

Research Article

# The use of a flocculant additive and its effect on biofloc formation, nitrification, and zootechnical performance during the culture of Pacific white shrimp *Penaeus vannamei* (Boone, 1931) in a BFT system

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**ABSTRACT.** Biofloc technology is a culture system that intensively uses microbial communities. Most bacteria can form aggregates around organic or inorganic surfaces. The high adsorption capacity, surface area, and flocculant properties of clay minerals make these compounds good candidates to stimulate biofloc formation, increase bacterial biomass, and improve nitrification. The objective of this study was to evaluate the addition of clay minerals to biofloc formation and its influence on the microbial community, nitrification, and zootechnical performance of *Penaeus vannamei* cultivated in a biofloc technology system. Experiment 1 involved the following treatments: control (without adding clay minerals), 10, 50, and 100 mg L<sup>-1</sup>, applied daily in the water. The treatments in experiment 2 were: control without product and inoculum, control without product and with inoculum, 50 mg L<sup>-1</sup> clay mineral without inoculum and 50 mg L<sup>-1</sup> clay mineral with inoculum. Both experiments with a density of 400 ind m<sup>-3</sup>. NO<sub>2</sub> levels, total suspended solids, turbidity, differed significantly ( $P < 0.05$ ) between treatments in experiment 1 and zootechnical performance parameters, where higher flocculant concentrations negatively affected these indices. In experiment 2, statistical differences were verified for NH<sub>3</sub> and NO<sub>2</sub> concentrations and the proximate composition of the bioflocs ( $P < 0.05$ ), the addition of the inoculum of bioflocs influenced these results. In summary, the addition of clay minerals influenced biofloc formation, proximal composition, and the zootechnical performance of the cultivated shrimp but did not affect the abundance of microorganisms and nitrification.

**Keywords:** *Penaeus vannamei*; nitrification; clay minerals; bacteria; substrate; aquaculture

## INTRODUCTION

The use of biofloc technology culture systems (BFTs) allows intensive production of aquatic organisms. Several studies have previously highlighted the benefits of bioflocs in shrimp culture (Wasielesky et al. 2006, Kruppenauer et al. 2011, Xu & Pan 2012, Lara et al. 2017). The basic principle of BFT systems is forming a microbial chain that consists of several microorganisms, including microalgae, protozoa, fungi, and bacteria, which attach to debris and organic particles (Avnimelech 2012).

These microbial aggregates can serve as food complement and probiotic sources for cultured animals, decreasing the need for protein in artificial feed. Moreover, aggregates are important for improving and maintaining water quality (Kruppenauer et al. 2014, Kumar et al. 2017). The intensity with which these effects are achieved may depend on the type and microbial composition of the system bioflocs (Samocha et al. 2017).

Due to the introduction of nitrogen into water, either through animal excretion or the provided artificial food, it is necessary to maintain good water quality. In the

BFT system, phytoplankton and bacteria are the most relevant microorganisms to achieve this function. The first group absorbs nitrogen and uses it for growth. The disadvantages of a system with a predominance of microalgae are the high variations in pH and dissolved oxygen throughout the day (Burford et al. 2003). The second group, bacteria, is subdivided into chemoheterotrophic and chemoautotrophic bacteria. The addition of organic carbon sources is essential in this system. Chemoheterotrophs synthesize proteins from ammonia-nitrogen and organic carbon, and for this to occur effectively, the carbon: nitrogen ratio must be appropriate for their use (Burford et al. 2003, Hargreaves 2013). Ebeling et al. (2006) determined that 6 g of carbon is required to convert 1 g of ammonia into bacterial biomass.

The other group of bacteria is chemoautotrophic bacteria, which metabolize ammonia into less toxic compounds (Hagopian & Riley 1998). Unlike microalgae and chemoheterotrophic bacteria, which have much faster ammonia cycling, chemoautotrophic bacteria are responsible for the nitrification process and the conversion of most of the nitrogen that enters an aquaculture system. Through ammonia-oxidizing bacteria (AOB), ammonia is metabolized to nitrite, and through nitrite-oxidizing bacteria (NOB), nitrite is metabolized to nitrate, the latter being the least toxic form. These bacteria have a slower growth ratio than chemoheterotrophic bacteria (Ebeling et al. 2006). Nitrifying bacteria use inorganic carbon for their development, and this whole process is limited by temperature, pH, dissolved oxygen, and nitrogen concentration (Timmons et al. 2002).

Bacteria, both in the natural environment and in a culture system, live in aggregate forms around an organic surface (Grossart 2010) but can also use inorganic particles (minerals) as substrates (Lind et al. 1997, Attramadal et al. 2012). Their abundance is significantly higher in aggregates than in suspension (Prosser 2007). Increasing the surface area for the microbial community development as a source of nutrients for its growth can stimulate the formation of these aggregates.

Due to their high specific surface area, sorption capacity, and ion exchange (Mello et al. 2011, Mueller 2015), clay minerals can become a suitable substrate, contributing to an increase in bacterial biomass. Moreover, nitrifying bacteria have their highest cellular activity when linked to surfaces that attract  $\text{NH}_4^+$  and  $\text{NO}_2^-$  ions, as in clay minerals (Prosser 2007). Stotzky (1966), Lind et al. (1997), and Mueller (2015) reported the use of clay minerals for bacterial aggregation and their interaction with these inorganic particles. In the BFT system, these aggregates are suspended due to the

mechanical movement caused by aeration, which is highly important for maintaining water quality and feeding cultured animals. As aggregation increases, the sinking ratio increases, and these bacteria are no longer suspended (Alldredge & Silver 1988).

The use of clay minerals has already been applied in the water treatment of industrial waste due to its coagulant capacity, ability to absorb nutrients, flocculation aid, and ability to form aggregates. Aguiar & Novaes (2002) used aluminum silicate to remove heavy metals from industrial waste. Choi & Yun (2002) reported a faster decrease in impurities from an effluent treatment plant with clay minerals. These clay minerals efficiently remove colloidal particles from various industrial effluents due to their ease of forming aggregates.

In aquaculture, particularly in biofloc systems, there is little information on inorganic sources used as substrate and flocculant agents, especially clay minerals. Thus, the metabolization of compounds may prove to be more efficient and, consequently, increase the animal's zootechnical performance. In this context, the objective of this study was to analyze the effect of supplementation with a mineral clay compound on the formation of bioflocs, microbial community, the quality of water, and the zootechnical performance in cultivated shrimp. This technique can guarantee the maintenance of water quality with increased bacterial biomass production.

## MATERIALS AND METHODS

### Culture conditions

The present study was conducted at the Marine Station of Aquaculture of the Federal University of Rio Grande (FURG), Institute of Oceanography, Brazil. *Penaeus vannamei* juveniles used in this study were obtained from the commercial hatchery Aquatec®-RN, Brazil, in the nauplius stage. The shrimp were cultured at the Aquaculture Marine Station throughout the hatchery and nursery stages until reaching the sizes used to start the experiments.

### Experimental design

Before juvenile storage, each experimental unit was filled with filtered seawater pumped from Casino Beach, Rio Grande, RS, Brazil. The water used was previously treated with  $10 \text{ mg L}^{-1}$  sodium hypochlorite solution for the possible elimination of microorganisms, and ascorbic acid powder at a concentration of  $1.0 \text{ mg L}^{-1}$  was used for the residual chlorine removal. Water losses due to evaporation were replaced with chlorinated and dechlorinated freshwater. The

experimental units were protected with a cover that reduced light intensity by 50% and a heater to keep the temperature constant to control and prevent temperature and light intensity fluctuations during the culture period. An Aero-tube<sup>®</sup> aeration system consisting of a pair of microperforated hoses powered by a 2HP power blower was installed in all experimental units. The tanks used in the experiments were circular-shaped and made of polyvinyl chloride material with 200 L. The stocking density used was 400 ind m<sup>-3</sup> in both experiments (80 ind per 200 L tank).

