



# The potential of rearing juveniles of bluegill, *Lepomis macrochirus*, in a biofloc system

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## ABSTRACT

Biofloc technology (BFT) can potentially enhance growth and provide supplemental nutrition to some fish species, but this has not been investigated in bluegill *Lepomis macrochirus* juveniles. In this study, *L. macrochirus* juveniles were cultured in a clear-water (CW) system versus BFT when using either corn starch (BFT-Sta) or sucrose-sugar (BFT-Sug) for 32 days at a carbon (C) to nitrogen (N) ratio of 15. After 32 days, water composition of cultivable bacteria were identified using molecular techniques while the survival, growth, histopathology, biochemical composition, and contribution of C and N from bioflocs (based on stable isotope analysis) to *L. macrochirus* were determined. In the CW system, *Escherichia coli* was detected but not in the BFT tanks, whereas *Aeromonas hydrophila* was present in the BFT tanks but not in CW. Fish obtained C and N from bioflocs at 4.11 % and 9.5 % in the BFT-Sta treatment, respectively, and 18.8 % and 1.4 % in the BFT-Sug treatment, respectively. A temporary loss of aeration in the BFT treatments led to dissolved oxygen (DO) plummeting, leading to 50 % mortality, which is indicative of the risk involved when using BFT but also the tolerance of *L. macrochirus* to temporary low DO. BFT also significantly reduced *L. macrochirus* growth, which was likely compounded by consistently elevated ammonia and reduced feed intake/feeding activities. The nutritional contribution of bioflocs to *L. macrochirus* along with no abnormal gill histopathology suggests BFT could have great potential for this species.

## 1. Introduction

Bluegill, *Lepomis macrochirus*, is a popular sportfish that are often stocked in ponds for recreational fishing, especially in the south of the United States (U.S.). In particular, Arkansas has a long history of *L. macrochirus* aquaculture where they are commercially produced. Moreover, the Arkansas Game & Fish Commission hatcheries produced and stocked over 500,000 juveniles in lakes throughout Arkansas for the purpose of recreational fishing in 2018 (AGFC (Arkansas Game and Fish Commission) (2020)). There is also interest in *L. macrochirus* as foodfish, which takes two years for the desired market size of around 300 g to be achieved in ponds (Dundenhoeffer et al., 2012). Although *L. macrochirus* are native to mainland U.S., they have been introduced throughout the world including Europe, Africa and Asia. Since the introduction of *L. macrochirus* to China in 1987, they have been farmed as a foodfish (Yang et al., 2016), although worldwide production statistics are not yet available.

The most common method for *L. macrochirus* culture is in earthen ponds, where a complete diet is unnecessary because they are opportunistic and voracious predators that feed on various small prey including insects, zooplankton, and benthic invertebrates (NCRAC, 2002; Uchii et al., 2006). While *L. macrochirus* can also be cultured in tank systems to allow for year round production (Mischke and Morris, 1997; Dundenhoeffer et al., 2012), they would require complete diets that could increase operating costs (Morris et al., 2002).

One way to combine tank culture with supplemental feed is through biofloc technology (BFT). Bioflocs are created by the addition of extra organic carbon that subsequently encourages heterotrophic bacterial growth that converts dissolved nitrogenous waste into potentially consumable microbial biomass. This biomass contains both macro- and micro-nutrients that are constantly available to animals capable of consuming small particles (Dauda, 2020). In contrast to clearwater systems, biofloc-based systems are substantially more microbe rich that can contain a mixture of potentially pathogenic, non-pathogenic

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bacteria and probiotic bacteria (Cardona et al., 2016; Liu et al., 2019).

Due to the higher abundance of microbes as well as organic particles, this increases the biological oxygen demand of the system and consequently rigorous aeration is typically required to sustain the extra respiration and keep the bioflocs in suspension. Any interruptions to aeration may result in ammonia spikes, reduced dissolved oxygen levels and, if prolonged, high mortalities of the cultured animal. Therefore, substantial risks are involved when culturing animals with BFT (Furtado et al., 2014), and farmers must consider this before implementation. Typically, species that benefit the most from BFT are those that are capable of collecting and consuming bioflocs, especially crustaceans (Cardona et al., 2015; Ray and Lotz, 2017). However, some fish species have also benefited in terms of growth and/or immunity including tilapia, *Oreochromis niloticus* (Avnimelech and Kochba, 2009), silver carp, *Hypophthalmichthys molitrix*, bighead carp, *Aristichthys nobilis* (Zhao et al., 2014) and lemon fin barb hybrids, *Hypsibarbus wetmorei* ♂ × *Barboides gonionotus* ♀ (Dauda et al., 2018a). Currently, there is no information regarding the feasibility of culturing *L. macrochirus* with BFT, which may be applicable for indoor tank culture.

The aim of this study was to investigate the possibility of culturing *L. macrochirus* with BFT (using either corn starch or sugar as the main carbon source) by assessing their growth, feeding and health as well as water quality compared to a clear-water (CW) system.

## 2. Materials and methods

### 2.1. Source of experimental animals and experimental set up

Bluegill juveniles (500 in total) were obtained from a local hatchery (F & L Anderson, Lonoke, Arkansas) and upon arrival to the research station at University of Arkansas at Pine Bluff (UAPB). The temperature of the holding water was slowly raised to the culture water over 15 min., and then once the temperatures were similar, the fish were added to an outdoor fiberglass tank (1000 L) located underneath a shed (roof but no walls). The tank was managed with reservoir water flowing through, (1 L/min.) and blown air was delivered through two Alumina air stones (1.5" × 1.5" × 3"; air flow of 0.3 cubic feet per min.) to maintain adequate dissolved oxygen (DO) levels. Over the next six days, the fish were fed to apparent satiation with sinking pellets (crude protein 32 %) designed for goldfish. During the acclimation period, all experimental tanks and airstones were thoroughly cleaned with chlorine, rinsed and allowed to dry. Afterwards, water in the experimental tanks were filled and 25 mL of chlorine was added and allowed to bubble off. The fish were fasted one day prior to transfer into their experimental tanks.

### 2.2. Experimental design and set up

After one week, a total of 270 fish (mean weight = 5.30 ± 0.01 g) were equally distributed among nine 100-L circular tanks, and then were randomly assigned to one of the three treatments which each had three replicate tanks (30 fish/replicate tank). The two biofloc technology (BFT)-based treatments received either corn starch (BFT-Sta) or sugar (BFT-Sug), while the clear-water (CW) treatment tanks was configured as a flow-through system (same as mentioned in 2.1). The CW tanks had two air diffusers while the BFT tanks had four air diffusers (same as mentioned in 2.1) to support the bioflocs. Additionally, 1 L of pond water was added into each BFT tank to act as an inoculant. The following day, the ammonia-N levels were tested and corn starch or sugar was added to achieve a C/N ratio of 15 based on the ammonia-N level of each tank. This was done because the fish were not yet fed. Afterwards, a C/N ratio of 15 was used to guide carbohydrate additions based on the amount of food provided the previous day, which was provided to apparent satiation once daily. However, if the ammonia-N level approached around 1 mg/l then additional carbon was added at a C/N ratio of 15 based on the ammonia-N level instead of feeding rates.

