

Research Article

Bioremediation and biocontrol of commercial probiotic in marine shrimp culture with biofloc

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ABSTRACT. The use of probiotics within shrimp farms has increased as an alternative to antibiotic use because of an increasing demand for more environment-friendly aquaculture. This has improved growth performance and health of shrimp, and improved pathogen control as well as water and soil quality in culture systems. However, efficacy of probiotics in intensive systems using biofloc remains uncertain. Here, bioremediation and biocontrol of a commercial probiotic was investigated through analysis of water quality and main bacterial groups that influence a *Litopenaeus vannamei* culture using biofloc. Furthermore, additional knowledge was gained on phytoplankton and shrimp performance. Treatments consisted of four different probiotic concentrations (*Bacillus subtilis* and *B. licheniformis*): 0.5 g m⁻³ (P0.5), 1 g m⁻³ (P1.0), 2 g m⁻³ (P2.0), 3 g m⁻³ (P3.0), and a control without probiotic (CTL). All variables were shown to be balanced and within the recommended limits for shrimp farming. Under the culture conditions adopted in this study, the commercial probiotic did not result in a significant effect ($P \geq 0.05$) on water quality, bacteria, phytoplankton, or shrimp performance. Bacteria naturally present in biofloc were sufficient for maintaining the balance of culture and continued to exert excellent bioremediation and biocontrol when management was conducted properly.

Keywords: *Litopenaeus vannamei*, shrimp, nursery phase, intensive system, aquaculture.

INTRODUCTION

Aquaculture has developed environment-friendly systems to integrate productivity, expansion, minimal environmental impact, and minimal production of wastes such as excess nutrients, toxic compounds, and pathogens. Culture systems using high density and minimal or zero water exchange have been used to increase aquaculture sustainability (Crab *et al.*, 2012). These systems use biofloc technology (BFT), which facilitates degradation of organic wastes and assimilation and nitrification of nitrogen by a wide microbial community that form biofloc under intense aeration and mixing (Avnimelech, 2009). Biofloc comprises various microorganisms (phytoplankton, bacteria, rotifers, nematodes, and protozoa), uneaten

feed, dead cells, detritus, and feces (Emerenciano *et al.*, 2011). In a BFT system, an additional organic carbon source (*e.g.*, molasses) under a controlled carbon to nitrogen ratio (C:N) is used to induce both growth and multiplication of bacteria in the water culture (Ebeling *et al.*, 2006). The bacteria then maintain water quality and constitute a supplemental source of proteins, lipids, vitamins, and minerals for the reared animals (Avnimelech, 2009).

Another method to achieve sustainable aquaculture has been the use of microorganisms with a probiotic function as an alternative to antibiotics. Probiotics act in several modes of action, which can provide protection against pathogens and prevent diseases (*e.g.*, biocontrol avoiding both quorum sensing and transfer of resistance genes), interact with phytoplankton, increa-

se performance of cultured species (with digestive enzymes and nutrients), and improve water and soil qualities (bioremediation) (Verschuere *et al.*, 2000; Balcazar *et al.*, 2006; Defoirdt *et al.*, 2011). *Bacillus* spp. have been widely used as a probiotic because they are naturally found in the environment and have several mechanisms to compete against pathogens (mainly *Vibrio* spp.); they also increase animal performance, improve water quality, and can tolerate changes in pH, temperature, and salinity (Ochoa-Solano & Olmos-Soto, 2006; Decamp *et al.*, 2008).

Nevertheless, in biofloc systems with limited or no water exchange, the efficacy of probiotics remains uncertain. Conflicting results for the same variables across multiple studies have been observed in crops (McIntosh *et al.*, 2000; Devaraja *et al.*, 2002; Patnaik *et al.*, 2007; Aguilera-Rivera *et al.*, 2014). Moreover, the main bacteria declared to be present in commercial probiotics (see Noor-Uddin *et al.*, 2015) are very common in biofloc (*e.g.*, *Bacillus* spp.) and provide an infinity of other heterotrophic and autotrophic bacteria that also contribute to the maintenance of system equilibrium (Zhao *et al.*, 2012; Ferreira *et al.*, 2015). Hence, the assessment of the efficacy of probiotic in BFT systems is a challenge due to conflicting information. The aim of the current study was to investigate bioremediation and biocontrol under different concentrations of a commercial probiotic through analysis of water quality and main bacterial groups that influence an intensive nursery culture of *Litopenaeus vannamei* in a biofloc system. Results of the current study are useful for further understanding the relative efficacy of probiotic in BFT systems. Furthermore, additional information concerning phytoplankton and shrimp performance is gained.

