Fish & Shellfish Immunology 64 (2017) 202–209

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Effects of carbon sources and plant protein levels in a biofloc system on growth performance, and the immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*)

Abdallah Tageldien Mansour [a, *], Maria Ángeles Esteban [b]

[a] Fish and Animal Production Department, Faculty of Agriculture (Saba Basha), Alexandria University, Alexandria, Egypt
[b] Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 16 January 2017
Received in revised form
7 March 2017
Accepted 10 March 2017
Available online 14 March 2017

Keywords:
Biofloc
Carbon source
Plant protein level
Growth
Innate immunity
Antioxidant
Oreochromis niloticus

ABSTRACT

The efficacy of different biofloc treatments (BFTs) to compensate for a reduction in dietary protein level under zero-water exchange systems was studied during a 10 weeks experiment, assessing the effect on water quality, growth, immune and antioxidant status of Nile tilapia (*Oreochromis niloticus*) fingerlings. Six groups were established and fed the same plant-based feed containing 20 or 30% crude protein: two groups in clear water conditions with no added carbon source, two biofloc groups given a wheat milling by-product (WMB) as additional carbon source and two biofloc groups given rice bran (RB). The results showed that biofloc volume was higher when WMB was used as carbon source. The highest growth performance were obtained with the biofloc system and the higher dietary protein level. Fish fed 20% crude protein and stocked in WMB biofloc significantly outperformed the fish fed 30% crude protein and stocked in clear water. Significant improvements in hematocrit, white blood cells, lymphocytes, plasma proteins, and humoral (immunoglobulin, lysozyme, myeloperoxidase and ACH_{50}) and cellular (phagocytosis activity and respiratory burst) immune parameters were observed in all BFT fish. BFT also increased superoxide dismutase and catalase activities. Moreover, the fish fed 20% dietary protein and reared in both biofloc conditions showed equal or superior levels of the immunological criteria to fish fed 30% protein in clear water conditions. In conclusion, using WMB as carbon source could make up for a reduction in dietary protein levels of 10% and improve growth performance, and the immune and antioxidant status of *O. niloticus*.

1. Introduction

The aquaculture of tilapia has increased to occupy second place in the list of farmed fish species worldwide and the first species in Egypt [1]. Furthermore, it is expected that this increase will continue at an even faster rate in the near future, giving rise to new challenges with respect to aquacultural conditions, including environmental issues [2]. Indeed, it is well known that the environmental impact of aquaculture is the major constraint in the sustainable development of this sector [2,3]. There are two main problems. The first is the "pre-effect" of aquaculture as natural resources in the form of fishing stock are depleted for use by fish meal and fish oil production industry [4], and the second is the "post-effect" in the form of drainage water, which is a rich source of organic matter, nitrogen and phosphorus, and which causes severe pollution and frequent harmful algal blooms in aquatic ecosystems [5,6].

Numerous potential solutions have been proposed to alleviate the ecological effects of aquaculture, among them water treatment [3], the integration of aquaculture-aquaculture or aquaculture-agriculture systems [7], or the use of recirculating aquaculture systems [8]. Furthermore, new efforts are being directed towards innovative solutions and investment in systems that involve minimal water expenditure, lower protein use in diets, smaller feed amounts and lower power consumption than are required at present while attaining similar growth efficiency levels in the farmed fish [9]. In this line, a modern approach is represented by a symbiotic process that includes the farmed animals, heterotrophic

* Corresponding author. 22 Tag El-Roasa St. Saba Basha, Boulkly, P.O. Box 21531, Alexandria, Egypt.
E-mail address: a_taag@yahoo.com (A.T. Mansour).

http://dx.doi.org/10.1016/j.fsi.2017.03.025
1050-4648/

bacteria and other microbial species in the water, which is referred to as biofloc [10].

Biofloc treatment (BFT) is one of the most recent environmentally friendly aquaculture systems to have been developed. This technology started as a minimization or zero-water exchange system and has been adapted over the last three decades by several researchers to improve the stimulation of bacterial growth and formation of biofloc suspended particles [11–13]. By adjusting the carbon-to-nitrogen ratio (C:N) in the aquaculture water through carbohydrate supplementation, the ability of some heterotrophic bacteria to assimilate the inorganic nitrogen can be promoted, resulting in an improvement of water quality and greater production of the microbial protein [11]. The increased growth of bacteria and other microbial organisms in BFT systems leads to the coagulation of suspended biofloc particles in the water, and these can be easily eaten by omnivores and filter feeder fish [14]. In this sense, BFT represent an integrated biofilter system in fish aquaculture systems for treating farm water through the removal of different nitrogen compounds and the breakdown of the organic matter [6,12] without using chemical agents.

Moreover, the high cost of aqua-feed, which mainly refers protein sources in the diet, especially fish meal [15], has led to animal protein being used as replacer in diet formulas in order to mitigate the above mentioned negative pre-effects of aquaculture on the environment. Although plant-based protein diets are perhaps the most practical choice for producers [16], their use could involve certain limitations, such as the inadequate level of key amino acids they contain, or the possible presence of endogenous anti-nutrient factors, which may negatively affect their nutritional value [16,17]. The above represent some of the reasons for the suggestion that bioflocs could be used as a supplementary feed to improve the nutritional quality of feed for farmed fish species [18,19]. The biofloc process produces feed with a high content of easy digestible proteins [18] because the nutritional composition of the biofloc particles includes 38–54% of crude protein [20,21]. For this reason, the suspended biofloc particles could replace part of the feed used to feed farmed specimens, while recycling and/or recovering a significant amount of the *N*-ammonia nitrogen excreted by fish [22,23]. Moreover, bacteria are usually associated to the suspended biofloc particles, providing extra nutrients and exogenous digestive enzymes [18,19] and stimulating growth and survival [24,25]. In addition, it has also been demonstrated that animals grown under a BFT system showed higher immune competency, antioxidant status and disease resistance compared to those reared in clear water [26,27]. Taking into account all these considerations, the present study was designed to evaluate the effect of two different BFTs to compensate the low dietary protein levels in a zero water-exchange system on growth performance, water quality, immune and antioxidant status of Nile tilapia (*O. niloticus*) fingerlings.

2. Materials and methods

2.1. Fish

A total of 756 Nile tilapia (*O. niloticus*) fingerlings (48.0 ± 1.10 g mean body weights) were purchased from a local farm (Kafr El-Sheikh Governorate, Egypt). Fish were allowed to acclimate to laboratory conditions for two weeks. The experiment was conducted at the Fish Nutrition Laboratory, Faculty of Agriculture (Saba Basha), Alexandria University (Egypt), starting in March 2016.

Fish specimens were distributed into eighteen indoor circular fiberglass tanks (1000 L water capacity) at an initial stocking density of 2 kg fish m^{-3} of water. The temperature was maintained in the range of 27.0–28.0 °C using an electric heater during the whole experimental period. The light regime was set at 12 h light/12 h dark and each tank was supported by two airstones (5 cm) to maintain appropriate dissolved oxygen and vigorous water agitation using an air blower.

