25 and CN590) compared to the control group ($p<0.05$). There was a tendency to increase cholesterol and calcium of hepatopancreas along with an increase in dietary CN supplementation.

Table 4. Metabolic response of prawns fed nano-calcium carbonate in biofloc system for 28 days

	Control	CN25	CN50	CN100
Protein (mg g^{-1} Tissue)	47.70± 0.40^{d}	55.36± 0.55^{b}	51.54± 1.50^{c}	57.82± 0.22^{a}
Hemocyanin (m mol kg^{-1})	1.14± 0.03^{d}	1.25± 0.04^{c}	1.71± 0.03^{a}	1.34± 0.01^{b}
Glucose (mg g^{-1} Tissue)	3.76± 0.07^{d}	4.99± 0.07^{a}	4.22± 0.06^{c}	4.85± 0.06^{b}
AST (u mg^{-1} Protein)	0.138± 0.0005^{a}	0.074± 0.0017^{c}	0.069± 0.0043^{c}	0.102± 0.0055^{b}
ALT (u mg^{-1} Protein)	0.024± 0.0017^{a}	0.013± 0.0005^{c}	0.012± 0.0005^{c}	0.016± 0.0006^{b}
Cholesterol (mg g^{-1} Tissue)	5.37± 0.05^{a}	4.11± 0.01^{c}	4.46± 0.06^{b}	5.29± 0.01^{a}
Calcium (mg g^{-1} Tissue)	1.22± 0.02^{d}	1.30± 0.04^{c}	1.45± 0.01^{b}	1.59± 0.02^{a}

Data are presented as mean ± SD. Data were analyzed through one-way ANOVA besides Duncan comparisons. Data with different superscript letters (a, b and c) in the same row mean significant differences among experimental groups ($p < 0.05$).

Discussion

The environment and farming system are main factors that affect the growth performance and health status of aquatic organisms and water quality of culture (M'balaka *et al.,* 2012). Biofloc or microbial flocs provides nutrients such as protein (essential amino acids), poly-unsaturated fatty acids, vitamins, and minerals (Azim and Little, 2008). Biofloc is a technology to improve water quality by adding extra carbon to the water environment or feed. If the ratio of carbon and nitrogen is regulated and balanced in the culture environment, nitrogen compounds are converted into bacterial biomass (Schneider *et al.,* 2005). In our study, water parameters were appropriate for Macrobrachium rearing (Ballester *et al.,* 2017). Water factors such as temperature, pH, and dissolved oxygen showed that no

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significant difference was observed between the treatments. Also, water nitrogen contents (TAN, NO_2, and NO_3) were not significantly different in BFT groups. Total ammonia nitrogen (TAN) is combination of ionised ammonia (NH_4^+) and unionised ammonia (NH_3) exist simultaneously in the water (Purwono *et al.,* 2017), which is produced by the dissociation and organic matter excretion and can affect cultured organisms action or leading to death in high concentrations (Lin and Chen, 2001).

Ionic compounds such as calcium can affect the size, stability, formation, and structure of bioflocs (De Schryver *et al.,* 2008). High calcium concentrations in water lead to an increase in the biofloc density (Luo *et al.,* 2013). Therefore, in this study, different levels of calcium carbonate nanoparticles (25, 50, and 100) were added to the shrimp diet. In the treatments containing calcium carbonate also the decrease in total ammonia concentration demonstrated no significant statistical difference compared to the control. This study showed that total ammonia nitrogen concentrations can be effectively controlled in biofloc treatments by absorbing ammonia to heterotrophic bacteria or autotrophic nitrification (Adineh *et al.,* 2019). Similar to our results, the researchers reported that the water quality of the culturing environment improved for the shrimp/prawn in the biofloc system. For example, Khanjani *et al* (2017) reported that adding carbon sources in zero-water exchange system of biofloc could help to recycle nitrogen waste and improve water quality. Similarly, the effects of biofloc different levels on *L. vannamei* post larvae cultured in zero-water exchange system showed that replacement of 25% biofloc with feed can improve water quality and growth performance of shrimp (Adineh and Harsij, 2019).