### Experiments

This study was conducted in two complementary experiments. A 5% fraction of biofloc inoculum was used in both experiments, according to Krummenauer et al. (2014). The inoculum came from an ongoing culture from the Marine Aquaculture Station grow-out units. The inoculum was added to the water before shrimp storage, except in treatments without inoculum inclusion. In experiment 1, 1.9 ± 0.57 g juvenile shrimp were stocked with the following treatments: 1) control without the addition of flocculant (Ctrl), 2) 10 mg L<sup>-1</sup> flocculant (T1), 3) 50 mg L<sup>-1</sup> flocculant (T2), and 4) 100 mg L<sup>-1</sup> flocculant (T3). The product was applied to the water daily. This experiment lasted 37 days. For experiment 2, treatments were applied as follows: 1) control without inoculum of bioflocs (C), 2) control with an inoculum of bioflocs (CI), 3) flocculant without inoculum of bioflocs (F), and 4) flocculant with an inoculum of bioflocs (FI). The concentration of flocculant agent used in this experiment was 50 mg L<sup>-1</sup> in the F and FI treatments. The product was applied to the water daily. This experiment lasted 28 days. The initial weight of the shrimp was 4.08 ± 0.81 g. All treatments had three replicas each in all experiments (12 tanks/each).

### Characterization of flocculant additive

Both experiments' additive used for flocculation (Inve Aquaculture) was a modified clay mineral compound and an organic carbon fraction. The flocculant was in powder form, and before being added to the tank, the compound dosage was diluted in a sample of water from the culture tank itself. Its ingredients and percentage of inclusion are described in Table 1.

### Biofloc formation

The carbon: nitrogen ratio of water was maintained at 15:1 according to the methodology proposed by Avnimelech (1999) and Ebeling et al. (2006) to stimulate biofloc formation. Organic fertilization was performed using sugar cane molasses with 37% carbon, calculated according to Ebeling et al. (2006). In addition,

**Table 1.** Composition of the flocculant additive.

Ingredient	Inclusion (%)
Aluminum silicates (Al <sub>2</sub> SiO <sub>5</sub> )	50.00
Wheat (wheat powder)	20.00
Iron sulfate Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub>	10.00
Magnesium sulfate (MgSO <sub>4</sub> )	7.50
Calcium sulfate (CaSO <sub>4</sub> )	7.50
Manganese sulfate (MnSO <sub>4</sub> H <sub>2</sub> O)	5.00
Total	100.00

once a week, a commercial probiotic (INVE<sup>®</sup> PROW) was used at a proportion of 1.0 ppm to contribute to water quality maintenance in all treatments in both experiments.

### Monitoring of water quality parameters

Temperature, dissolved oxygen (YSI<sup>®</sup> pro 20), and pH (pH meter<sup>®</sup> FE 20/FG2) were monitored twice daily. Likewise, total ammoniacal nitrogen (T-AN) (N-(NH<sub>3</sub><sup>+</sup>NH<sub>4</sub><sup>+</sup>)) and nitrite-nitrogen (N-NO<sub>2</sub><sup>-</sup>) were monitored daily following the methodology of UNESCO (1983) and Strickland & Parsons (1972), respectively. When T-AN levels exceeded 1 mg L<sup>-1</sup>, organic carbon was applied to maintain a C:N ratio of 6:1 (Ebeling et al. 2006). When NO<sub>2</sub>-N levels exceeded the recommended safety level for the species (Lin & Chen 2003), a water exchange of 20% of the useful volume of the tank was carried out. Salinity was checked twice a week (Hach<sup>®</sup> HQ40 d). Alkalinity was analyzed following the APHA 2012 methodology every three days. When the alkalinity values were below 150 mg L<sup>-1</sup> CaCO<sub>3</sub> or when the pH was below 7.3, corrections were made with the application of hydrated lime according to the methodology proposed by (Furtado et al. 2011, Zhang et al. 2017).

Turbidity was determined by a turbidity meter (Hach<sup>®</sup> 2100P) once a week. Nitrate (N-NO<sub>3</sub><sup>-</sup>) and phosphate (P-PO<sub>4</sub><sup>3-</sup>) were checked every week using the methodology proposed by Strickland & Parsons (1972). For analysis of total suspended solids (TSS), water samples were filtered through GF50-A glass fiber filters (pore: 0.7 μm; diameter: 47 mm. Macherey-Nagel) using a vacuum suction pump (Primatec<sup>®</sup>). According to Gaona et al. (2011), a clarification method was performed using decanters, where the water from each tank was pumped to that decanter and returned by gravity to the tank.

### Feeding management and growth performance

In both experiments, shrimp were fed with commercial feed containing 38% protein content twice a day. (Potimar GUABI<sup>®</sup>). Feeding ratios were adjusted according to the methodology suggested by Garza de Yta et al. (2004). Weekly, 20 shrimp from each tank

were weighed (biometry) to assess weekly weight gain and growth parameters and thus adjust feeding ratios. According to the following calculations, weekly weight gain, apparent food conversion, survival, and productivity were evaluated weekly. Weight gain (WG) = (final weight - initial weight) / number of weeks of culture. Apparent feed conversion rate (FCR) = feed offered / biomass increase. Survival: (final biomass / individual mean weight) / number of individuals stocked) × 100. Productivity = (final biomass - initial biomass) / tank volume.

### Microbial community assessment

Samples of 18 mL of water were collected once a week from each experimental unit and fixed with 2 mL of 40% formalin to estimate the microorganisms present in the culture water. Samples were filtered through polycarbonate membrane filters (Nuclepore, pore: 0.2 µm and diameter: 25 mm) and stained with 1% acridine orange at a concentration of 1 µg mL<sup>-1</sup> (Hobbie et al. 1977) to quantify bacterial abundance. At the end of the staining process, the slides were photographed using a photographic camera connected to an Axioplan-Zeiss epifluorescence microscope at 1000x magnification, and 30 random fields were counted. An inverted microscope model Olympus IX51 with a final magnification of 200x was used to quantify protozoa and other groups. Aliquots of 2.1 mL were used, and 20 random fields were counted (Utermohl 1958). Counting was performed at the Phytoplankton and Marine Microorganisms Ecology Laboratory of the Institute of Oceanography, FURG, Brazil.

### Proximate composition of bioflocs

At the end of experiment 2, bioflocs were collected from each experimental unit and filtered through a 50 µm filter for proximal composition analysis. Samples were dried in an oven at 60°C for 72 h. For the quantification of ashes, bioflocs samples were incinerated in a muffle at 600°C for 5 h. The gray value was obtained by determining the weights before and after incineration. Crude protein analysis was performed according to the Kjeldahl method, and the ether extract content was obtained using the Soxhlet extraction method using petroleum ether as a solvent. The Wendee method was used to quantify the fiber. All analyses were carried out at the Aquatic Organisms Nutrition Laboratory of the Institute of Oceanography (FURG), according to AOAC (2000) methodology protocols.

### Statistical analysis

The present study results were subjected to homoscedasticity (Levene) and normality analysis in the data

distribution (Shapiro Wilk). When the assumptions were not accepted, the values went through mathematical transformations. Then, unidirectional ANOVA ( $\alpha = 0.05$ ) was applied in experiment 1, and factorial ANOVA ( $\alpha = 0.05$ ) in experiment 2. In both experiments, Tukey's *post-hoc* test was used when significant differences were found ( $P < 0.05$ ) (Zar 2010).