2.2. Experimental design

Six groups (with three replicates of each one) were established and fed the same plant-based feed containing 20 or 30% crude protein: two groups in clear water conditions with no added carbon source, two biofloc groups given a wheat milling by-product (WMB) as additional carbon source and two biofloc groups given rice bran (RB). At the beginning of the experiment, all the tanks were filled with fresh water. No organic carbon was added to the tanks of the clear water groups, in which a daily water exchange rate of 30% was applied. For their part, each of the tanks containing the fish exposed to the BFT treatments was inoculated with 100 ml of concentrated biofloc from old biofloc tanks on the first day of the experiment. Furthermore, the BFT tanks were supplied daily with one of the tested carbon sources, two hours after feeding to maintain the C:N ratio of 15:1 [28]. Fresh water was regularly added to the BFT tanks to compensate for water loss due to evaporation, thus maintaining a fixed volume of water.

The diets were formulated using plant protein sources with no added fish meal (Table 1). Fish were hand-fed with the experimental diets twice daily for 10 weeks at 3% of wet body weight per day, adjusting the daily amount of feed every two weeks according to the fish biomass present in each tank.

2.3. Water quality monitoring

The water quality parameters were monitored weekly throughout the experimental period. Temperature and dissolved

Table 1
Ingredients and chemical composition (% dry matter basis) of the experimental diets.

Ingredients	Diets (% protein)	
	20	30
Soya bean meal	200	400
Corn gluten	300	500
Wheat bran	200	200
Yellow corn	920	369
Wheat bran	280	440
L-lysine	10	10
DL- methionine	10	10
Sun flower oil	40	40
Vitamin premix[a]	20	20
Minerals premix[a]	20	20
Chemical composition (% DM basis)		
Dry matter (DM)	89.21	89.96
Crude protein (CP)	19.98	28.82
Ether extract	7.37	8.39
Crude fiber	5.54	7.21
Ash	4.15	5.38
Nitrogen-free extract (NFE)[b]	62.96	50.20
GE (KJ1 g^{-1} DM)[c]	18.45	18.74
P/E ratio (mg CP: kJ)[d]	10.83	15.38

[a] Vitamins and minerals in 1 kg: vitamin A–50 00 000 IU; vitamin D3–10 00 000 IU; vitamin B2–2.0 g; vitamin E – 750 units; vitamin K–1.0 g; calcium pantothenate 2.5 g; nicotinamide–10.0 g; vitamin B12–6.0 g; choline chloride–150.0 g; calcium–750.0 g; manganese–27.5 g; iodine–1.0 g; ion–7.5 g; zinc–15.0 g; copper–2.0 g; cobalt-0.45 g, calcium carbonate up to (1000 g).

[b] NFE (nitrogen free extract) = 100-(crude protein + ether extract + crude fiber + ash).

[c] GE (gross energy) calculated on the basis of 23.6, 39.4 and 17.2 kJ gross energy g^{-1} protein, ether extract and NFE, respectively [66].

[d] P/E ratio (protein energy ratio) (mg crude protein kJ^{-1} gross energy) = CP/GE × 1000.

oxygen (DO, mg L^{-1}) were measured at 9:00 a.m. using a portable DO meter (Crison, model OXI 45 P, Spain). The pH, salinity (g L^{-1}), total ammonia-nitrogen (TAN, mg L^{-1}), nitrate-nitrogen (NO_3, mg L^{-1}) were measured using a digital multimeter (Crison, model MM41, Spain). Biofloc volume was determined using an Imhoff cone, where the biofloc volume was registered after 30 min of one liter water sedimentation of each BFT tank [29], therefore the number of observation is 30 treatment^{-1} (10 values replicate^{-1} and three replicate treatment^{-1}).

2.4. Fish growth performance and survival

Fish growth performance was determined by studying the weight gain percentage (wt. gain%), thermal unit growth coefficient (TGC), total yield, feed conversion, feed intake and survival ratio (FCR) which were determined as follows:

Wt. gain (%) = 100 × (final weight -initial weight/initial weight) [30]

TGC = $(W_D^{1/3}\text{-}W_0^{1/3}/\sum_{i=1}^{D} Ti) \times 1000$ [31]

Total yield (kg m^{-3}) = total weight of fish/cubic meter

FCR = the total feed intake (g)/weight gain (g).

Feed intake (g diet kg^{-1} fish) = total weight of feed provided (g)/kg of fish.

where: W_f is the final weight (g), W_0 is the initial weight (g), T_i is the mean daily temperature (°C) and D is the number of days.

Survival (%) = 100 × (final fish number/initial stocked number)

2.5. Hematological parameters

Blood was collected from the caudal vein of previously anaesthetized fish (50 mg clove oil L^{-1}) using a sterile insulin syringe containing one drop of heparin (Amoun Pharmaceutical Co. S.A.E., Egypt). Blood samples were taken from five fish per treatment, pooled and divided into two portions. The first half was used to develop the hematological assays, and cellular immune parameters (phagocytosis and respiratory burst activities), while the other part was centrifuged (1075 × g, 10 min, 4 °C) to obtain plasma. The plasma samples were stored at −80 °C until used in the biochemical and immunological assays.

The white blood cells count (WBC; 10^3 mm^{-3}) was made using a standard Neubauer hemocytometer chamber with Shaw's solution as diluting fluid. Moreover, a differential leukocyte count was made by light microscopy (Optika, Via Rigla, Ponteranica, Italy) using Giemsa-stained smears. The hematocrit (Ht; %) was determined by filling hematocrit capillary tubes which were centrifuged (8400 × g, 10 min) using a Micro-hematocrit centrifuge (Krebs, Bunsen, EU). The hematocrit values were recorded by means of a centrifuge combo-reader.

2.6. Humoral immune parameters

The total protein (g dL^{-1}) was determined in plasma samples from fish of the different experimental groups by Biuret's method according to Gornall et al. [32]. Albumin (g dL^{-1}) was determined using the bromocresol green method [33], while globulin (g dL^{-1}) was calculated as the difference between total protein and albumin.

The lysozyme activity (U mg^{-1} protein) in plasma was measured by turbidimetric assay according to [34] with some modifications. Briefly, aliquots of 25 μl of plasma were added to 1 ml suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1} in a 0.05 M sodium phosphate buffer (pH 6.2) and the absorbance was measured at 670 nm after 30 s and 180 s by spectrophotometer (Spectrophotometer PD-303 UV, APEL, Japan). Total immunoglobulin (Ig, mg dL^{-1}) was evaluated according to Siwicki et al. [35], whereas total protein was determined using a micro protein determination method (C-690; Sigma, USA) before and after Ig molecules were precipitated by a 12% polyethylene glycol solution (Sigma, USA). The difference in protein content before and after Ig molecule precipitation was considered as the Ig content.

The alternative complement activity (ACH_{50}) was determined using sheep red blood cells as a target and the absorbance of the lysed cells was measured at 540 nm in a spectrophotometer [36]. The volume of plasma producing 50% hemolysis was determined and the ACH_{50} was obtained for each experimental group as follows:

ACH_{50} value (unit ml^{-1}) = 1/Y x (reciprocal of the plasma dilution).

where Y is the amount of plasma (ml) giving 50% lysis.