Each day, the DO, ammonia-N, pH, biofloc volume and temperature were measured. The DO, pH and temperature were measured using a multimeter probe (YSI Professional Plus). Each day, the total ammonia-nitrogen was measured colorimetrically using the salicylate-hypochlorite method (Verdouw et al., 1978), while the nitrite-nitrogen and nitrate-nitrogen were measured using API test kits every three days and each week, respectively. The biofloc volume was measured based on the volume settling out from 1 L after 30 min. in an Imhoff cone. Every week the alkalinity was determined using the titration method from Model AL-AP, HACH.

After 32 days, 50 mL of water from each tank were collected in sterile containers, stored at -20 °C and the bacterial composition was evaluated (section 2.3). Isotope analyses were also performed on the biofloc, fish muscle, and food (section 2.4). Fish were euthanized with an overdose of MS-222, and then weighed to calculate the specific growth rates (SGR) and condition factor, according to the following equations,

$$SGR (weight) = \frac{[\ln final weight (g) - \ln initial weight (g)]}{initial weight (g)} \times 100$$

$$SGR (length) = \frac{[\ln final length (cm) - \ln initial length (cm)]}{initial length (cm)} \times 100$$

$$Condition factor = \frac{Weight (g)}{Length (cm)^3}$$

The fish were then dissected to remove the liver to calculate the hepatosomatic index (HSI), using the following equation,

$$HSI = \frac{Liver weight (g)}{Body weight (g)} \times 100$$

The gills from 3 fish/replicate of each treatment were placed in Bouin's solution for 18 h for later histology, as this fixative softens the cartilage. Both the livers and intestines from the same 3 fish/replicate in each treatment were removed and placed in 10 % phosphate buffered formalin for 18 h as a general fixative for soft tissues (Section 2.5). The muscle moisture content of the fish was measured, and the protein and lipid content were measured according to Bradford's method (Bradford, 1976) using bovine serum albumin as a standard. The lipid content was measured with a tripalmitin standard following the method of Bligh and Dyer (1959). All procedures for measuring protein and lipid were conducted while samples were preserved on ice.

### 2.3. Bacterial composition of the culture water

Water samples (200 µL) were inoculated on Brain Heart infusion agar as well as non-selective agar plates under a sterile laminar flow cabinet. The agar plates were covered and incubated at room temperature (20 °C) for 24 h and all the grown bacterial colonies were pooled from the agar plates and added to a sterile saline solution. Next, 3 mL of the culture was centrifuged at 10,000 rpm for 5 min and the supernatant discarded. The pellet was resuspended in 200 µL of ATL buffer and the bacterial DNA was extracted using Qiagen DNeasy Blood and Tissue kit (Cat No.: 69,506). The bacterial DNA was isolated using the QIAcube robotic workstation system (Qiagen, Germantown, MD, USA), which is designed for fully automated extraction and purification processes. All steps up to elution of highly pure DNA were performed according to the manufacturers' instructions. The quantity of the DNA was evaluated by using Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA).

Genotypic bacterial identification was done from the pooled bacterial colonies using the 16S rRNA identification method using two primer sets and a GyraseB primer set. A primer set to amplify approximately 1300 bp of a consensus 16S rRNA gene was used (Marchesi et al., 1998) with a forward primer 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse primer 1387R (5'-GGG CGG WGT GTA CAA

GGC-3’).

Another primer set (Wang et al., 2015) from the bacterial hypervariable V3 region of the 16S rRNA genes was amplified using a set of primers; 8 F (5’-AGAGTTTGATCCTGGCTCAG -3’) and reverse primer 533R (5’-TTACCGCGGCTGCTGGCAC-3’). The gyraseB Primer set was GyrB F: (5’ TCCGGCGGTCTGCACGGCGT- 3’), GyrB R: (5’ TTGTCCGG GTTGTACTCGTC -3’).

The qPCR analyses were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions containing 2.5 µL of DNA (10 ng/µL), 0.5 µL each of forward (0.4 µM) and reverse (0.4 µM) primers, and 6.25 µL Taqman Universal Fast Master Mix 2X (Applied Biosystems, Foster City, CA, USA) were performed in a three-step experimental run protocol: a denaturation program (2 min at 95 °C); an amplification and extension repeated 40 cycles (15 s at 95 °C, 1 min at 55 °C, 1 min at 72 °C) and finally a cooling step. The amplified PCR product was purified using QIAquick PCR purification kit (Qiagen, Germany). Four microliter of purified amplicons were sent to a commercial company (GENEWIZ LLC, South Plainfield, NJ) for DNA sequencing. The DNA sequence was then compared with those in the GenBank database using the BLAST server by the National Center for Biotechnology Information (NCBI).

#### 2.4. Isotope analysis

Centrifuged bioflocs and fish muscle (n = 5) were oven dried at 50 °C and then ground to a fine powder using a mortar and pestle in triplicate. Samples were analyzed using a Costech Model 4010 Elemental Combustion System connected to a Thermo, Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). Stable isotope values were expressed using the following equation:

$$\delta = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 100$$

where R is the ratio of heavier to lighter isotopes (<sup>13</sup>C/<sup>12</sup>C and <sup>14</sup>N/<sup>15</sup>N). The values in the CW treatment were used to calculate the fractionation factors (Δ), which was the difference in δ values between the fish muscle and feed according to the following equation (Fry 2006),

$$\Delta = \delta_{\text{SOURCE}} - \delta_{\text{PRODUCT}}$$

Where the source is the commercial feed and the product is the fish muscle from the CW treatment, which assumes all nutrition from the fish in the CW was obtained from the commercial feed.

A two-source mixing model was used to estimate the nutritional contribution from the feeds and bioflocs to the fish in the BFT treatments using the following equations,

$$f1 = \frac{(\delta_{\text{SAMPLE}} - \delta_{\text{SOURCE2}})}{(\delta_{\text{SOURCE1}} - \delta_{\text{SOURCE2}})}$$

$$f2 = 1 - f1$$

where f1 is the portion of C or N contributed by source 1 (commercial feeds), f2 is the portion contributed by source 2 (bioflocs) and the sample was fish muscle in the BFT treatments.