## RESULTS

### Experiment 1

#### Physical and chemical parameters of water

The mean values ( $\pm$  standard deviation; SD) of water physical and chemical parameters are shown in Table 2. Variables such as dissolved oxygen, nitrite (NO<sub>2</sub>-N), turbidity, and the total suspended solids (TSS) showed significant differences among treatments ( $P < 0.05$ ). The other water quality parameters (temperature, pH, alkalinity, salinity, ammoniacal nitrogen, nitrate, and phosphate) were not significantly different ( $P > 0.05$ ).

The temperature varied, remaining in the range of 22-32°C throughout the experiment. The pH varied from 7.5 to 8.5. Alkalinity ranged from 110-270 mg L<sup>-1</sup>. The content of total ammonia nitrogen (T-AN) varied between 0.02 and 3 mg L<sup>-1</sup>, presenting its highest concentration in the Ctrl treatment on the ninth day of the experiment (Fig. 1a). Nitrate concentrations accumulated throughout cultivation in all treatments, reaching 69.89 mg L<sup>-1</sup> in treatment T2 (Fig. 1c). The phosphate content ranged from 0.25 to 6.5 mg L<sup>-1</sup>.

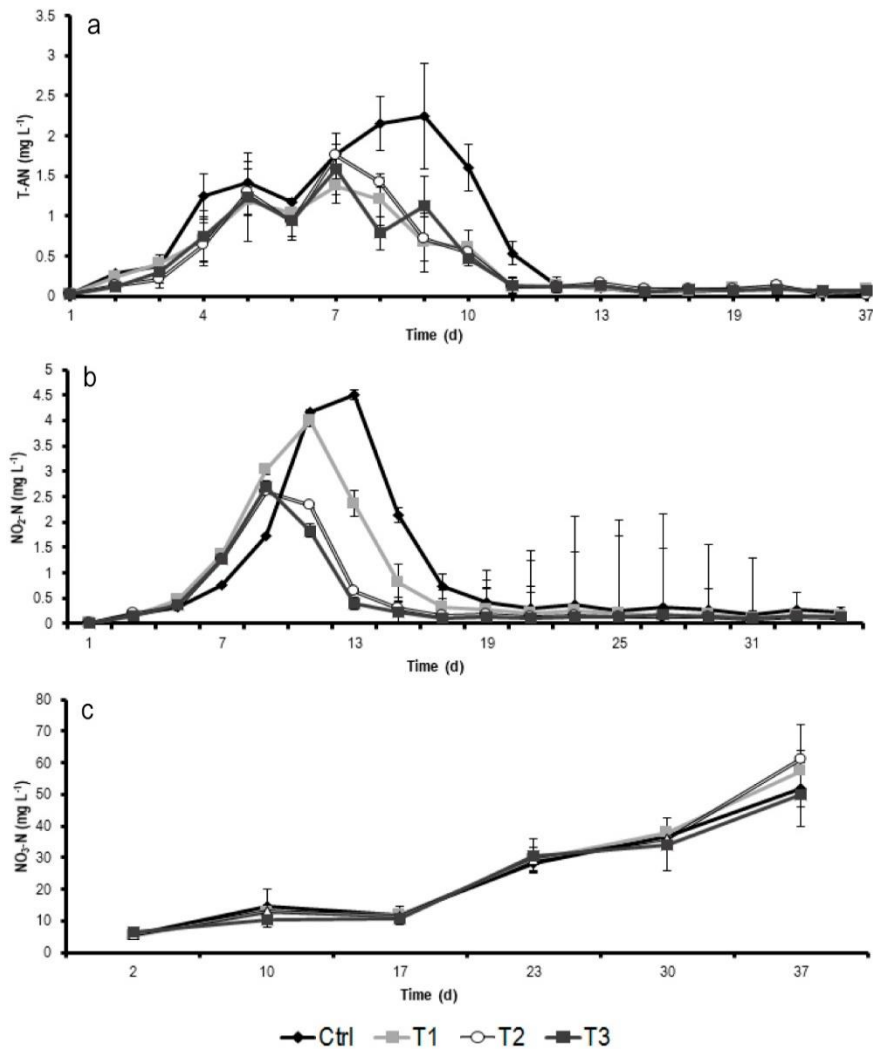
Among the parameters that showed significant differences, nitrite varied from 0.01-6 NO<sub>2</sub>-N mg L<sup>-1</sup>, reaching the maximum concentration on the day 13 of the experiment in the control treatment. In treatments with the addition of flocculant, the highest concentrations were reached on the day 10 of the experiment, but they were lower than the concentrations found in the control treatment (Fig. 1b). The TSS and turbidity concentrations showed similar trends throughout the study (Fig. 2b). Turbidity ranged from 7.86-751 NTU. Treatment T2 showed the highest average turbidity concentration. Likewise, for TSS, treatments with greater inclusion of flocculants showed higher this parameter levels than the control (Ctrl). The maximum level of TSS was reported for treatment T3, with 1030 mg L<sup>-1</sup> TSS on the last day of culture (Fig. 2a).

#### Microbial community assessment

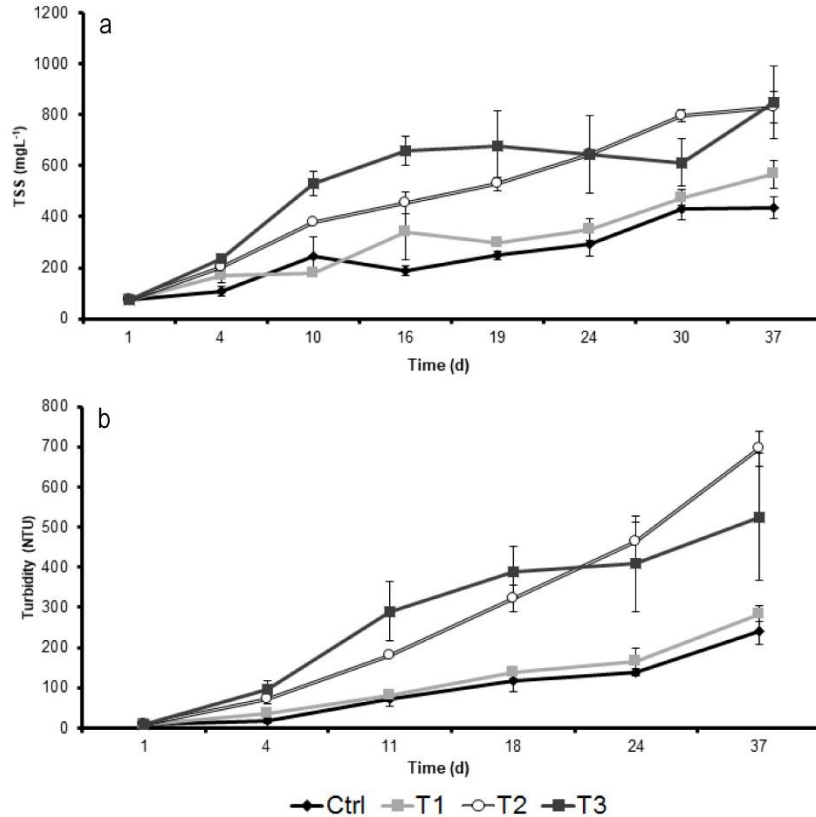
The microorganisms identified in the culture water belong to three groups: bacteria, protozoa, and rotifers. The first group identified coccoid, filamentous, bacilli, and vibrio bacteria. The identified protozoa were ciliated, flagellate, and amoeba, and the third group were rotifers. The microbial density count was separated into three stages of the experiment: initial (1

**Table 2.** Mean values and standard deviation of the physical and chemical parameters of the water in the treatments: control (Ctrl), 10 mg L<sup>-1</sup> (T1), 50 mg L<sup>-1</sup> (T2), and 100 mg L<sup>-1</sup> (T3) (concentrations of flocculant) during the 37 days of culture. Different letters superscripted in the same line indicate significant differences between treatments (*P* < 0.05).