The total myeloperoxidase (MPO) content in plasma was measured according to Sahoo et al. [37]. Briefly, serum (20 μl) was diluted with HBSS (Hanks balanced salt solution without Ca^{2+} or Mg^{2+}, Sigma, USA) in 96-well plates. Then, 35 μl of 20 mM 3,3′−5,5′-tetramethyl benzidine hydrochloride (Sigma, USA) and 5 mM H_2O_2 were added. The color change reaction was stopped after 2 min by adding 35 μl of 4 M sulfuric acid. Finally, OD was read at 450 nm.

2.7. Cellular immune parameters

Cellular immune parameters (phagocytic and respiratory burst activities) were determined in fresh blood samples obtained from the caudal vein using a syringe with heparin as anticoagulant. Phagocytic activity (PA) was determined using *Candida albicans*as target particles according to [38]. Briefly, 200 μl of blood and 10 μl of yeast (1 g *C. albicans*ml^{-1} saline) were incubated in a water bath and shaken for 3 h at 25 °C to facilitate the action of leucocytes. Blood smears were then prepared, stained with Giemsa and studied under an oil-lens light microscope (OPTIKA, Via Rigla, Ponteranica, Italy). Phagocytosis was estimated by determining the proportion of macrophages which contained intracellular yeast cells in a random count of 300 phagocytes, and the results were expressed as percentage of PA (%).

Finally, respiratory burst activity of leucocytes was determined from the reduction of nitroblue tetrazolium (NBT) to formazan as a measure of superoxide anion production [39]. Briefly, 200 μl of blood was mixed with 100 μl of NBT (0.2% in PBS, Sigma USA) and superoxide dismutase (SOD, Sigma, 300 U ml^{-1}). After incubation at room temperature for 60 min with regular mixing, plates were centrifuged at 500 × g for 3 min and the supernatants were discarded. Cells were washed twice with HBSS and fixed in 70% methanol. Formazan crystals were dissolved by adding a 120 μl of 2 M KOH and 140 μl dimethyl sulfoxide (DMSO). After the formation of the turquoise-blue-colored solutions, absorbance values were read at 620 nm using KOH/DMSO (120 μl of 2 M KOH/140 μl DMSO) as blank.

2.8. Antioxidant parameters

Catalase activity (CAT, U/mg protein) was measured according to Ref. [40]. Briefly, 10 μl plasma sample was added to 1.25 ml of freshly prepared buffer containing 50 μl of H_2O_2 10 ml^{-1} Na-K phosphate buffer (0.15 M, pH 7, El-gomhoria Co., Egypt). The difference in absorbance was recorded after 20 s (A1) and after 80 s (A2) of incubation at 240 nm against air. The CAT value calculated as A1-A2/0.0008.

Superoxide dismutase (SOD; U/mg protein) was evaluated according to Misra et al. [41]. Briefly, 20 μl of plasma was added to 940 μl sodium carbonate buffer (pH 10.2, 0.05 M, El-gomhoria Co.,

Egypt) and 40 μl epinephrine (30 mmol l^{-1} dissolved by adding 30 μl of HCL, Sigma, USA). The inhibition of epinephrine auto-oxidation in the alkaline medium to adrenochrome was recorded after 30 and 90 s at 480 nm. A control was prepared as 960 μl sodium carbonate buffer and 40 μl epinephrine.

The percent of inhibition (%) = 100- [(ΔA control-ΔA sample/ΔA control) × 100].

SOD activity in plasma (U/ml) = % inhibition × 3.75.

2.9. Statistical analyses

The statistical analyses were performed using SAS v9.3 for Windows (Cary, NC, USA). Data were subjected to two-way ANOVA to test the effect of carbon sources and protein levels. Duncan's multiple range test was used as a post hoc test to compare means at $P < 0.05$ [42]. The results were presented as means ± standard error. The following general liner model was used:

$$Y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + e_{ijk}$$

where *Yijk* is the mean value of the tank, μ is the mean population, a_i is the fixed effect of carbon sources effect, b_j is the fixed effect of protein levels, $(ab)_{ij}$ is the interaction between fixed effects, and e_{ijk} is the random error.

3. Results

3.1. Water quality

Water quality parameters (DO_2, temperature, pH and salinity) were not affected by any of the treatments, but, TAN, NO_2, NO_3 and biofloc volume were significantly ($P \leq 0.05$) affected by the different carbon sources added (Table 2). The nitrogen content increased in the biofloc treatments in a zero-water exchange system more than in the control with daily water change. However, the levels remained within an acceptable range for *O. niloticus*. The biofloc volume was greater when WMB was used as carbon source rather than RB.

3.2. Growth performance

The greatest increase in growth parameters (Wt. gain, TGC and yield) and best FCR were also recorded using WMB as carbon source. The 30% dietary plant protein diets in general led to appreciably higher growth and FCR than the lower protein level (Table 3). However, the growth of fish reared in biofloc tanks and fed 20% protein still surpassed that of the fish cultured in clear water, and fed the 20 or 30% protein diet. Feed intake (g $fish^{-1}$) was significantly ($P \leq 0.05$) higher with WMB than in the other treatments, and increased, but not significantly ($P > 0.05$), with the 30% protein level. Survival ranged from 96 to 100% at the end of the experiment, without any significant ($P > 0.05$) effect of carbon sources, protein level or their interaction.

3.3. Hematological changes

The Ht, WBCs and lymphocytes were significantly higher using RB and WMB as carbon source than in the fish stocked in the control tanks (Table 4). The higher protein level improved the WBC count, Ht and lymphocytes, significantly so in the case of the WBC. The interaction effect of carbon source and protein level on hematological parameters was significant ($P < 0.05$) in the case of the WBC count. The highest WBC count was recorded with fish reared in the WMB biofloc tanks. The effect of BFT produced by both RB and WMB on the WBC of fish fed 20% dietary protein levels was greater than in the group fed 30% protein and reared in clear water tanks.

3.4. Humoral non-specific immune parameters

Plasma total protein, albumin, globulin and humoral factors of innate immunity (Ig, lysozyme, MPO and ACH_{50}) improved significantly ($P < 0.05$) with the biofloc treatments and associated higher protein levels (Table 5). The significant interaction ($P < 0.05$) was clear from the Ig and MPO levels, and the highest values were recorded with WMB biofloc and 30% dietary protein. The two produced biofloc compensated the reduction of dietary protein levels by maintaining higher Ig levels than the two control groups. For its part, the MPO compensated for the lower protein content of the 20% protein diet only in the WMB biofloc treatment.

3.5. Cellular non-specific immune parameters

Rearing *O. niloticus* in biofloc systems using RB and WMB significantly increased PA and NBT activities compared with the fresh water group (control). The same trend was observed for the higher protein level (Table 6). The two-way ANOVA showed a significant interaction effect of different carbon sources and protein levels on the PA, the highest value being obtained with WMB.

3.6. Antioxidant enzyme activities

The different carbon sources, protein levels and their interaction significantly ($P < 0.05$) affected CAT and SOD (Table 6), the highest

Table 2
Water quality parameters of Nile tilapia (*O. niloticus*) fingerlings reared in tanks with or without biofloc treatments for 10 weeks.