#### 2.5. Histological analysis

After the samples were fixed in their formalin-based solutions, they were transferred to 70 % (v/v) ethanol. The gills were washed several times in ethanol prior to processing, which occurred the following day. During processing, the samples were progressively dehydrated at increasing ethanol concentrations, then cleared in xylene and finally embedded in paraffin wax.

Sections (5µm) were made using a rotary microtome (HM 340E, Thermo Scientific) and at least 4 slides were made for each tissue

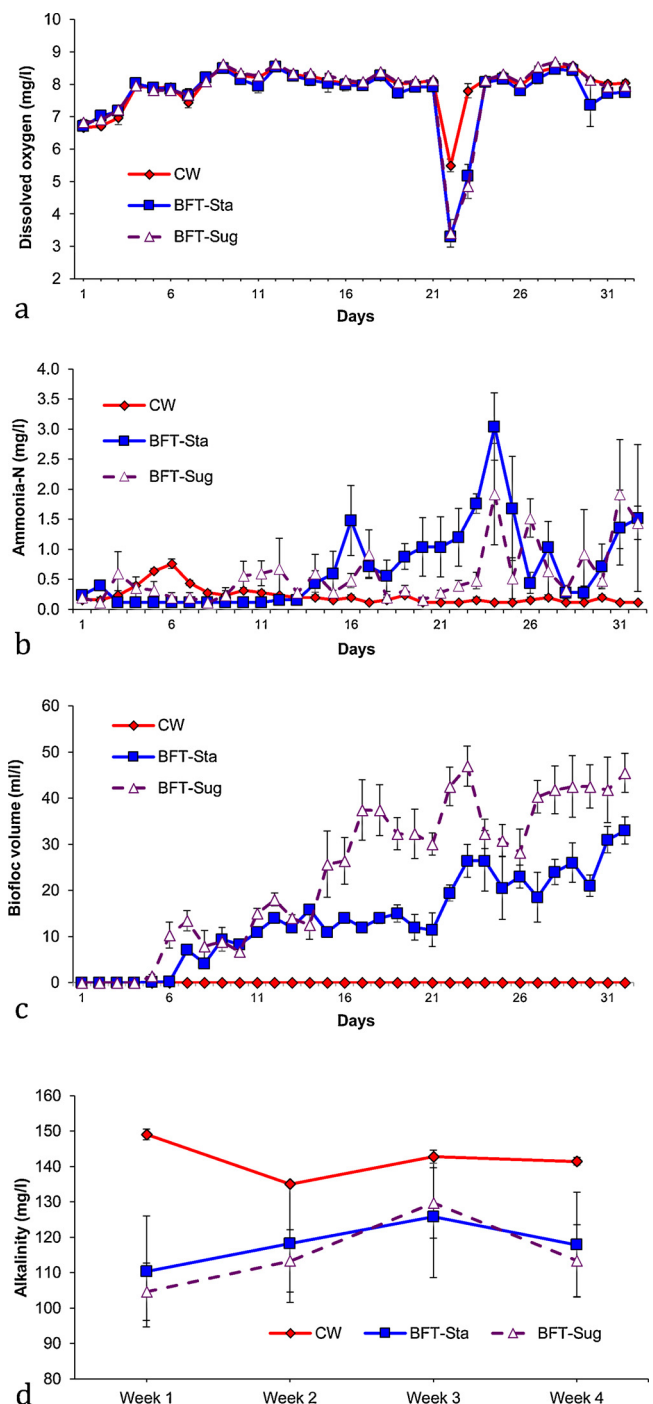


Fig. 1. Mean ( ± SE) dissolved oxygen levels (mg/l) (a), ammonia-N (mg/l) (b), biofloc volume (ml/l) (c) and alkalinity (mg/l) (d) in a clear-water (CW) system versus biofloc-based systems using starch (BFT-Sta) or sugar (BFT-Sug).

sample from each replicate. Two slides were then stained with hematoxylin and eosin (H&E) and the other two slides were stained with Periodic-acid Schiff (PAS) according to Romano et al., 2018a,b. Pictures of the slides were taken with a microscope mounted with a camera (Leica DM3000 LED). The PAS staining intensity was quantified according to Karami et al. (2016).

### 3. Results

#### 3.1. Water quality and amount of carbon used

The dissolved oxygen (DO) levels were similar among all treatments, except on day 22 in the BFT treatments when the DO significantly reduced to 0.2–1.5 mg/l compared to 5.2–5.8 mg/l in the CW treatment. This change in DO was the same day the aeration was temporarily disrupted and the DO values presented in Fig. 1a are means from two samples (morning and evening). This was due to unusually high rainfall and subsequent flooding in the area tripped the electrical fuse (thus negating the backup generator) and caused a temporary stoppage to aeration as well as water flow to the CW tanks. By the time the area could be accessed and electricity brought back, the DO reached as low as 0.2 mg/l. In contrast, the DO level only dropped to 5.59 mg/L in the CW tanks (Fig. 1a).

Similarly, on days 22–23, the ammonia levels spiked on the BFT tanks. Moreover, the ammonia-N levels in the CW treatment were generally lower than the BFT treatments, except on days 4–8. Among the BFT treatments, the ammonia-N levels consistently remained below 1 mg/L for the first 2 weeks. Afterwards, the ammonia-N tended to increase, which was generally higher in the corn starch treatment (Fig. 1b).

Biofloc volume gradually increased over time, but became higher in the BFT-Sug from day 15 onwards, which was often significant (Fig. 1c). Once the bioflocs were established after 14 days, the mean biofloc volume in the BFT-Sta was significantly lower at 19.71 ( $\pm$  5.9) ml/l compared to 35.3 ( $\pm$  4.6) ml/l in the BFT-Sug treatments. Water alkalinity was consistently higher in the CW treatment compared to the BFT treatments (Fig. 1d).

The mean water quality parameters in all the treatments along with the total amount of carbon added over 32 days are shown in Table 1. The overall temperature and DO among the treatments were not significantly different. In contrast, both the pH and alkalinity were significantly lower in the BFT treatments compared to the CW treatment. Meanwhile, both ammonia-N and nitrite-N were significantly lower in the CW treatment compared to the BFT treatments. Nitrate-N was undetectable throughout the study among all treatments.

#### 3.2. Bacterial composition of the water

The bacterial composition of the water from the CW, BFT-Sta and BFT-Sug on day 32 is shown in Table 2. There was some similarity among the BFT treatments, which included *Aeromonas* spp. being present, but was not cultured in the CW treatment. Moreover, in the CW treatment there were *Citrobacter* spp., *Klebsiella* spp., and *Escherichia coli*, but these were not cultivated from the BFT treatments. However, there was also some dissimilarity between BFT treatments, with *Pseudomonas* spp. being detected in the BFT-Sta, but not in the BFT-Sug

**Table 1**

Mean ( $\pm$  SE) water quality parameters and amount of carbon added among the clear-water (CW) systems versus biofloc-based systems using starch (BFT-Sta) or sugar (BFT-Sug) after 32 days.