Parameter	Treatment			
	Ctrl	T1	T2	T3
Temperature (°C)	28.47 ± 1.42	28.49 ± 1.01	28.23 ± 1.30	28.21 ± 1.56
Dissolved oxygen (mg L <sup>-1</sup> )	5.64 ± 0.31 <sup>a</sup>	5.68 ± 0.27 <sup>ab</sup>	5.76 ± 0.33 <sup>b</sup>	5.72 ± 0.34 <sup>ab</sup>
pH	7.98 ± 0.14	7.97 ± 0.11	7.98 ± 0.11	7.97 ± 0.11
Salinity	33.24 ± 0.54	33.15 ± 0.51	33.57 ± 0.61	33.19 ± 0.76
Alkalinity (mg of CaCO <sub>3</sub> L <sup>-1</sup> )	174.00 ± 37.50	162.89 ± 27.21	164.67 ± 26.21	155.33 ± 19.50
T-AN (mg L <sup>-1</sup> )	0.71 ± 0.80	0.43 ± 0.47	0.45 ± 0.54	0.43 ± 0.52
NO <sub>2</sub> <sup>-</sup> N (mg L <sup>-1</sup> )	1.01 ± 1.44 <sup>a</sup>	0.85 ± 1.32 <sup>ab</sup>	0.52 ± 0.82 <sup>b</sup>	0.49 ± 0.82 <sup>b</sup>
NO <sub>3</sub> <sup>-</sup> N (mg L <sup>-1</sup> )	24.83 ± 16.31	25.74 ± 18.21	26.09 ± 19.43	23.75 ± 16.76
PO <sub>4</sub> <sup>3-</sup> P (mg L <sup>-1</sup> )	1.77 ± 1.69	2.04 ± 1.40	2.37 ± 1.46	2.18 ± 1.08
Turbidity (NTU)	98.84 ± 81.54 <sup>a</sup>	118.79 ± 93.41 <sup>ab</sup>	290.31 ± 238.15 <sup>ab</sup>	285.98 ± 201.86 <sup>b</sup>
Total suspended solids (mg L <sup>-1</sup> )	253.13 ± 130.13 <sup>a</sup>	306.88 ± 161.59 <sup>ab</sup>	480.17 ± 253.06 <sup>b</sup>	536.25 ± 257.77 <sup>b</sup>



**Figure 1.** Mean values of a) total ammonia nitrogen, b) nitrite, and c) nitrate throughout the rearing of *Penaeus vannamei* in the biofloc technology culture system exposed to different flocculant concentrations. In the four experimental treatments: control (Ctrl), 10 mg L<sup>-1</sup> (T1), 50 mg L<sup>-1</sup> (T2), and 100 mg L<sup>-1</sup> (T3) during the 37 days of rearing. The vertical bars indicate the standard deviation.



**Figure 2.** Mean values of a) total suspended solids (TSS), and b) turbidity throughout the rearing of *Penaeus vannamei* in biofloc technology culture system exposed to different concentrations of flocculant. In the four experimental treatments: control (Ctrl), 10 mg L<sup>-1</sup> (T1), 50 mg L<sup>-1</sup> (T2), and 100 mg L<sup>-1</sup> (T3) during the 37 days of rearing. The vertical bars indicate the standard deviation.

day of experiment), middle (18 days of experiment), and final (37 days of experiment). The abundances of coccoid bacteria, filamentous bacteria, bacilli, vibrios, ciliates, flagellates, amoebae, and rotifers were not significantly different between treatments at the three experimental times analyzed ( $P > 0.05$ ). The abundance of microorganisms in the three cultures was shown (Fig. 3).

### Zootechnical performance of shrimp

The application of the flocculant agent in treatments T2 and T3 led to significantly lower average body weight (g), weekly growth (g week<sup>-1</sup>), biomass (g), apparent feed conversion ratio, and productivity (kg m<sup>-3</sup>) compared to Ctrl and T1 ( $P < 0.05$ ). No significant differences ( $P > 0.05$ ) were observed in survival for all treatments (Table 3).

### Experiment 2

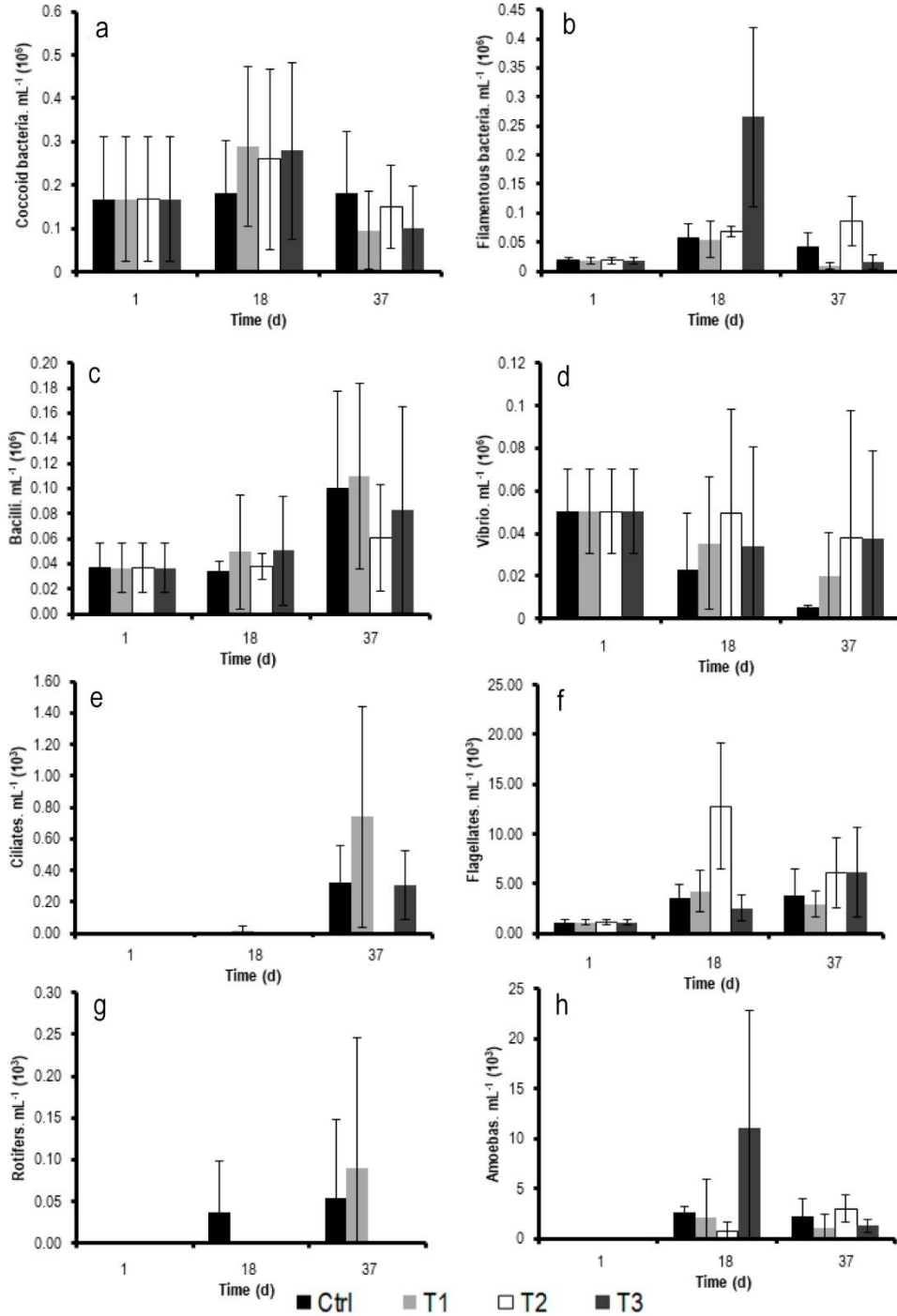
#### Physical and chemical parameters of water

The mean values ( $\pm$  SD) of the physical and chemical parameters of the water are shown (Table 4). During the

experiment, the water quality values (temperature, dissolved oxygen, pH, alkalinity, salinity, nitrate, phosphate, turbidity, and TSS) were similar between the three treatments, with no significant differences ( $P > 0.05$ ). Significant differences were found in total ammoniacal nitrogen and nitrite parameters ( $P < 0.05$ ).