Treatments	Temperature (°C)	Dissolved oxygen (mg L^{-1})	pH	Salinity (g L^{-1})	Ammonia-N Nitrogen (mg L^{-1})	Nitrite (mg L^{-1})	Nitrate (mg L^{-1})	Biofloc volume (ml)
Carbon source (C)								
Control	26.97 ± 0.22	5.50 ± 0.45	8.02 ± 0.10	1.30 ± 0.00	0.49 ± 0.15^{b}	0.33 ± 0.05^{b}	1.28 ± 0.25^{b}	0.83 ± 0.11^{c}
Rice bran	27.03 ± 0.22	5.62 ± 0.62	8.00 ± 0.09	1.50 ± 0.00	1.61 ± 0.05^{a}	1.37 ± 0.11^{a}	12.58 ± 0.64^{a}	18.58 ± 3.84^{b}
Wheat milling by-product	27.00 ± 0.20	5.28 ± 0.56	7.92 ± 0.07	1.50 ± 0.00	1.62 ± 0.06^{a}	1.55 ± 0.04^{a}	12.33 ± 0.67^{a}	29.92 ± 4.10a
Protein levels (P)								
20%	26.97 ± 0.18	26.97 ± 0.22	7.96 ± 0.07	1.43 ± 0.03	1.18 ± 0.21	1.02 ± 0.20	8.67 ± 1.91	15.44 ± 4.81
30%	27.03 ± 0.16	27.03 ± 0.22	8.00 ± 0.07	1.43 ± 0.03	1.30 ± 0.19	1.14 ± 0.20	8.80 ± 1.92	17.44 ± 5.06
Two-way ANOVA								
P values								
C	0.98	0.93	0.73	0.98	0.001	0.00	0.00	0.00
P	0.81	0.80	0.69	0.99	0.28	0.15	0.85	0.63
C × P interaction	0.94	0.98	0.61	0.89	0.63	0.20	0.68	0.82

Different superscript letters in the same column (a, b and c for carbon sources) and (A and B for protein levels) indicate significant differences ($P < 0.05$).

Table 3
Growth performance of Nile tilapia (*O. niloticus*) fingerlings reared in tanks with or without biofloc treatments for 10 weeks.

Treatments	Weight gain (%)	Thermal growth coefficient	Total yield (kg m^{-1})	Survival (%)	Feed intake (g $kgfish^{-1}$)	Feed conversion ratio
Carbon source (C)						
Control	67.00 ± 3.92^{c}	5.67 ± 0.33^{c}	3262.68 ± 111.67^{c}	96.83 ± 1.59	1002.33 ± 37.02^{a}	2.46 ± 0.17^{a}
Rice bran	88.23 ± 1.81^{b}	7.47 ± 0.15^{b}	3732.980 ± 25.72^{b}	98.41 ± 1.00	859.47 ± 22.18^{b}	1.80 ± 0.05^{b}
Wheat milling by-product	100.02 ± 3.92^{a}	8.47 ± 0.33^{a}	4032.345 ± 79.06^{a}	100.00 ± 0.00	872.41 ± 26.34^{b}	1.72 ± 0.08^{b}
Protein levels (P)						
20%	79.14 ± 5.49^{B}	6.70 ± 0.47^{B}	3540.432 ± 133.44^{B}	97.88 ± 1.15	933.23 ± 41.85^{A}	2.13 ± 0.18^{B}
30%	91.02 ± 4.68^{A}	7.70 ± 0.40^{A}	3811.569 ± 103.75^{A}	98.94 ± 0.70	889.58 ± 15.34^{B}	1.85 ± 0.07^{A}
Two-way ANOVA *P* values						
C	0.001	0.001	0.001	0.18	0.001	0.001
P	0.001	0.001	0.002	0.43	0.03	0.001
C × P interaction	0.04	0.04	0.03	0.53	0.001	0.001

Different superscript letters in the same column (a, b and c for carbon sources) and (A and B for protein levels) indicate significant differences ($P < 0.05$).

Table 4
Hematological parameters of Nile tilapia (*O. niloticus)* fingerlings reared in tanks with or without biofloc treatments for 10 weeks.

Treatments	Hematocrit (%)	White blood cells (10^{3} mm^{-3})	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)
Carbon source (C)					
Control	27.33 ± 0.49^{c}	23.67 ± 0.56^{c}	30.67 ± 0.92^{c}	4.00 ± 0.37	63.50 ± 0.92^{a}
Rice bran	33.00 ± 0.37^{b}	30.29 ± 0.61^{b}	34.50 ± 0.22^{b}	4.83 ± 0.31	59.50 ± 0.50^{b}
Wheat milling by-product	35.00 ± 0.45^{a}	37.00 ± 1.24^{a}	37.33 ± 0.33^{a}	4.67 ± 0.33	57.17 ± 0.31^{c}
Protein levels (P)					
20%	31.44 ± 1.33	28.98 ± 1.70^{B}	33.67 ± 1.15	4.33 ± 0.24	60.78 ± 1.15^{A}
30%	32.11 ± 1.03	31.66 ± 2.23^{A}	34.67 ± 0.94	4.67 ± 0.33	59.33 ± 0.87^{B}
Two-way ANOVA *P* values					
C	0.0001	0.0001	0.0001	0.26	0.0001
P	0.15	0.0001	0.11	0.44	0.04
C × P interaction	0.08	0.02	0.15	0.81	0.23

Different superscript letters in the same column (a, b and c for carbon sources) and (A and B for protein levels) indicate significant differences ($P < 0.05$).

Table 5
Non-specific immune parameters of Nile tilapia (*O. niloticus*) fingerlings reared in tanks with or without biofloc treatments for 10 weeks.

Treatments	Total protein (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})	Immunoglobulin (mg dl^{-1})	Lysozyme (U mg^{-1} protein)	Total myeloperoxidase (OD at 450 nm)	ACH_{50} U ml^{-1}
Carbon source (C)							
Control	3.68 ± 0.08^{c}	2.24 ± 0.03^{b}	1.44 ± 0.08^{b}	2.25 ± 0.15^{c}	25.00 ± 2.02^{c}	1.31 ± 0.19^{c}	18.97 ± 1.60^{c}
Rice bran	3.92 ± 0.07^{b}	2.37 ± 0.03^{a}	1.55 ± 0.05^{b}	3.03 ± 0.07^{b}	29.67 ± 1.41^{b}	2.28 ± 0.06^{b}	30.30 ± 1.02^{b}
Wheat milling by-product	4.29 ± 0.07^{a}	2.37 ± 0.03^{a}	1.92 ± 0.05^{a}	3.20 ± 0.09^{a}	32.42 ± 1.39^{a}	2.63 ± 0.06^{a}	37.05 ± 1.74^{a}
Protein levels (P)							
20%	3.83 ± 0.09^{B}	2.29 ± 0.03^{B}	1.54 ± 0.08^{B}	2.64 ± 0.18^{B}	25.56 ± 1.31^{B}	1.88 ± 0.26^{B}	25.77 ± 2.70^{B}
30%	4.09 ± 0.10^{A}	2.36 ± 0.03^{A}	1.73 ± 0.08^{A}	3.01 ± 0.13^{A}	32.50 ± 0.96^{A}	2.27 ± 0.15^{A}	31.78 ± 2.67^{A}
Two-way ANOVA *P* values							
C	0.001	0.01	0.001	0.001	0.001	0.001	0.001
P	0.001	0.05	0.01	0.001	0.001	0.001	0.001
C × P interaction	0.94	0.96	0.98	0.05	0.12	0.001	0.25

Different superscript letters in the same column (a, b and c for carbon sources) and (A and B for protein levels) indicate significant differences ($P < 0.05$).