	CW	BFT-Sta	BFT-Sug
Temperature (°C)	25.4 $\pm$ 0.1	24.7 $\pm$ 0.1	24.8 $\pm$ 0.2
Dissolved oxygen (mg/l)	7.8 $\pm$ 0.1	7.6 $\pm$ 0.1	7.7 $\pm$ 0.0
pH	8.15 $\pm$ 0.02 <sup>a</sup>	7.95 $\pm$ 0.04 <sup>b</sup>	7.96 $\pm$ 0.03 <sup>b</sup>
Alkalinity (mg/l)	142 $\pm$ 3 <sup>a</sup>	118 $\pm$ 3 <sup>b</sup>	115 $\pm$ 5 <sup>b</sup>
Ammonia-N (mg/l)	0.23 $\pm$ 0.01 <sup>a</sup>	0.69 $\pm$ 0.09 <sup>b</sup>	0.57 $\pm$ 0.07 <sup>b</sup>
Nitrite-N (mg/l)	0.0 <sup>a</sup>	0.2 $\pm$ 0.2 <sup>b</sup>	0.1 $\pm$ 0.1 <sup>b</sup>
Nitrate-N (mg/l)	0.0	0.0	0.0
Carbon added (g)	0.0	517.3	471.6

Different superscripted letters in each row indicate significant differences ( $p < 0.05$ ).

**Table 2**

Bacterial composition of the water in the clear-water (CW) system versus biofloc-based systems using starch (BFT-Sta) or sugar (BFT-Sug) when culturing bluegill, *Lepomis macrochirus*, juveniles after 32 days.

Identified cultivable bacteria	CW	BFT-Sta	BFT-Sug
<i>Aeromonas veronii</i>	-	+	+
<i>Aeromonas sobria</i>	-	+	+
<i>Aeromonas hydrophila</i>	-	+	+
<i>Achromobacter xylosoxidans</i>	-	+	-
<i>Arenimonas daejeonensis</i>	-	+	-
<i>Bordetella genomosp. 8</i>	-	+	-
<i>Bordetella bronchialis</i>	+	-	-
<i>Citrobacter freundii</i>	+	-	-
<i>Citrobacter braakii</i>	+	-	-
<i>Citrobacter portucalensis</i>	+	-	-
<i>Enterobacter cloacae</i>	+	-	-
<i>Enterobacter hormaechei</i>	+	-	-
<i>Escherichia coli</i>	+	-	-
<i>Klebsiella aerogenes</i>	-	-	+
<i>Klebsiella michiganensis</i>	+	-	+
<i>Klebsiella pneumoniae</i>	-	-	-
<i>Klebsiella grimontii</i>	+	-	-
<i>Klebsiella oxytoca</i>	+	-	-
<i>Pseudomonas arsenicoxydans</i>	-	+	-
<i>Pseudomonas asplenii</i>	-	+	-
<i>Pseudomonas cremoricolorata</i>	-	+	-
<i>Pseudomonas chlororaphis</i>	-	+	-
<i>Pseudomonas entomophila</i>	-	+	-
<i>Pseudomonas fluorescens</i>	+	+	-
<i>Pseudomonas fulva</i>	-	+	-
<i>Pseudomonas koreensis</i>	+	+	-
<i>Pseudomonas kribbensis</i>	-	+	-
<i>Pseudomonas mandelii</i>	+	+	-
<i>Pseudomonas moraviensis</i>	-	+	-
<i>Pseudomonas mosselii</i>	+	-	-
<i>Pseudomonas putida</i>	+	+	-
<i>Pseudomonas stutzeri</i>	-	+	-
<i>Pseudomonas tolaasii</i>	+	-	-
<i>Stenotrophomonas acidaminiphila</i>	+	-	-
<i>Stenotrophomonas maltophilia</i>	+	+	-
<i>Stenotrophomonas rhizophila</i>	+	+	-
<i>Staphylococcus pseudintermedius</i>	-	+	-
<i>Staphylococcus aureus</i>	-	+	-

treatments. Overall, there was substantially more species diversity in the CW and BFT-Sta treatments with 19 and 23 being identified, respectively, compared to only 5 in the BFT-Sug treatment.

#### 3.3. Animal performance

Survival was similar among the treatments and no mortality observed until the mechanical failure on day 22, when survival significantly decreased in the BFT-Sta and BFT-Sug treatments, compared to the CW treatment (Table 3). Only one mortality occurred in the CW

**Table 3**

Survival, feed intake and body indices ( $\pm$  SE) of bluegill, *Lepomis macrochirus*, juveniles cultured in a clear-water (CW) system versus biofloc-based systems using corn starch (BFT-Sta) or sugar (BFT-Sug) after 32 days.

	CW	BFT-Sta	BFT-Sug
Initial weight (g)	5.34 $\pm$ 0.01	5.30 $\pm$ 0.02	5.31 $\pm$ 0.02
Initial length (mm)	65.4 $\pm$ 0.0	65.1 $\pm$ 0.0	65.7 $\pm$ 0.0
Final weight (g)	8.16 $\pm$ 0.07 <sup>a</sup>	7.24 $\pm$ 0.27 <sup>b</sup>	7.34 $\pm$ 0.11 <sup>b</sup>
Final length (mm)	78.6 $\pm$ 0.1	77.0 $\pm$ 0.1	78.0 $\pm$ 0.0
SGR weight (%/day)	1.47 $\pm$ 0.03 <sup>a</sup>	0.94 $\pm$ 0.18 <sup>b</sup>	1.15 $\pm$ 0.10 <sup>b</sup>
SGR length (%/day)	0.41 $\pm$ 0.11 <sup>a</sup>	0.25 $\pm$ 0.07 <sup>b</sup>	0.36 $\pm$ 0.04 <sup>b</sup>
Total feed intake (g)	402 $\pm$ 8 <sup>a</sup>	360 $\pm$ 13 <sup>b</sup>	345 $\pm$ 7 <sup>b</sup>
Condition factor	1.68 $\pm$ 0.05	1.59 $\pm$ 0.04	1.56 $\pm$ 0.02
Survival (%)	98.3 $\pm$ 0.8 <sup>a</sup>	63.3 $\pm$ 1.7 <sup>b</sup>	65.0 $\pm$ 10.1 <sup>b</sup>

Different superscripted letters indicate significant differences ( $p < 0.05$ ) within each row.