The temperature varied but remained between 22.3 and 31.3°C during the experiment. The dissolved oxygen concentration ranged 5.2-7.5 mg L<sup>-1</sup>, pH 7.4-8.1, alkalinity 100-260 mg L<sup>-1</sup>, and salinity 27.5-31.1 during 28 days of cultivation. The concentration of nitrate accumulated over the experimental time, remaining in the range of 0.01-47.9 mg L<sup>-1</sup>, the latter being obtained on the last day of the experiment with the FI treatment. The phosphate ranged within 0.01-7.2 mg L<sup>-1</sup>. Turbidity ranged from 3.26 to 646 NTU, and TSS ranged within 40-905 mg L<sup>-1</sup>. The turbidity parameters and TSS showed the highest concentrations in the F and FI treatments, in which the flocculant was added.

The concentration of T-AN varied at 0.01-7.8 mg L<sup>-1</sup> between treatments. The highest values found were in treatments C and F (Fig. 4a), whereas the FI treatment



**Figure 3.** Mean values of a) coccoid bacteria, b) filamentous bacteria, c) bacilli, d) vibrio, e) ciliates, f) flagellates, g) rotifers, and h) amoebas throughout the culture of *Penaeus vannamei* in biofloc technology culture system exposed to different concentrations of flocculant. In the four experimental treatments: control (Ctrl), 10 mg L<sup>-1</sup> (T1), 50 mg L<sup>-1</sup> (T2), and 100 mg L<sup>-1</sup> (T3) during the 37 days of rearing. The vertical bars indicate the standard deviation.

had the lowest average parameter concentration. The CI and FI treatments showed significantly different from the C and F treatments ( $P < 0.05$ ). The nitrite concentration between treatments ranged from 0.01-28 mg L<sup>-1</sup>, where the highest concentrations were found

in treatments C and F (Fig. 4b), differing significantly from treatments CI and FI ( $P < 0.05$ ). In treatments C and F, water changes were made to reduce nitrite concentrations.

**Table 3.** Mean values and standard deviation of the zootechnical performance of *Penaeus vannamei* shrimp cultivated in biofloc technology culture system with the addition of flocculant in treatments: control (Ctrl), 10 mg L<sup>-1</sup> (T1), 50 mg L<sup>-1</sup> (T2), and 100 mg L<sup>-1</sup> (T3) (concentrations of flocculant) during the 37 days of study. Different letters superscripted in the same line show significant differences ( $P < 0.05$ ).

Parameter	Treatment			
	Ctrl	T1	T2	T3
Initial weight (g)	1.90 ± 0.57	1.90 ± 0.57	1.90 ± 0.57	1.90 ± 0.57
Final weight (g)	5.76 ± 1.26 <sup>a</sup>	5.68 ± 1.27 <sup>a</sup>	4.68 ± 0.94 <sup>b</sup>	4.89 ± 1.01 <sup>ab</sup>
Weekly growth (g week <sup>-1</sup> )	0.62 ± 0.33 <sup>a</sup>	0.63 ± 0.20 <sup>a</sup>	0.46 ± 0.25 <sup>b</sup>	0.5 ± 0.30 <sup>ab</sup>
Survival (%)	96.67 ± 4.73	94.58 ± 1.44	95.00 ± 5.73	91.25 ± 3.75
Final biomass (g)	433.88 ± 14.31 <sup>a</sup>	429.92 ± 7.39 <sup>a</sup>	354.86 ± 14.67 <sup>b</sup>	357.62 ± 33.68 <sup>b</sup>
Productivity (kg m <sup>-3</sup> )	2.17 ± 0.07 <sup>a</sup>	2.15 ± 0.04 <sup>a</sup>	1.77 ± 0.07 <sup>b</sup>	1.79 ± 0.17 <sup>b</sup>
Feed conversion ratio	1.49 ± 0.06 <sup>a</sup>	1.50 ± 0.05 <sup>a</sup>	2.02 ± 0.14 <sup>b</sup>	1.92 ± 0.24 <sup>b</sup>

**Table 4.** Mean values and standard deviation of the physical and chemical parameters of the water in the treatments: control without inoculum of bioflocs (C), control with an inoculum of bioflocs (CI), flocculant without inoculum of bioflocs (F), and flocculant with an inoculum of bioflocs (FI) during the 28 days of rearing. Different letters superscripted in the same line indicate significant differences between treatments ( $P < 0.05$ ).

Parameters	Treatment			
	C	CI	F	FI
Temperature (°C)	26.90 ± 1.60	26.38 ± 1.57	26.92 ± 1.57	26.79 ± 1.32
Dissolved oxygen (mg L <sup>-1</sup> )	6.22 ± 0.34	6.15 ± 0.37	6.02 ± 0.41	6.05 ± 0.30
pH	7.79 ± 0.15	7.76 ± 0.16	7.75 ± 0.17	7.77 ± 0.16
Salinity	28.38 ± 0.88	29.05 ± 1.55	28.35 ± 0.85	29.00 ± 1.50
Alkalinity (mg of CaCO <sub>3</sub> L <sup>-1</sup> )	174.36 ± 37.06	162.56 ± 30.53	163.59 ± 27.69	166.41 ± 33.85
T-AN (mg L <sup>-1</sup> )	2.38 ± 2.15 <sup>a</sup>	0.79 ± 0.93 <sup>b</sup>	1.82 ± 1.89 <sup>a</sup>	0.67 ± 0.81 <sup>b</sup>
NO <sub>2</sub> <sup>-</sup> N (mg L <sup>-1</sup> )	7.31 ± 8.17 <sup>a</sup>	2.87 ± 4.03 <sup>ab</sup>	6.25 ± 7.77 <sup>ab</sup>	1.33 ± 1.94 <sup>b</sup>
NO <sub>3</sub> <sup>-</sup> N (mg L <sup>-1</sup> )	11.75 ± 14.79	18.64 ± 17.19	14.85 ± 18.11	21.58 ± 17.66
PO <sub>4</sub> <sup>3-</sup> P (mg L <sup>-1</sup> )	1.42 ± 1.87	1.65 ± 1.89	1.99 ± 2.04	2.14 ± 1.47
Turbidity (NTU)	152.09 ± 113.91	184.20 ± 157.97	239.55 ± 183.56	229.74 ± 188.52
Total suspended solids (mg L <sup>-1</sup> )	389.00 ± 221.08	416.00 ± 235.46	530.67 ± 292.63	497.67 ± 259.74

### Microbial community assessment

Figure 5 shows the abundance of microorganisms at the three analyzed times: initial (1 day of experiment), middle (15 days of experiment), and final (28 days of experiment). The microorganisms identified in the culture water belong to three groups: bacteria, protozoa, and rotifers. Coccoid, filamentous bacteria, bacilli, and vibrios were identified in the first group. The identified protozoa were ciliates, flagellates, and amoebae, and the third group was rotifers. The coccoid and filamentous bacteria showed statistically significant differences in the initial time (Figs. 5a-b). These bacteria showed the greatest abundances in the middle of the experiment. The bacillus concentrations are shown (Fig. 5c). All treatments showed significant differences ( $P < 0.05$ ) at the beginning of the culture, decreasing their abundance at the end of the experiment.