Table 6
Cellular innate immune parameters and antioxidant enzymes of Nile tilapia (*O. niloticus*) fingerlings reared in tanks with or without biofloc treatments for 10 weeks.

Treatments	Phagocytic activity (%)	Nitroblue tetrazolium (mg/mL)	Catalase (U/mg protein)	Super oxide dismutase (U/mg protein)
Carbon source (C)				
Control	22.22 ± 1.12^{c}	0.56 ± 0.04^{c}	22.78 ± 1.89^{b}	47.49 ± 3.20^{c}
Rice bran	25.10 ± 0.55^{b}	0.80 ± 0.04^{b}	33.72 ± 0.61^{a}	56.10 ± 3.27^{b}
Wheat milling by-product	26.83 ± 0.31^{a}	0.84 ± 0.03^{a}	35.03 ± 1.25^{a}	60.95 ± 1.66^{a}
Protein levels (P)				
20%	23.49 ± 1.00^{B}	0.67 ± 0.05^{B}	28.71 ± 2.56^{B}	49.07 ± 2.46^{B}
30%	25.94 ± 0.46^{A}	0.80 ± 0.04^{A}	32.31 ± 1.57^{A}	60.62 ± 1.79^{A}
Two-way ANOVA*P* values				
C	0.001	0.001	0.001	0.001
P	0.001	0.001	0.004	0.001
C × P interaction	0.02	0.27	0.02	0.03

Different superscript letters in the same column (a, b and c for carbon sources) and (A and B for protein levels) indicate significant differences ($P < 0.05$).

values of both enzyme activities being obtained with WMB at 30% protein. The compensating effect of biofloc was evident in the case of CAT using both carbon sources and at both protein levels (20 or 30%), fish outperforming those fed 30% dietary protein and reared in clear water.

4. Discussion

In tilapia farming, 30% dietary protein is the ideal level to cover the requirements of growth, and the general metabolic and physiological responses of growing fish [16], while lower levels affect normal functions. At the same time, the role of biofloc as a supplementary feed component of high nutritional quality, especially in terms of protein, has been demonstrated in several aquaculture species [13,18,43,44]. The present study determines the efficacy of different biofloc treatments to compensate for a reduction in protein levels under zero-water exchange conditions in fingerlings of *O. niloticus*. Two dietary plant-based protein levels (20% and 30% protein) were used without or with the addition of two carbon sources (WMB or RB). In shrimp, BFT has been seen to be effective in compensating for a decrease in protein levels from 35 to 20% [43].

Moreover, the organic carbon source used for stimulating biofloc formation is a detrimental factor for production in culture systems. In the present work, WMB and RB were tested as cheap and widely available carbon sources. The results demonstrated that the use of WMB as a carbon source is preferable to RB for farmed *O. niloticus*. The effect of a carbon source on the growth of cultured species depends on some characteristics of the biofloc produced, such as its volume, chemical composition, and ability to store bioactive compounds (e.g. polymers, carotenoids, phytosterols and extracellular enzymes) [6,18,45,46].

The experiment described lasted 10 weeks in order to determine the possible effect of biofloc on fish growth and nutrient utilization. As regard, water quality parameters (DO, pH and salinity), no significant differences with respect to the parameters tested in the fresh water tank were observed and all values were always within a range considered acceptable for tilapia production [16]. Although the TAN and NO_2 tended to increase in BFT treatments, it is known that the heterotrophic and ammonium-oxidizing bacteria formedin BFT take part in the oxidation of ammonia in the water to produce NO_3. Accordingly, the nitrification that occurred in the BFT treatments maintained the ammonia and nitrite at safe levels for *O. niloticus* [11,13]. These findings agree with previous ones demonstrating that the *in situ* biofloc formation accelerates the nitrification process in the water of tanks [47,48]. The greater effect of WMB in improving the water quality and forming biofloc particles observed in the current study may be attributed to both the degradability of its components and to the particle size formed, which would have increased the surface area for bacterial growth, hence an increased biofloc volume. This view is supported by Ferreira et al. [48], who found that increasing the substrate surface area in biofloc tanks improved water quality and increased food availability.

Furthermore, the results of our experiment revealed that the best growth performance and highest FCR were obtained for fish reared with BFT and fed the higher protein level (30%). Interestingly, the growth of fish fed 20% crude protein and reared in biofloc conditions with WMB significantly surpassed that of the fish fed 30% crude protein and stocked in clear water. Biofloc systems therefore represent a suitable culture condition for growth and feed utilization of *O. niloticus* without any obvious negative effect on water quality or fish survival, which reflects previous findings concerning the positive effect of biofloc on the growth performance of cultured fish and shrimp [18,45,46,49].

The improvement in growth performance in the present study might be due to the formed biofloc also used by the fish as a source of protein to compensate for the reduction in dietary protein levels and to provide a proper amino acid profile when plant-based protein diets are used. These results are in consistence with those obtained with shrimps fed different dietary protein levels (20–35%) and reared under BFT. In this study, no significant differences in terms of final body weight, weight gain and specific growth rate and FCR were observed between the animal fed different protein levels [43]. Also, *O. niloticus* fed different dietary protein 24 and 35% and reared under biofloc systems did not showed any significant changes in growth performance [50]. Interestingly, in the present study significant changes in growth performance among different biofloc treatments were recorded. Perhaps, the different results may be relay to the use of higher C:N ratio (15:1) and higher feed intake (3%) in the present study, in comparison with the C:N ratio (10:1) and feed intake (1.5%) used in the study of Azim et al. [50]. Rather than the differences in the used carbon sources in the two studies, whereas carbon source is the most determinate factor in the chemical composition and characteristic of the biofloc produced [6,18,45,46].

Similarly, the suitability of using biofloc at 4% level of dietary supplementation for improving growth, FCR and digestive enzyme activities has been demonstrated in *Penaeus monodon* [51]. Our results show that the use of low levels of dietary protein with BFT systems can have similar effects on fish growth as demonstrated in shrimps. The improvement in growth and FCR afforded by BFT in the present study would be due to the abundance of active heterotrophic bacteria, which can assimilate the waste nitrogen and produce new cellular protein for fish consumption, as previously indicated [11,12,18]. The observed retardation of fish growth in groups fed 20% dietary plant-based protein and reared in clear water in the present study, could be attributed to the reduction in protein levels together with the unsuitable amino acid profile of both diets using a plant protein mixture [26]. On the other hand, biofloc represents an efficient recycling process for feed nutrients and a rich source of essential amino acids [52], as well as an external source of digestive enzymes [6,45], rather than stimulating indigenous digestive enzyme activities (amylase, cellulase, protease and lipase) of reared animals [43,51]. For these reasons, biofloc could make up for the reduction of dietary protein and enhance the amino acid profile of fish diets, hence offsetting the continually increasing price feed cost and increasing production efficiency.