**Table 4**

Mean proximate composition (% wet weight basis) of the muscle, liver glycogen (Periodic-acid Schiff staining intensity %) and isotope values ( $\pm$  SE) of bluegill, *Lepomis macrochirus*, juveniles cultured in a clear-water system (CW) versus biofloc-based systems using corn starch (BFT-Sta) or sugar (BFT-Sug) after 32 days.

	CW	BFT-Sta	BFT-Sug
Moisture (%)	65.59 $\pm$ 0.25	64.54 $\pm$ 0.86	64.35 $\pm$ 0.38
Crude protein (%)	14.68 $\pm$ 0.45 <sup>a</sup>	12.85 $\pm$ 0.44 <sup>b</sup>	14.50 $\pm$ 1.05 <sup>ab</sup>
Crude lipid (%)	10.40 $\pm$ 0.61 <sup>a</sup>	9.40 $\pm$ 0.65 <sup>b</sup>	9.53 $\pm$ 0.36 <sup>b</sup>
Liver glycogen (%)	49.83 $\pm$ 0.92 <sup>a</sup>	32.81 $\pm$ 0.57 <sup>b</sup>	29.41 $\pm$ 0.82 <sup>b</sup>
Muscle $\delta$ C <sup>13</sup>	-20.47 $\pm$ 0.11 <sup>b</sup>	-20.01 $\pm$ 0.06 <sup>a</sup>	-20.39 $\pm$ 0.07 <sup>ab</sup>
Muscle $\delta$ N <sup>15</sup>	9.70 $\pm$ 0.12	9.82 $\pm$ 0.05	9.88 $\pm$ 0.04

Different superscripted letters indicate significant differences ( $p < 0.05$ ) within each row.

tank on day 26. Growth was also significantly lower in the BFT-Sta and BFT-Sug treatments, but there was no significant effect on the condition factor (Table 3).

### 3.4. Proximate composition and isotope analysis

The muscle protein content was significantly higher in the CW treatment compared to that of the BFT-Sta treatment. The muscle lipid content was significantly lower in both of the BFT treatments compared to the CW treatment. The moisture content was similar among the treatments (Table 4). The PAS staining intensity was significantly higher in the CW treatment compared to those in either the BFT-Sta or BFT-Sug treatments (Table 4).

The muscle of fish in the CW treatment had significantly lower  $\delta$  C<sup>13</sup> values compared to BFT-Sta (Table 4). No significant difference in the  $\delta$  N<sup>15</sup> values was detected. Based on the two-sample isotope mixing model, fish obtained significantly more nitrogen from bioflocs in the BFT-Sta treatment (9.55 %) compared to BFT-Sug (1.46 %), but the reverse was found for the contribution of carbon at 18.89 % in the BFT-Sug treatment compared to 4.11 % in the BFT-Sta treatment (Fig. 2).

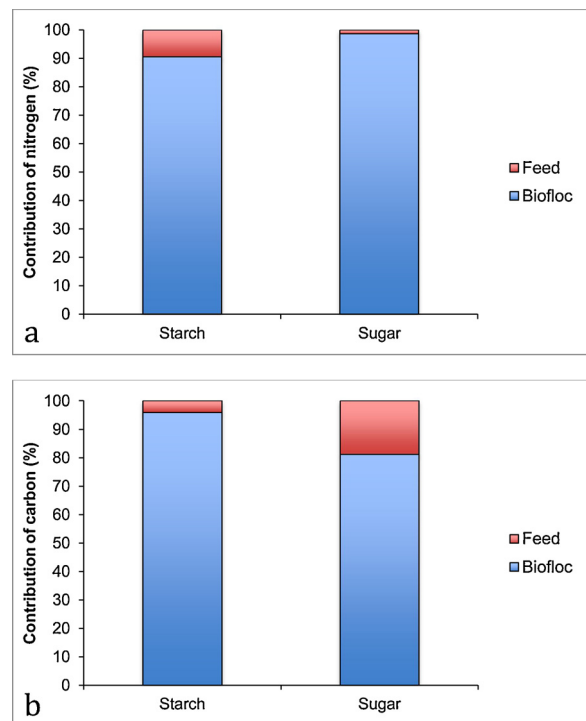
### 3.5. Histopathology

The gills of *L. macrochirus* juveniles appeared similar among the treatments, with no observable signs abnormalities or injury. In particular, the secondary lamellae of the gills showed no evidence of inflammation, increased interlamellae cell masses, sloughing or necrosis (pictures not shown). Similarly, the distal intestine appeared similar among treatments with a similar distribution of goblet cells and lymphocytes with no evidence of inflammation, such as shortened villi or wider villi/lamina propria (pictures not shown).

The livers showed no indications of damage or inflammation, such as necrosis or infiltrations of white blood cells, respectively. However, the majority of livers from fish in CW showed extensive basophilia, less vacuolization and more intense staining for Periodic-acid Schiff (glycogen) (Fig. 3).

## 4. Discussion

Farmers are often hesitant to implement BFT due to the additional required management such as performing solids management, ensuring aeration is constant, and often needing to closely monitor water quality. In the case of the latter, this is because BFT can be less stable in terms of water quality compared to recirculating or flow through aquaculture systems. Additionally, BFT is sometimes viewed as being “dirty” or unhygienic due to the accumulation of bioflocs that makes the water turbid and often appear muddy (Crab et al., 2007). Interestingly, however, some of the bacterial compositional results seem to counter this. For example, cultivable *Escherichia coli* was found in the CW