The vibrios, flagellates, ciliate, and rotifers showed no significant differences between treatments, as shown

(Figs. 5d-g). The abundance of amoebae showed significant differences between treatments at the beginning of the culture and showed the highest abundance in the middle of the culture (Fig. 5h).

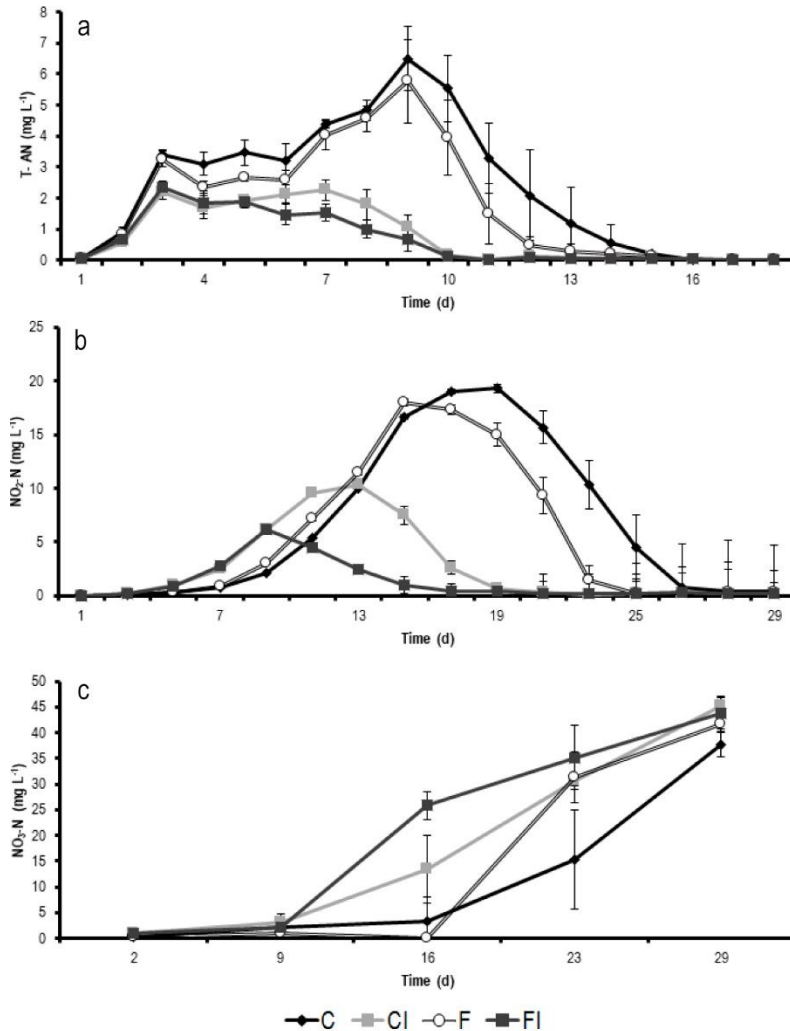
### Proximate composition of bioflocs

The results obtained for the proximate composition of the bioflocs are shown in Table 5. The percentages of protein, lipids, and ash showed significant differences ( $P < 0.05$ ) between treatments. The fiber content was statistically equal for all treatments ( $P > 0.05$ ). The highest percentage of protein and lipids was found in the CI treatment, and the lowest percentages of these nutrients were found in the F treatment. Treatment F obtained a higher percentage of ash, followed by FI treatment.

### Zootechnical performance of shrimp

The results of shrimp growth performance during the 28 days of the experiment are presented in Table 6, with





**Figure 4.** Mean values of a) total ammonia nitrogen (T-AN), b) nitrite and c) nitrate throughout the culture of *Penaeus vannamei* in biofloc technology culture system in the four experimental treatments: control without inoculum of bioflocs (C), control with an inoculum of bioflocs (CI), flocculant without inoculum of bioflocs (F) and flocculant with an inoculum of bioflocs (FI) during the 28 days of rearing. The vertical bars indicate the standard deviation.

no significant differences verified among treatments ( $P > 0.05$ ).

## DISCUSSION

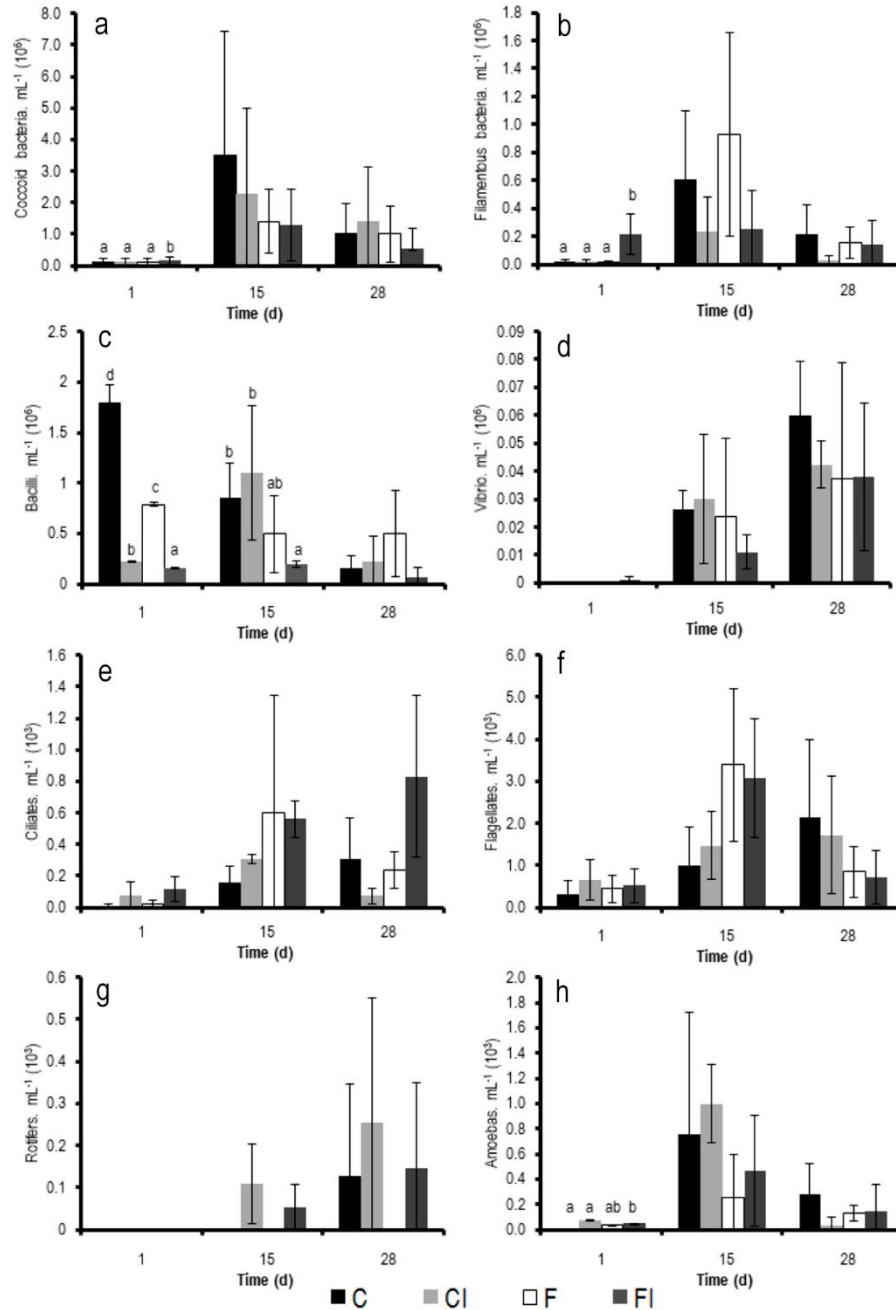
### Water physical and chemical parameters