Recent approaches in aquaculturеare have used high quality feeds not only for optimal growth but also for maintaining the physiological, immunological and antioxidant status of fish [53]. As regard hematological parameters, in the present study fish reared under BFT showed higher WBC counts than fish maintained in clear water. Furthermore, the increased number of WBC was due to the increase in both neutrophils and lymphocytes, while monocyte numbers remained unchanged. Leucocytes play an important role in innate immunity during inflammation and their numbers are considered to be an indicator of fish health status [54]. However, *O. niloticus* reared in BFT conditions but using glucose as a carbon source showed no significant differences in WBC, red blood cell, hemoglobin or hematocrit levels compared to the values obtained for fish reared in clear water [44]. Such different results are probably due to the different carbon sources used in the studies (glucose or WMB/RB). In agreement with the present results, it BFT maintained the total haemocyte count of shrimps even with the dietary protein level was reduced from 35 to 20% [48].

A strong innate immune status is associated with increasing levels of proteins, albumin, and globulin, which represent the major proteins in plasma [55]. In the present study, all three proteins improved significantly in BFT reared fish. Also, total

immunoglobulin, lysozyme, MPO and ACH_{50} levels markedly increased in fish reared with both biofloc treatments compared with the levels recorded for the same activities in fish reared in clear water. The interaction of carbon source and protein levels demonstrates that rearing fish in biofloc conditions can make up for a 10% reduction in dietary protein in terms of non-specific immune parameters. The notable increase in Ig levels in the biofloc-based treatments is consistent with previous results obtained for *Labeo rohita* reared in BFT systems using different carbon sources [47]. Similar results were obtained in shrimps reared in BFT, which showed an increase in gene expression (pro-phenoloxidase, lysozyme, and serine proteinase) in the hepatopancreas, compared with the expresion observed in the hepatopancreas of shrimps reared in clear water [55]. Lysozyme and MPO are immune enzymes involved in defence against bacterial infection. In fish, lysozyme is produced by leucocytes and causes bacterial cell wall lysis, thus stimulating and activating the complement system and phagocytosis of different pathogens [34,56]. Also, MPO is one of the microbial enzymes expressed and stored in neutrophils, and plays a role in respiratory burst via peroxide to produce hypochlorous acid [57]. On the other hand, complement activity plays a crucial role in the antibacterial defence mechanism in teleosts [58], a function that can be activated by immunostimulants [59]. The present results indicate that the immune status of *O. niloticus* reared in biofloc conditions is stronger than that of fish reared in clear water. Future studies might look at whether the increase observed in these immune parameters is accompanied by improved defense against disease or stressful situations.

In phagocytes, several sequenced processes, referred as respiratory burst activity, destroy invading pathogens. Such activity is used as an important indicator of the innate immune defence mechanism in fish [60], which is measured as NBT activity in the present work. Respiratory burst and the phagocytic activities of blood leucocytes improved in fish reared in BFT conditions and fed the 30% protein level; furthermore, leucocytes of BFT fish reared in the same conditions but fed the lower protein level showed higher phagocytic activity than fish maintained in clear water. Similar results regarding increased phagocytic activity were reported in hemolymph of shrimp reared in biofloc systems [43].

The effect of bioflocs as immunostimulants seemed to be carbon source-dependent, and BFT fish given WMB showed a higher immune status than those given RB in the current study. Furthermore, the improvement recorded in the immunological parameters may be due to microbial components, to unknown growth factors or even to some probiotic microorganisms like *Bacillus*, *Lactobacillus* present in the biofloc [46,51]. Moreover, it is known that immune responses are modulated in different ways by nutrients like proteins, lipids, antioxidants, vitamins, carotenoids and minerals [61]. The digestion of biofloc in the intestine could increase the quantity and/or quality of such nutrients and potentially stimulate the fish cellular defences in the form of phagocytosis [43] or respiratory burst. Furthermore, the complementary protein source rich in key amino acids provided by biofloc systems [52] might also contribute to the optimum growth and immune function in BFT reared fish [62]. New studies on biofloc composition could help throw light on the exact compounds involved in this immunostimulant effect as well as in growth promotion in fish reared in BFT systems.

Besides the increased immune competency of fish reared in BFT conditions, the antioxidant status of such fish was also higher than that of *O. niloticus* reared in clear water. More specifically, CAT and SOD enzymatic activities increased significantly with the biofloc treatments. In agreement with the current findings, increased SOD and glutathione activities were previously reported in *O. niloticus* reared in biofloc systems compared with fish reared in clear water [44]. SOD and CAT are enzymes associated with the prevention of lipid peroxidation. SOD catalyzes superoxide anion to produce hydrogen peroxide [63], which, in turn, is decomposed by CAT to water and oxygen, preventing the beginning of lipid peroxidation [64]. The increasing of SOD and CAT activity observed in the present study might reflect increased fish welfare and reduced oxidative stress [65]. Indeed, a relationship between stress and the BFT treatments has been described. For example, BFT reduced the physiological stress (cortisol and glucose levels) of *Labeo rohita* [49]. Besides this, shrimps fed low levels of protein had a low total antioxidant capacity, while it modulated in shrimps reared under BFT system [43].

To conclude, the use of *in situ* biofloc production systems involving two different carbon sources (WMB and RB) significantly improved growth performance, FCR and some haematological parameters of *O. niloticus* compared to fish reared in clear water. Their immune and antioxidant status also improved. WMB seems to be a more suitable carbon source than RB for *O. niloticus* biofloc rearing systems. Moreover, the biofloc technique can compensate for a reduction in protein levels from 30 to 20% by increasing the immune and antioxidant status of reared fish. The reduction in the percentage of protein required in feed and the improvement seen in water quality add to the benefits of BFT.

Acknowledgements

We would like to thank all staff member of Fish and Animal Department, Faculty of Agriculture, Alexandria University (Egypt) for their help and support during the running, sampling and statistical analysis of the experiment. This work was partly supported by the *Fundación Séneca de la Región de Murcia* (Spain) (*Grupo de Excelencia.*Grant no. 19883/GERM/15).