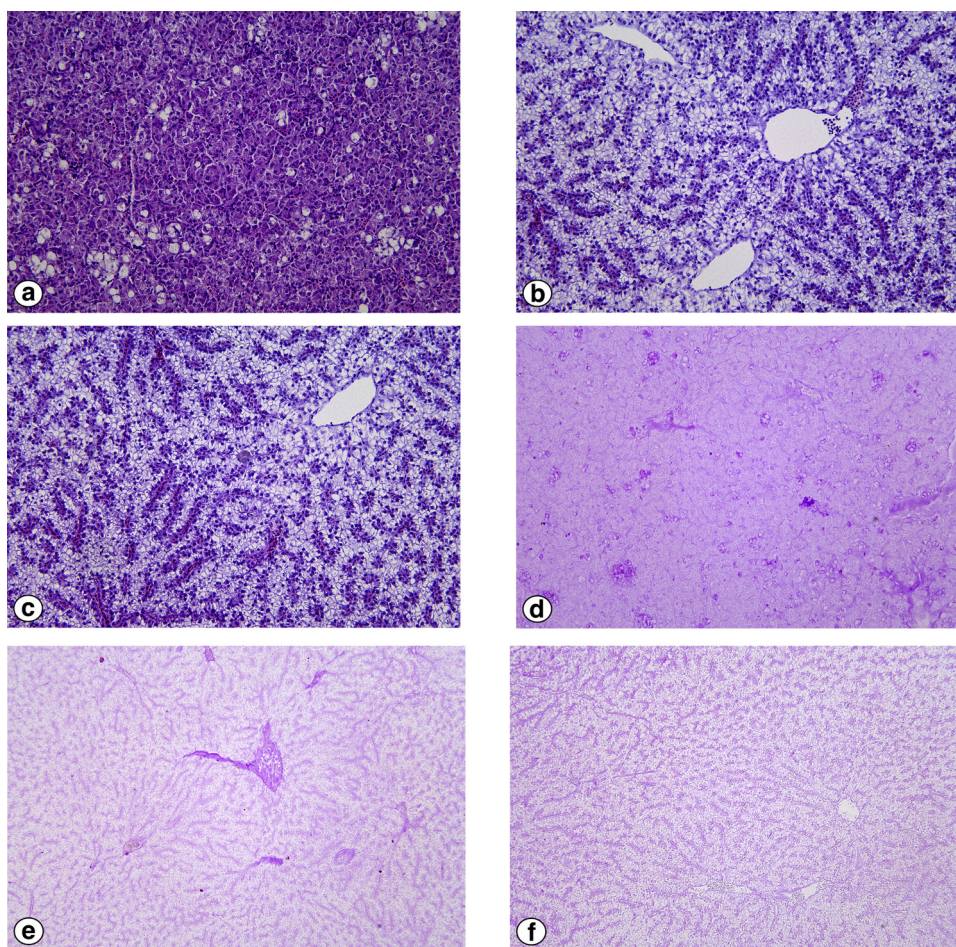


**Fig. 2.** Percentage contribution of feed and bioflocs to the carbon content (a) and nitrogen content (b) to bluegill, *Lepomis macrochirus*, juveniles based on a two-sample isotope mixing model. Differences in the contribution of carbon and nitrogen from biofloc were each significantly different between treatments ( $p < 0.05$ ).

treatments, but not in the BFT treatments. It is well known that *E. coli* in water is from fecal contamination from humans or animals, which can cause intestinal distress in humans (Kolenda et al., 2015). The water source in this study was from a nearby reservoir and various animals are known to inhabit the area, especially at night, which was likely the source of contamination. It seems likely that *E. coli* was not present in the BFT treatments due to other bacteria outcompeting for organic material. Additionally, several bacterial species that can be pathogenic to humans, including *Enterobacter cloacae* and *Bordetella bronchialis* were cultured from the CW tanks but not from BFT ones.

Another interesting finding was the presence of *Aeromonas hydrophila* in the BFT treatments, which is an opportunistic pathogen to various fish species, including *L. macrochirus* (Reed and Fracis-Floyd, 2011). Although other studies have also found *Aeromonas* spp. (Gou et al., 2019) and *A. hydrophila* in biofloc-based systems (Pérez-Fuentes et al., 2018), other potentially probiotic bacteria were also detected including *C. freundii*, *Enterobacter* spp., and *Bacillus* spp. (Hai, 2015). In this study, however, probiotic bacteria were not detected in the BFT tanks, but rather only in the CW treatment. Additionally, in the BFT treatments, many species of *Pseudomonas* were found, which can be pathogenic to humans or fish. Although BFT can be effective at protecting against diseases (Ekasari et al., 2014; Aguilera-Rivera et al., 2019), including to *A. hydrophila* (Ahmad et al., 2016; Dauda et al., 2018b), it would be interesting to know whether such protection depends on the presence of probiotics.

The use of BFT is often first viewed as a water quality management strategy where toxic nitrogenous waste can be reduced, and although little to no water exchange is normal, it does require more aeration to support the additional respiration by bacteria and keep the bioflocs suspended. In this study, ammonia-N and nitrite-N tended to be higher in the BFT treatments after about two weeks, despite adding more carbon when ammonia-N levels reached or exceeded 1 mg/l. Elevated ammonia-N in BFT systems has also been reported when compared to



**Fig. 3.** Liver histopathology of bluegill, *Lepomis macrochirus*, juveniles in a clear-water system (a, d) or in biofloc systems when using starch (b, e) or sugar (c, f) after 32 days. The majority of livers from fish in the CW treatment showed increased basophilia, and greater staining intensity for glycogen and less vacuolization compared to those in the BFT treatments. Hematoxylin and eosin (a, b, c)  $\times$  200 magnification; and Periodic-acid Schiff (d, e, f);  $\times$  100 magnification.

CW systems (e.g. Azim and Little, 2008; Kamilya et al., 2017; Fleckenstein et al., 2018b). However, the ammonia-N spiked on days 22 and 23 due to a temporary stoppage to aeration as result of rare flooding in the area. This led to mortality in approximately half of the population in the BFT tanks, whereas all fish survived in the CW system due to the DO being substantially higher. This was a highly unfortunate event, but it also underscores the risk of culturing animals with BFT where the duration to reestablish aeration before mass mortalities occurs is much shorter compared to more traditional clear water systems.

It is likely that this temporary stoppage to aeration, along with consistently elevated ammonia-N levels and lower feed intake, contributed to significantly reducing *L. macrochirus* growth in the BFT treatments. This has similarly been suggested as the cause for reduced growth when piracanjuba, *Brycon orbignyanus*, and tilapia, *Oreochromis niloticus*, were cultured with BFT (Sgnaulin et al., 2018; Fleckenstein et al., 2018b). In fact, reduced feeding activities of fish in the BFT tanks were observed within 5 days, compared to those in the CW tanks that actively fed right below the surface. The reduced feeding activity at the surface along with the elevated water turbidity in the BFT tanks made it difficult to ensure fish were being fed to satiation throughout the study. This can be a challenge in any biofloc-based system housing fish to closely monitor activity and health, which have been noted by other researchers as well (Green et al., 2018). Based on the histological examinations, the gills, livers and intestine showed no evidence of damage or restructuring, which seems to indicate the higher turbidity was not inherently detrimental to *L. macrochirus*. It was similarly shown that when using sucrose or glycerol to create biofloc conditions, there were

no adverse effects to the livers of African catfish, *Clarias gariepinus* (Dauda et al., 2017). In contrast, BFT caused some histopathological abnormalities that included interlamellar cell masses on the gills and liver granulomas in largemouth bass, *Micropterus salmoides* that could indicate that excessive suspended solids were being an irritant and inadequate nutrition, respectively (Romano et al., 2020). In this study, it was observed that the livers from the majority of fish in the CW treatment showed increased energy storage based on increased basophilia as well as staining intensity for glycogen while those in the BFT treatments had extensive vacuolization. It may be possible that such vacuolization was the result of non-lipid cytoplasmic vacuolation and appears consistent with fish in the BFT treatments eating less than those in the CW treatment. Another factor may have included more aeration and turbulence in the BFT tanks to necessitate additional energy by the fish to maintain their position in the water, but more research is required to substantiate this suggestion.