Research on the effect of calcium on water quality in the biofloc system is very limited. In this regard, Furtado *et al* (2014) reported that calcium hydroxide can be used daily between 10 and 20% of the total feed of *L. vannamei* or doses of 0.05 g L^{-1} to correct alkalinity and pH in a biofloc system. Research with shrimp and prawn indicates that culture water in biofloc system contains growth enhancing factors such as microbial proteins that boost production. Flocs are a supplemental food resource that can be effective for the growth of aquatic animals (Ballester *et al.,* 2017; Negrini *et al.,* 2017; Nguyen *et al.,* 2019; Pinto *et al.,* 2020). The final weight, weight gain, and specific growth rate were significantly ($p<0.05$) improved in experiment treatments fed diets containing 25 and 50 mg CN kg^{-1} diet compared to the control group in the biofloc system. The highest growth and the lowest FCR were obtained in CN25 and CN50 groups. Similarly, the highest growth factors of shrimp and the lowest feed conversion ratio were obtained in the biofloc system (Xu *et al.,* 2020). The combination of microorganisms such as bacteria, fungi, algae, etc. as microbial proteins in the biofloc environment can enhance growth. In addition, the use of dietary supplements in these environments can stimulate growth. According to Kaya *et al* (2019), the use of carbon source (corn starch)

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in biofloc system with 3 g kg^{-1} mannan oligosaccharides led to the strengthening and stimulation of growth in the *Metapenaeus monoceros*. In this experiment, the addition of 25 and 50 mg kg^{-1} calcium carbonate nanoparticles in the *M. nipponense* diet in the biofloc system increased feed and growth efficiency. The conditions for the production of microbial biomass and improvement of water quality are provided in the biofloc environment, due to the uptake of ammonia by bacteria (Avnimelech, 1999). Also, biofloc is a suitable medium for nutrition, growth, and resistance to stress in aquatic animals (Adineh *et al.,* 2019). Moreover, crustaceans need calcium for some important physiological activities such as molting and growth. The calcium carbonate is an interesting mineral for biofloc cultivation. The reason for this is that both sources are Ca^{2+}, which is important for changing the bioflocs composition (Luo *et al.,* 2013). In a published study, water quality and growth performance of *Oreochromis niloticus* in the biofloc system improved in $NaHCO_3$ and Ca $(OH)_2$ groups compared to the $CaCO_3$ group (Martins *et al.,* 2017). According to Furtado *et al* (2014), 0, 10%, and 20% treatments had significantly better growth performance than 40% calcium hydroxide in the biofloc system. Calcium is one of the essential elements for calcification in the cuticle, which plays a key role in the growth of crustaceans (Li and Cheng, 2012).

In our study, protein, glucose, and hemocyanin of hepatopancreas were significantly increased in experiment groups compared to the control group in the biofloc system. Homocyanin is a respiratory pigment that makes up about 80 to 90% of the total protein concentration in the crustaceans hemolymph, which is involved in immune functions including phenolioxidase, hemolytic, antiviral, antitumor, and antimicrobial (Coates and Nairn, 2014). Liver enzymes (ALT and AST) can be used as a marker of invertebrate aquatic stress. In our experiment, the hepatopancreas ALT and AST activities were significantly increased in the control group than other experiment groups. Also, the increase of calcium carbonate nanoparticles supplementation levels lead to an increase in the calcium of hepatopancreas. Li and Cheng (2012) examined changes in calcium levels in hemolymph and shrimp tissues at different stages of molting and salinity. Their results showed the calcium in crustaceans stored in the hemolymph and then released to support mineralization in the post-melting. By storing calcium in the molting cycle, muscles may participate in the growth of shrimp.

Generally, it can be stated that the presence of calcium carbonate nanoparticles in the diet and its storage in the hepatopancreas can accelerate the growth of prawn. Also, the nutrient-rich biofloc environment introduced as an anti-stress site, so the addition of nano-carbon calcium to the diet of *Macrobrachium nipponense* in the biofloc system can improve growth and metabolic activity.

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Conflicts of interest

None of the authors has any conflicts of interest to declare.

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