References

[1] GAFRD, General Authority for Fish Resources Development. The General Authority for Fishery Resources Development: Fisheries Statistics Year Book 2012, Ministry of Agriculture and Land Reclamation, Cairo, Egypt, 2013.
[2] N.F. Soliman, D.M. Yacout, Aquaculture in Egypt: status, constraints and potentials, Aquacult Int. 24 (5) (2016) 1201–1227.
[3] R.H. Piedrahita, Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation, Aquaculture 226 (1) (2003) 35–44.
[4] R.L. Naylor, R.J. Goldburg, J.H. Primavera, N. Kautsky, M.C. Beveridge, J. Clay, C. Folke, J. Lubchenco, H. Mooney, M. Troell, Effect of aquaculture on world fish supplies, Nature 405 (6790) (2000) 1017–1024.
[5] F. Páez-Osuna, A. Gracia, F. Flores-Verdugo, L.P. Lyle-Fritch, R. Alonso-Rodrı, A. Roque, A.C. Ruiz-Fernández, Shrimp aquaculture development and the environment in the Gulf of California ecoregion, Mar. Pollut. Bull. 46 (7) (2003) 806–815.
[6] P. De Schryver, W. Verstraete, Nitrogen removal from aquaculture pond water by heterotrophic nitrogen assimilation in lab-scale sequencing batch reactors, Bioresour. Technol. 100 (3) (2009) 1162–1167.
[7] C.K. Lin, Y. Yi, Minimizing environmental impacts of freshwater aquaculture and reuse of pond effluents and mud, Aquaculture 226 (1) (2003) 57–68.
[8] J. Van-Rijn, Waste treatment in recirculating aquaculture systems, Aquacult Eng. 53 (2013) 49–56.
[9] E.M. Turra, F.L.B. Toral, É.R. de Alvarenga, F.S.S. Raidan, A.F. de Araújo Fernandes, G.F. de Oliveira Alves, S.C.M. de Sales, E. de Alencar Teixeira, L.G. Manduca, T.S. de Brito, Genotype× environment interaction for growth traits of Nile tilapia in biofloc technology, recirculating water and Cage systems, Aquaculture 460 (2016) 98–104.
[10] H. Manan, J.H.Z. Moh, N.A. Kasan, S. Suratman, M. Ikhwanuddin, Identification of biofloc microscopic composition as the natural bioremediation in zero water exchange of Pacific white shrimp, *Penaeus vannamei*, culture in closed hatchery system, Appl. water Sci. (2016), http://dx.doi.org/10.1007/s13201-016-0421-4.
[11] Y. Avnimelech, Biofloc Technology: a Practical Hand Book, EUA, Baton Rouge (, 2009.
[12] R. Crab, T. Defoirdt, P. Bossier, W. Verstraete, Biofloc technology in aquaculture: beneficial effects and future challenges, Aquaculture 356 (2012) 351–356.
[13] S.B. Day, K. Salie, H.B. Stander, A growth comparison among three commercial tilapia species in a biofloc system, Aquacult Int. 24 (5) (2016) 1309–1322.
[14] J. Ekasari, S. Maryam, Evaluation of biofloc technology application on water