To ensure that the growth of *Penaeus vannamei* is not affected, the temperature must remain between 24 and 32°C (Van Wyk & Scarpa 1999). In this study, the mean temperature remained above 26°C in both experiments and showed no significant differences between treatments. According to Van Wyk & Scarpa (1999), dissolved oxygen should be kept between 5 and 9 mg L<sup>-1</sup> because it is necessary for animal breathing, which generates energy to metabolize feed nutrients. Despite the significant differences in experiment 1, the

concentrations in both experiments remained above 5 mg L<sup>-1</sup>.

In closed systems, such as BFT, the pH of water during rearing tends to decrease due to decreased alkalinity and increased CO<sub>2</sub> caused by increased biomass and respiration of organisms (Decamp et al. 2007, Furtado et al. 2011). During this study, pH and alkalinity were in accordance with Van Wyk & Scarpa (1999), and Furtado et al. (2011) recommended range for penaeid shrimp.

The removal of ammoniacal nitrogen from water can occur in two ways: heterotrophic and chemoautotrophic mechanisms (Ebeling et al. 2006). The first occurs through heterotrophic bacteria, which transform nitrogen into bacterial biomass. The second occurs through nitrifying bacteria that convert ammonia to less



**Figure 5.** Mean values of a) coccoid bacteria, b) filamentous bacteria, c) bacilli, d) vibrio, e) ciliates, f) flagellates, g) rotifers, and h) amoebas (h) throughout the culture of *Penaeus vannamei* in biofloc technology culture system in the four experimental treatments: control without inoculum of bioflocs (C), control with an inoculum of bioflocs (CI), flocculant without inoculum of bioflocs (F) and flocculant with an inoculum of bioflocs (FI) during the 28 days of rearing. The vertical bars indicate the standard deviation.

toxic compounds. In experiment 1, ammonia levels were equal at all application dosages of the flocculant agent. On the other hand, in experiment 2, the addition of biofloc inoculum influenced the lower concentration found in the treatments. According to Krummenauer et al. (2014), water reuse in the BFT system at an inoculum percentage of 25% of the working tank

volume was already sufficient to achieve a more effective removal of nitrogen compounds. Even though in the present study, the percentage of inoculum was lower (5%) in the CI and FI treatments, the same efficiency in nitrogen removal was observed. Such efficiency can be attributed to nitrifying bacteria in the mature biofloc inoculum. Santos et al. (2019), when

**Table 5.** Mean values and standard deviation of the proximal biofloc composition in the treatments: control without inoculum of bioflocs (C), control with an inoculum of bioflocs (CI), flocculant without inoculum of bioflocs (F), and flocculant with an inoculum of bioflocs (FI) (experiment lasting 28 days). Different letters superscripted in the same line indicate significant differences between treatments ( $P < 0.05$ ). \*Estimated through the difference [100- (PB + L + FB + Ash)]. All analyses were performed with dry matter from the bioflocs.

	Treatment			
	C	CI	F	FI
Crude protein (%)	20.55 ± 3.42 <sup>ab</sup>	23.53 ± 1.35 <sup>a</sup>	14.77 ± 1.54 <sup>b</sup>	19.38 ± 1.78 <sup>ab</sup>
Lipids (%)	0.89 ± 0.37 <sup>ab</sup>	1.63 ± 0.42 <sup>a</sup>	0.34 ± 0.21 <sup>b</sup>	0.65 ± 0.32 <sup>b</sup>
Ashes (%)	57.33 ± 2.06 <sup>ab</sup>	53.96 ± 0.55 <sup>a</sup>	66.77 ± 1.21 <sup>c</sup>	62.44 ± 2.94 <sup>bc</sup>
Raw fiber (%)	5.77 ± 0.24	6.74 ± 0.65	5.26 ± 1.10	5.55 ± 0.69
Non-nitrogen extracted*(%)	15.46 ± 6.44	14.15 ± 2.59	12.85 ± 1.10	11.98 ± 0.94

**Table 6.** Mean values and standard deviation of the zootechnical performance of *Penaeus vannamei* shrimp in the treatments: control without inoculum of bioflocs (C), control with an inoculum of bioflocs (CI), flocculant without inoculum of bioflocs (F), and flocculant with an inoculum of bioflocs (FI) during the 28 days of rearing. Different letters superscripted in the same line indicate significant differences between treatments ( $P < 0.05$ ).

Parameter	Treatment			
	C	CI	F	FI
Initial weight (g)	4.08 ± 0.81	4.08 ± 0.81	4.08 ± 0.81	4.08 ± 0.81
Final weight (g)	7.32 ± 0.15	7.30 ± 0.15	7.54 ± 0.45	7.57 ± 0.06
Weekly growth (g week <sup>-1</sup> )	0.81 ± 0.31	0.80 ± 0.25	0.87 ± 0.32	0.87 ± 0.27
Survival (%)	92.50 ± 3.31	96.67 ± 3.15	94.17 ± 6.88	97.08 ± 2.60
Final biomass (g)	541.67 ± 30.14	563.31 ± 5.33	575.18 ± 22.32	588.30 ± 21.51
Productivity (kg m <sup>-3</sup> )	2.70 ± 0.15	2.80 ± 0.03	2.89 ± 0.11	2.90 ± 0.11
Feed conversion ratio	1.97 ± 0.17	1.89 ± 0.06	1.83 ± 0.20	1.73 ± 0.08

evaluating the nitrification process with the addition of biofloc inoculum, obtained results showing that the addition of inoculum is a factor that can decrease the concentration of nitrogen byproducts in the culture system.

Nitrite is an intermediate compound of nitrification and is the result of ammonia metabolization, and the effect of its toxicity varies according to salinity (Lin & Chen 2003, Ramírez-Rochin et al. 2017). In experiment 1, the addition of an already mature biofloc inoculum contributed to maintaining the levels in an acceptable range for shrimp culture. The addition of argilomineral at ratios of 50 mg L<sup>-1</sup> (T2) and 100 mg L<sup>-1</sup> (T3) resulted in a lower concentration of nitrite compared to the control (Ctrl). The addition of clay also influenced the concentration of total suspended solids in the system. Similarly, higher concentrations of TSS were followed by a more efficient nitrification process, corroborating the study conducted by Gaona et al. (2016).

Clay minerals have a high specific surface area, representing surfaces for bacterial adherence. Jiang et al. (2007) observed higher adsorption ratios of bacteria in the presence of clay minerals in the soil. Clay mineral colloids and flakes formed by the presence of this

flocculant may have constituted an efficient substrate for nitrifying bacteria, which already were present in the biofloc inoculum, to fix and carry out the nitrification process more efficiently. Koops & Pommeranig-Roser (2002) reported that some species of nitrifying bacteria in aquatic environments could colonize existing flocs and biofilms. In addition, clay minerals can absorb some nutrients, including nitrite, resulting in lower nutrient concentrations in the water. Öztürk & Köse (2008) reported using clay minerals to remove nitrite from drinking water.