Despite these findings indicating BFT is sub-optimal for culturing *L. macrochirus* juveniles, isotope analysis showed that the fish were obtaining some nutrients from bioflocs. Interestingly, however, the carbon source influenced the contribution of nitrogen and carbon, with significantly more nitrogen and carbon being obtained from bioflocs cultured from BFT-Sta and BFT-Sug, respectively. This could be due to differences in the nutritional composition of the bioflocs, but requires more research to elucidate this finding. However, the contribution of carbon (4.1–18.8%) and especially nitrogen by bioflocs (1.4–9.5%) was substantially less in *L. macrochirus* juveniles compared to the shrimps *Litopenaeus stylirostris* at 39.8 % and 36.9 %, respectively (Cardona



et al., 2015) and *L. vannamei* at 27.7 and 58.5 %, respectively (Ray and Lotz, 2017). It was also found that bioflocs could contribute up to 25 % of nitrogen in *O. niloticus*, compared to a normal ration of commercial food (Avnimelech and Kochba, 2009). Due to the similarities in feeding between *O. niloticus* and *L. macrochirus* further research should explore ways to better enhance the nutritional contribution of bioflocs to *L. macrochirus*. Perhaps with more time, the fish would be able to assimilate a larger portion of the carbon and nitrogen in their tissues from the biofloc.

Inferences on the productivity of *L. macrochirus* to BFT cannot be confidently made due to the high mortality event. However, there does appear to be great potential for the culture of *L. macrochirus* with BFT based on bioflocs providing additional supplemental nutrition. Further research could focus on culturing *L. macrochirus* on a longer time frame until market size as well as the bioeconomic analysis of BFT compared to clearwater systems. Such research could provide greater flexibility to the culture methods, site selection and grow-out/maintenance of *L. macrochirus* to a wider population of farmers.

## 5. Author statement

Hayden Fischer: conducted experiment including measuring water quality and preparing samples for later analysis

Nicholas Romano: provided funding/supervision, performed histology, primary writer, assisted with data analysis and final sampling

Nilima Renukdas: performed all bacterial analysis and provided technical assistance

Nathan Egnew: assisted in each aspect of this study, especially for biochemical analysis

Amit Kumar Sinha: assisted with water quality analysis, biochemical analysis, final sampling and editing this manuscript

Andrew Ray: provided valuable manuscript editing as well as technical assistance

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests of personal relationships that could have appeared to influence the work reported in this paper.

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## References

- AGFC (Arkansas Game & Fish Commission), 2020. AGFC Hatcheries Produce More Than 7.4 Million Fish in 2018. <https://www.agfc.com/en/news/2018/12/19/agfc-hatcheries-produce-more-than-7-4-million-fish-in-2018/>.
- Aguilera-Rivera, D., Escalante-Herrera, K., Gaxiola, G., Prieto-Davó, A., Rodríguez-Fuentes, G., Guerra-Castro, E., Hernández-López, J., Chávez-Sánchez, M.C., Rodríguez-Canul, R., 2019. Immune response of the Pacific white shrimp, *Litopenaeus vannamei*, previously reared in biofloc and after an infection assay with *Vibrio harveyi*. *J. World Aquacult. Soc.* 50, 119–136.
- Ahmad, I., Verma, A.K., Rani, A.M.B., Rathore, G., Saharan, N., Gora, A.H., 2016. Growth, non-specific immunity and disease resistance of *Labeo rohita* against *Aeromonas hydrophila* in biofloc systems using different carbon sources. *Aquaculture* 457, 61–67.
- Avnimelech, Y., Kochba, M., 2009. Evaluation of nitrogen uptake and excretion by tilapia in bio floc tanks, using <sup>15</sup>N tracing. *Aquaculture* 287, 163–168.
- Azim, M.E., Little, D.C., 2008. The biofloc technology (BFT) in indoor tanks: water quality, biofloc composition and growth and welfare of Nile tilapia (*Oreochromis niloticus*). *Aquaculture* 283, 29–35.
- Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 249–254.
- Cardona, E., Lorgeoux, B., Geffroy, C., Richard, P., Saulnier, D., Gueguen, Y., Guillou, G., Chim, L., 2015. Relative contribution of natural productivity and compound feed to

tissue growth in blue shrimp (*Litopenaeus stylirostris*) reared in biofloc: assessment of C and N stable isotope ratios and effects on key digestive enzymes. *Aquaculture* 448, 288–297.