quality and production performance of red tilapia *Oreochromis sp.* cultured at different stocking densities, HAYATI J. Biosci. 19 (2) (2012) 73–80.
[15] FAO, Impact of Rising Feed Ingredient Prices on Aquafeeds and Aquaculture Production, FAO of the United Nations., Food and Agriculture Organization, Rome, Italy, 2009.
[16] A. El-Sayed, Tilapia culture, first ed., CABI Publishing, Wallingford, 2006.
[17] R.W. Hardy, Utilization of plant proteins in fish diets. Effect of global demand and supplies of fishmeal, Aquacult Res. 41 (2010) 770–776.
[18] G. Wang, E. Yu, J. Xie, D. Yu, Z. Li, W. Luo, L. Qiu, Z. Zheng, Effect of C/N ratio on water quality in zero-water exchange tanks and the biofloc supplementation in feed on the growth performance of crucian carp, *Carassius auratus*, Aquaculture 443 (2015) 98–104.
[19] A. Moreno-Arias, J. López-Elías, A. Miranda-Baeza, M. Rivas-Vega, L. Martínez-Córdova, J. Ramírez-Suárez, Replacement of fishmeal by vegetable meal mix in the diets of *Litopenaeus vannamei* reared in low-salinity biofloc system: effect on digestive enzymatic activity, Aquacult Nutr 23 (2) (2017) 236–245.
[20] M. Rajkumar, P.K. Pandey, R. Aravind, A. Vennila, V. Bharti, C.S. Purushothaman, Effect of different biofloc system on water quality, biofloc composition and growth performance in *Litopenaeus vannamei* (Boone, 1931), Aquacult Res. 47 (11) (2016) 3432–3444.
[21] D.D. Kuhn, A.L. Lawrence, J. Crockett, D. Taylor, Evaluation of bioflocs derived from confectionary food effluent water as a replacement feed ingredient for fishmeal or soy meal for shrimp, Aquaculture 454 (2016) 66–71.
[22] M.A. Burford, P.J. Thompson, R.P. McIntosh, R.H. Bauman, D.C. Pearson, The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system, Aquaculture 232 (1) (2004) 525–537.
[23] R. Crab, B. Chielens, M. Wille, P. Bossier, W. Verstraete, The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae, Aquacult Res. 41 (4) (2010) 559–567.
[24] J. Ekasari, M. Zairin, D.U. Putri, N.P. Sari, E.H. Surawidjaja, P. Bossier, Biofloc-based reproductive performance of Nile tilapia *Oreochromis niloticus* L. broodstock, Aquacult Res. 46 (2) (2015) 509–512.
[25] A. Abduljabbar, A. Nour, T. Srour, N. El-Bermawy, W. Fayed, A. Mansour, Intensive Nile tilapia (*Oreochromis niloticus*) production under biofloc technology systems, G. J. Fish. Aquacult Res. 2 (2015) 64–80.
[26] J. Ekasari, M.H. Azhar, E.H. Surawidjaja, S. Nuryati, P. De Schryver, P. Bossier, Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources, Fish. Shellfish Immunol. 41 (2) (2014) 332–339.
[27] E. Cardona, D. Saulnier, B. Lorgeoux, L. Chim, Y. Gueguen, Rearing effect of biofloc on antioxidant and antimicrobial transcriptional response in *Litopenaeus stylirostris* shrimp facing an experimental sub-lethal hydrogen peroxide stress, Fish. Shellfish Immunol. 45 (2) (2015) 933–939.
[28] Y. Avnimelech, Carbon/nitrogen ratio as a control element in aquaculture systems, Aquaculture 176 (1999) 227–235.
[29] Y. Avnimelech, M. Kochba, Evaluation of nitrogen uptake and excretion by tilapia in bio floc tanks, using 15 N tracing, Aquaculture 287 (1) (2009) 163–168.
[30] D. Choudhury, A.K. Pal, N.P. Sahu, S. Kumar, S. Das, S.C. Mukherjee, Dietary yeast RNA supplementation reduces mortality by *Aeromonas hydrophila* in rohu, *Labeo rohita* L juveniles, Fish. Shellfish Immunol. 19 (2005) 281–291.
[31] C.Y. Cho, Fish nutrition, feeds and feeding: with special emphasis on salmonid aquaculture, Food Rev. Int. 6 (1990) 333–357.
[32] A.G. Gornall, C.J. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction, J. Biol. Chem. 177 (2) (1949) 751–766.
[33] B.T. Doumas, W.A. Watson, H.G. Biggs, Albumin standards and the measurement of serum albumin with bromocresol green, Clin. Chim. acta 258 (1) (1997) 21–30.
[34] A.E. Ellis, Lysozyme assay, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. Van, M. Winkel (Eds.), Techniques in Fish Immunology, SOS Publications, Fair Haven, NJ, USA, 1990, pp. 101–103.
[35] A.K. Siwicki, D.P. Anderson, Nonspecific Defense Mechanisms Assay in fish: II. Potential Kill. Activity Neutrophils Macrophages, Lysozyme Activity Serum Organs Total Immunoglobulin Level Serum, FAO project GCP/INT/JPA, IFI, Olsztyn, Poland, 1993, pp. 105–112.
[36] M. Alishahi, M. Ranjbar, M. Ghorbanpoor, R. Peyghan, M. Mesbah, Effects of dietary Aloe vera on some specific and nonspecific immunity in the common carp (*Cyprinus carpio*), Iran. J. Vet. Med. 4 (3) (2010) 189–195.
[37] P. Sahoo, J. Kumari, B. Mishra, Non-specific immune responses in juveniles of Indian major carps, J. Appl. Ichthyol. 21 (2) (2005) 151–155.
[38] E. Kawahara, T. Ueda, S. Nomura, In vitro phagocytic activity of white-spotted char blood cells after injection with *Aeromonas salmonicida* extracellular products, Fish. Pathol. 26 (4) (1991) 213–214.
[39] C.J. Secombes, Isolation of salmonid macrophages and analysis of their killing ability, in: J.S. Stolen, T.C. Fletcher, D.P. Anderson, B.S. Roberson, W.B. Van, M. Winkel (Eds.), Techniques in Fish Immunology, SOS Publications, Fair Haven NJ, 1990, pp. 137–152.
[40] H. Luck, Catalase, in: M.V. Bergmayer (Ed.), Method of Enzymatic Analysis, Verlag Chemic/Academic Press, New York, USA, 1974, p. 885.
[41] H. Misra, I. Fridovich, The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase, J. Biol. Chem. 247 (1972) 3170–3175.
[42] D.B. Duncan, Multiple range and multiple F tests, Biometrics 11 (1) (1955) 1–42.
[43] W.-J. Xu, L.-Q. Pan, Evaluation of dietary protein level on selected parameters of immune and antioxidant systems, and growth performance of juvenile *Litopenaeus vannamei* reared in zero-water exchange biofloc-based culture tanks, Aquaculture 426 (2014) 181–188.
[44] L. Long, J. Yang, Y. Li, C. Guana, F. Wu, Effect of biofloc technology on growth, digestive enzyme activity, hematology, and immune response of genetically improved farmed tilapia (*Oreochromis niloticus*), Aquaculture 448 (2015) 135–141.
[45] S.J. Arnold, F.E. Coman, C.J. Jackson, S.A. Groves, High-intensity, zero water-exchange production of juvenile tiger shrimp, Penaeus monodon: an evaluation of artificial substrates and stocking density, Aquaculture 293 (1) (2009) 42–48.
[46] D. Zhao, L. Pan, F. Huang, C. Wang, W. Xu, Effects of different carbon sources on bioactive compound production of biofloc, immune response, antioxidant level, and growth performance of *Litopenaeus vannamei* in zero-water exchange culture tanks, J. World Aquacult Soc. 47 (4) (2016) 566–576.
[47] D. Krummenauer, T. Samocha, L. Poersch, G. Lara, W. Wasielesky Jr., The reuse of water on the culture of pacific white shrimp, *Litopenaeus vannamei*, in BFT system, J. World Aquacult Soc. 45 (1) (2014) 3–14.
[48] L.M. Ferreira, G. Lara, W. Wasielesky Jr., P.C. Abreu, Biofilm versus biofloc: are artificial substrates for biofilm production necessary in the BFT system? Aquacult Int. 24 (2015) 921–930.
[49] A. Verma, A.B. Rani, G. Rathore, N. Saharan, A.H. Gora, Growth, non-specific immunity and disease resistance of *Labeo rohita* against *Aeromonas hydrophila* in biofloc systems using different carbon sources, Aquaculture 457 (2016) 61–67.
[50] M.E. Azim, D.C. Little, The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition, and growth and welfare of Nile tilapia (*Oreochromis niloticus*), Aquaculture 283 (1) (2008) 29–35.
[51] P.S. Anand, M. Kohli, S. Kumar, J. Sundaray, S.D. Roy, G. Venkateshwarlu, A. Sinha, G. Pailan, Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*, Aquaculture 418 (2014) 108–115.
[52] J. Ekasari, D. Angela, S.H. Waluyo, T. Bachtiar, E.H. Surawidjaja, P. Bossier, P. De Schryver, The size of biofloc determines the nutritional composition and the nitrogen recovery by aquaculture animals, Aquaculture 426 (2014) 105–111.
[53] V. Kiron, Fish immune system and its nutritional modulation for preventive health care, Anim. Feed Sci. Technol. 173 (2012) 111–133.
[54] C.J. Secombes, The non-specific immune system: cellular defenses, in: G. Iwama, T. Nakanishi (Eds.), The Fish Immune System: Organism, Pathogens and Environment, CA: Academic Press., San Diego, 1996, pp. 63–103.
[55] Y.V. Rao, B. Das, P. Jyotyrmayee, R. Chakrabarti, Effect of Achyranthes aspera on the immunity and survival of *Labeo rohita* infected with Aeromonas hydrophila, Fish. Shellfish Immunol. 20 (3) (2006) 263–273.
[56] S. Cecchini, G. Terova, G. Caricato, M. Saroglia, Lysozyme activity in embryos and larvae of sea bass (*Dicentrarchus labrax* L.), spawned by broodstock fed with vitamin C enriched diets, Bulletin-European Assoc. Fish Pathologists 20 (3) (2000) 120–124.
[57] R. Dalmo, K. Ingebrigtsen, J. Bøgwald, Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES), J. Fish. Dis. 20 (4) (1997) 241–273.
[58] A.E. Ellis, Innate host defense mechanisms of fish against viruses and bacteria, Dev. Comp. Immunol. 25 (8) (2001) 827–839.
[59] H. Matsuyama, R.E. Mangindaan, T. Yano, Protective effect of schizophyllan and scleroglucan against *Streptococcus sp.* infection in yellowtail (*Seriola quinqueradiata*), Aquaculture 101 (3) (1992) 197–203.
[60] T. Miyazaki, A simple method to evaluate respiratory burst activity of blood phagocytes from Japanese flounder, Fish. Pathol. 33 (3) (1998) 141–142.
[61] V.V. Trichet, Nutrition and immunity: an update, Aquacult Res. 41 (3) (2010) 356–372.
[62] P. Li, K. Mai, J. Trushenski, G. Wu, New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds, Amino Acids 37 (1) (2009) 43–53.
[63] Y. Tao, L. Pan, H. Zhang, S. Tian, Assessment of the toxicity of organochlorine pesticide endosulfan in clams Ruditapes philippinarum, Ecotoxicol. Environ. Saf. 93 (2013) 22–30.
[64] O.L. Tavares-Sánchez, G.A. Gómez-Anduro, X. Felipe-Ortega, M.A. Islas-Osuna, R.R. Sotelo-Mundo, C. Barillas-Mury, G. Yepiz-Plascencia, Catalase from the white shrimp Penaeus (Litopenaeus) vannamei: molecular cloning and protein detection, Comp. Biochem. Physiology Part B Biochem. Mol. Biol. 138 (4) (2004) 331–337.
[65] D.P. Parrilla-Taylor, T. Zenteno-Savín, F.J. Magallón-Barajas, Antioxidant enzyme activity in pacific whiteleg shrimp (*Litopenaeus vannamei*) in response to infection with white spot syndrome virus, Aquaculture 380 (2013) 41–46.
[66] NRC, National Research Council, Nutrient Requirements of Fishes, National Academy of Sciences, Washington, DC, USA, 1993.