In experiment 2, inoculum addition (CI and FI) treatments showed lower nitrite concentrations than treatments without inoculum addition (C and F). The presence of inoculum was a factor that influenced these results. However, contrary to experiment 1, the addition of clay minerals did not influence this parameter. Treatment with clay minerals + inoculum did not differ significantly from the control + inoculum treatment. The use of clay minerals positively influenced nitrification in experiment 1, which was not found in experiment 2, indicating that the BFT system's nitrification process is dynamic. Nitrite-oxidizing bacteria are sensitive to environmental factors such as ammonia,

temperature, pH, oxygen, and nitrite (Antoniou et al. 1990, Kim & Kim 2006, Munz et al. 2011, Isnansetyo et al. 2014), and variations in these parameters can influence their growth. Another influencing factor may be the inoculum maturation time between the two experiments, which may have affected the microbial community.

In this system, nitrate and phosphate accumulate during rearing (Samocha et al. 2010, Da Silva et al. 2013). In this study, nitrate and phosphate concentrations were not significantly different between treatments in both experiments and were below the lethal concentrations (Tsai & Chen 2002, Furtado et al. 2015 for nitrate). Phosphate toxicity is poorly reported. Samocha et al. (2017) reported that on commercial farms, concentrations of 32 mg L<sup>-1</sup> phosphate were found in the rearing water without showing apparent damage to the shrimp.

Concentrations of TSS increase due to high storage densities and water reuse from other cycles but, bacterial biomass primarily accumulates because of reduced water exchange (Krummenauer et al. 2011, 2014). Suspended solids can serve as a substrate for bacterial fixation and improve nitrification. On the other hand, high concentrations can damage shrimp, causing clogging of the gills (Schveitzer et al. 2013). In experiment 1, the addition of clay minerals favored an increase in TSS in the water, especially in the treatments with 50 (T2) and 100 mg L<sup>-1</sup> (T3) flocculant agents. Because clay minerals have adsorptive properties, in addition to the microorganisms present in the water, the feces and feed may have been trapped in the clay molecules, thus increasing the concentrations of TSS. Sharma (2003) and Li et al. (2011) used clay minerals for the adsorption and removal of contaminants from residual effluents, which resulted in a high ratio of adsorption of contaminants by the colloids of the clay. Even with high concentrations of TSS, the survival ratio was not affected. The turbidity presented the same behavior as the TSS in this experiment. Increasing organic matter during culture may contribute to increased turbidity.

In experiment 2, the TSS concentrations did not differ significantly between the treatments; however, in treatments with the addition of clay, TSS concentrations increased more rapidly than treatments without compost. This behavior also occurred in experiment 1. These results indicate the power of the clay minerals as flocc formers.

### Microbial community

Understanding microorganisms dynamics, metabolism, and ecology is fundamental in a culture system based on a microbial chain. In addition to contributing to the

diet (Burford et al. 2003), microorganisms also metabolize some compounds toxic to cultured species (Ebeling et al. 2006, Hargreaves 2013). In experiment 1, there were no significant differences between treatments concerning the abundance of microorganisms. In this experiment, nitrification took place more efficiently. Although the bacteria were not identified, the addition of clay minerals probably contributed to the increase in nitrifying bacteria in the culture system.

Clay mineral particles have a high specific surface area and can serve as a bacterial substrate. Xia et al. (2016) reported that the nitrification ratio could be improved by increasing the specific surface area for bacterial fixation. In addition, it is believed that fixing these bacteria on solid surfaces improves their development (Xia et al. 2009) because this type of surface possibly provides more stable physical support so that they can grow (Kholderbarin & Oertl 1977).

In experiment 2, coccoid bacteria, filamentous bacteria, bacilli, and amoebae were differentiated only at the beginning of the culture, unlike experiment 1; however, in this experiment, the addition of clay minerals did not influence the nitrification process. The result of the second experiment corroborates the findings of Zadinelo et al. (2016), who determined that the use of clay minerals in recirculation systems to decrease the ammonia concentration did not consistently attenuate the concentrations of this nutrient and did not contribute to better nitrification results.

According to Tietjen et al. (2005), the interaction of clay minerals and bacterial production can be variable. It may increase or decrease depending on the bioavailability of organic nutrients supplied and the adsorption processes performed by the clay particles. In addition, applying this product in aquaculture systems to help bacterial productivity is very rare, and further studies are needed to assess the interaction of microorganisms with clay minerals in this type of system.

### Proximate composition of bioflocs

In addition to contributing to the maintenance of water quality, bioflocs can also be used as a food supplement for shrimp (Burford et al. 2004, Kuhn et al. 2010). Their quality can be influenced by the size of the floc (Ekasari et al. 2014), the incidence of light and microbial community (Reis et al. 2019), and different sources of carbon (Wei et al. 2016). The addition of clay minerals affected the proximate composition of the biofloc in experiment 2. The percentages of protein, lipids, and ash differed between treatments. In the treatments with the addition of clay minerals (F and FI), the protein and lipid contents were lower than those in treatments that

were not supplemented with the flocculant agent (C and CI).

However, ash content was higher in these treatments -the factor attributed to the addition of minerals results in a higher percentage of inorganic matter. Protein and lipid values in all treatments were below those found by Wang et al. (2016) and Becerril-Cortés et al. (2018); 24-26 and 42.01% for protein and 3-4 and 2.50% for lipids, respectively, when fertilized with the same source of organic carbon as in that study. Hargreaves (2013), Da Silva et al. (2013), and Reis et al. (2019) found similar values of proteins, lipids, and ash to those found in treatments without the addition of clay minerals. The crude fiber content did not show significant differences between treatments and was close to that observed by Becerril-Cortés et al. (2018). The addition of clay minerals led to an increase in the content of inorganic matter in the flocs, which reduced the percentage of protein and lipids available for shrimp.

### Zootechnical performance

In experiment 1, treatments with higher concentrations of clay minerals (T2 and T3) showed lower results in weekly growth, apparent feed conversion, final weight, biomass, and productivity than the control (Ctrl) and T1 treatments. Although in this experiment, nitrification in the T2 and T3 treatments was more efficient. Moreover, the nitrite concentration was significantly lower than in the other treatments, and it did not lead to improved zootechnical performance, contrary to the results of Krummenauer et al. (2014), which showed that more efficient nitrification could contribute to better zootechnical performance. The high indices of zootechnical performance in the control treatment were similar to those found by Krummenauer et al. (2011) and Reis et al. (2019) for BFT systems with stocking densities similar to those in this study.

High concentrations of TSS did not affect shrimp survival, but it is likely that these high levels affected feed consumption and hence shrimp growth. Schweitzer et al. (2013) reported that high concentrations of TSS can cause gill clogging, affecting respiration and consequently compromising feed intake and animal growth. Gaona et al. (2011) recommend the removal of suspended solids from the culture tank for better shrimp performance.

In experiment 2, there were no significant differences between the treatments. However, in this experiment, ammonia, and nitrite concentrations were higher when there was no addition of biofloc inoculum. The nutritional quality of the bioflocs was lower in treatments with the addition of clay minerals with low concentrations of proteins, lipids, and high ash content.

However, in this experiment, it can be said that this factor did not influence the observed percentages of zootechnical performance.

## CONCLUSION

According to the results presented in this work, the use of mineral-based flocculants can significantly affect the proximal composition of bioflocs, with an increase in ash content; the water quality, especially with a increase in total suspended solids concentrations and the zootechnical performance of cultured shrimp, where high concentrations of flocculants may have effects on zootechnical indices. The addition of Biofloc inoculum in some treatments effectively controlled nitrogen. However, the actual interaction of this flocculant with the microbial community was not course, and further studies are needed to assess how these clay minerals influence microorganisms and presence of residual sludge in the BFT system.

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