- Cardona, E., Gueguen, Y., Magré, K., Lorgeoux, B., Piquemal, D., Pierrat, F., Noguier, F., Saulnier, D., 2016. Bacterial community characterization of water and intestine of the shrimp *Litopenaeus stylirostris* in a biofloc system. *BMC Microbiol.* 16, 157.
- Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., Verstraete, W., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture* 270, 1–14.
- Dauda, A.B., 2020. Biofloc technology: a review on the microbial interactions, operational parameters and implications to disease and health management of cultured aquatic animals. *Rev. Aquacult.* 12, 1193–1210.
- Dauda, A.B., Romano, N., Ebrahimi, M., Karim, M., Natrah, I., Kamarudin, M.S., Ekasari, J., 2017. Different carbon sources affects biofloc volume, water quality and the survival and physiology of African catfish *Clarias gariepinus* fingerlings reared in an intensive biofloc technology system. *Fish. Sci.* 83, 1037–1048.
- Dauda, A.B., Romano, N., Chen, W.W., Natrah, I., Kamarudin, M.S., 2018a. Differences in feeding habits influence the growth performance and feeding efficiencies of African catfish (*Clarias gariepinus*) and lemon fin barb hybrid (*Hypsibarbus wetmorei* ♂ × *Barboides gonionotus* ♀) in a glycerol-based biofloc technology system versus a recirculating system. *J. Aquac. Eng. Fish. Res.* 82, 31–37.
- Dauda, A.B., Romano, N., Ebrahimi, M., Teh, J.C., Ajadi, A., Chong, C.M., Karim, M., Natrah, I., Kamarudin, M.S., 2018b. Influence of carbon/nitrogen ratios on biofloc production and biochemical composition and subsequent effects on the growth, physiological status and disease resistance of African catfish (*Clarias gariepinus*) cultured in glycerol-based biofloc systems. *Aquaculture* 483, 120–130.
- Dundenhoeffer, G.A., Wetzel, J.E., Omara-Alwala, T.R., 2012. Weaning ages of bluegill and redear sunfish fry in indoor recirculating aquaculture systems. *Nor. Amer. J. Aquacult.* 74, 208–213.
- Ekasari, J., Azhar, M.H., Surawidjaja, E.H., Nuryati, S., De Schryver, P., Bossier, P., 2014. Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. *Fish Shellfish Immunol.* 41, 332–339.
- Fleckenstein, L.J., Tierney, T.W., Ray, A.J., 2018b. Comparing biofloc, clear-water, and hybrid recirculating nursery systems (Part II): tilapia (*Oreochromis niloticus*) production and water quality dynamics. *J. Aquac. Eng. Fish. Res.* 82, 80–85.
- Furtado, P., Serra, F.P., Poersch, L.H., Wasielesky, W., 2014. Acute toxicity of hydrogen peroxide in juvenile white shrimp *Litopenaeus vannamei* reared in biofloc technology systems. *Aquac. Int.* 22, 653–659.
- Gou, J., Hong, C.U., Deng, M., Chen, J., Hou, J., Li, D., He, X., 2019. Effect of carbon to nitrogen ratio on water quality and community structure evolution in suspended growth bioreactors through biofloc technology. *Water* 11, 1640.
- Green, B., Rawles, S.D., Webster, C.D., McEntire, M.E., 2018. Effect of stocking rate on growing juvenile sunshine bass, *Morone chrysops* × *M. saxatilis*, in an outdoor biofloc production system. *J. World Aquacult. Soc.* 49, 827–836.
- Hai, N.V., 2015. The use of probiotics in aquaculture. *J. Appl. Microbiol.* 119, 917–935.
- Kamalya, D., Debbarma, M., Pal, P., Kheti, B., Sarkar, S., Singh, S.T., 2017. Biofloc technology application in indoor culture of *Labeo rohita* (Hamilton, 1822) fingerlings: the effects of inorganic nitrogen control, growth and immunity. *Chemosphere* 182, 8–14.
- Karami, A., Romano, N., Hamzah, H., Simpson, S.L., Yap, C.K., 2016. Acute phenanthrene toxicity to juvenile diploid and triploid African catfish (*Clarias gariepinus*): molecular, biochemical, and histopathological alternations. *Environ. Pollut.* 212, 155–165.
- Kolenda, R., Burdukiewicz, M., Schierack, P., 2015. A systematic review and meta-analysis of the epidemiology of pathogenic *Escherichia coli* of calves and the role of calves as reservoirs for human pathogenic *E. Coli*. *Front. Cell. Infect. Microbiol.* 5, 23.
- Liu, H., Li, H., Wei, H., Zhu, X., Han, D., Jin, J., Yang, Y., Xie, S., 2019. Biofloc formation improves water quality and fish yield in a freshwater pond aquaculture system. *Aquaculture* 506, 256–269.
- Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* 64, 795–799.
- Mischke, C.C., Morris, J.E., 1997. Out-of-season spawning of sunfish *Lepomis* spp. In the laboratory. *Progress. Fish-culturist* 59, 297–302.
- Morris, J.E., Mischke, C.C., Garling, D.L., 2002. Sunfish culture guide. North Central Regional Aquaculture Center (NCRAC). NCRAC Culture Series 102.
- Pérez-Fuentes, J.A., Pérez-Rostro, C.I., Hernández-Vergara, M.P., Monroy-Dosta, M.C., 2018. Variation of the bacterial composition of biofloc and the intestine of Nile tilapia *Oreochromis niloticus*, cultivated using biofloc technology, supplied different feed rations. *J. Aquac. Res. Dev.* 49, 3658–3668.
- Ray, A.J., Lotz, J.M., 2017. Shrimp (*Litopenaeus vannamei*) production and stable isotope dynamics in clear-water recirculating aquaculture systems versus biofloc systems. *J. Aquac. Res. Dev.* 48, 4390–4398.
- Reed, P., Francis-Floyd, R., 2011. Red Sore Disease” in Game Fish. University of Florida. IFAS Extension.
- Romano, N., Dauda, A.B., Ikhsan, N., Karim, M., Kamarudin, M.S., 2018a. Fermenting rice bran as a carbon source for biofloc technology improved the water quality, growth, feeding efficiencies, and biochemical composition of African catfish *Clarias gariepinus* juveniles. *Aquac. Res.* 49, 3691–3701.
- Romano, N., Kanmani, N., Ebrahimi, M., Chong, C.M., Teh, J.C., Hoseinifar, S.H., Amin, S.M.N., Kamarudin, M.S., Kumar, V., 2018b. Combination of dietary pre-gelatinized starch and isomaltooligosaccharides improved pellet characteristics, subsequent feeding efficiencies and physiological status in African catfish, *Clarias gariepinus*, juveniles. *Aquaculture* 484, 293–302.
- Romano, N., Surratt, A., Renukdas, N., Monico, J., Egnew, N., Sinha, A.K., 2020. Assessing the feasibility of biofloc technology to largemouth bass *Micropterus salmoides* juveniles: insights into their welfare and physiology. *Aquaculture* 520, 735008.

- Sgnaulin, T., de Mello, G.L., Thomas, M.C., Garcia, J.R.E., de Oca, G.A.R.M., Emerenciano, M.G.C., 2018. Biofloc technology (BFT): an alternative aquaculture system for piracanjuba *Brycon orbignyanus*? Aquaculture 485, 119–123.
- Uchii, K., Matsui, K., Yonekura, R., Tani, K., Kenzaka, T., Nasu, M., Kawabata, Z., 2006. Genetic and physiological characterization of the intestinal bacterial microbiota of bluegill (*Lepomis macrochirus*) with three different feeding habitats. Microb. Ecol. 51, 277–284.
- Verdouw, H., Van Echteld, C.J.A., Dekkers, E.M.J., 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Res. 12, 399–402.
- Wang, N.F., Zhang, T., Zhang, F., Wang, E.T., He, J.F., Ding, H., Zhang, B.T., Liu, J., Ran, X.B., Zhang, J.Y., 2015. Diversity and structure of soil bacterial communities in the Fildes Region (maritime Antarctica) as revealed by 454 pyrosequencing. Front. Microbiol. 6, 1188.
- Yang, M., Wang, J., Han, T., Yang, Y., Li, X., Jiang, Y., 2016. Dietary protein requirement of juvenile bluegill sunfish (*Lepomis macrochirus*). Aquaculture 459, 191–197.
- Zhao, Z., Xu, Q., Luo, L., Wang, C., Li, J., Wang, L., 2014. Effect of feed C/N ratio promoted bioflocs on water quality and production performance of bottom and filter feeder carp in minimum-water exchanged pond polyculture system. Aquaculture 434, 442–448.