MATERIALS AND METHODS

Experimental design and management

The current study was conducted at the Aquaculture Station of the Federal Rural University of Pernambuco (UFRPE), located in Recife, Pernambuco, Brazil. A 61-day nursery study was conducted in 800-L round fiberglass outdoor tanks ($n = 20$), which were filled with seawater (salinity of 30) and covered with polyethylene screens to prevent the escape of shrimp. No water exchanges took place, only the adjustment of water level and salinity due to evaporation by adding fresh water. Commercial probiotic application occurred daily in the culture water. The probiotic consisted of *Bacillus subtilis* and *B. licheniformis* at a concentration of 5×10^{10} CFU g^{-1} (data provided by the manufacturer).

Four replicates were randomly assigned to the following treatments: 0.5 $g\ m^{-3}$ (P0.5), 1 $g\ m^{-3}$ (P1.0), 2 $g\ m^{-3}$ (P2.0), 3 $g\ m^{-3}$ (P3.0), and a control without probiotic (CTL). These concentrations were chosen from the concentration recommended by the manufacturer. To promote the biofloc development, sugarcane molasses was added in all tanks as an organic carbon source by daily application in water. The molasses was added through a C:N ratio of 6:1, using 6 g of carbon to convert 1 g of total ammonia nitrogen based on Ebeling *et al.* (2006) and Samocha *et al.* (2007). Molasses addition was suspended when total ammonia nitrogen was $<1\ mg\ L^{-1}$.

Post-larvae

All tanks were stocked with 10 days-old post-larvae (PL₁₀) of *L. vannamei* (initial weight approximately 2 mg), with a density of 2,100 PL m^{-3} . After stocking, newly hatched *Artemia* sp. at a concentration of 40 nauplii $PL^{-1}\ day^{-1}$ (105 nauplii L^{-1}) were the exclusive feed. During the first week, shrimp were fed with both *Artemia* sp. and a commercial feed [45% crude protein (CP)], followed by gradual replacement of a 40% CP diet until the end of the culture, four times a day. The initial feeding rate was 50% of the total estimated biomass and down to 5% of the estimated shrimp biomass towards the end of the culture. Specific growth rate {SGR = $100 [(\ln\ final\ weight - \ln\ initial\ weight) / culture\ time]$ }, survival [S = $100 (final\ population / initial\ population)$], yield (Yd = final biomass/tank volume), and feed conversion ratio (FCR = amount of food provided/biomass gain) were analyzed to evaluate shrimp performance.

Water quality

Temperature, dissolved oxygen (DO), and pH (multiparameter: YSI model 556 MPS) were measured daily during the morning (7:00 am) and during the afternoon (4:00 pm), and salinity was measured weekly (multiparameter: YSI model 556 MPS). Nitrite-nitrogen (NO_2-N), silicate (SiO_2), orthophosphate (PO_4-P), alkalinity, and settleable solids were analyzed weekly, while total ammonia nitrogen (TAN) was determined twice a week and nitrate-nitrogen (NO_3-N) was assessed monthly. TAN, nitrite, nitrate, orthophosphate, and silicate were measured using a spectrophotometer (Hach DR 2800), and methods 8038 (Nessler Method), 8507 (Diazotization Method; absorbance reading at 507 nm), 8039 (Cadmium Reduction Method; 500 nm), 8185 (Silicomolybdate Method; 452 nm), and 8048 (method PhosVer[®] 3; 880 nm), respectively. Settleable solids (SS) were obtained by Imhoff cones ($mL\ L^{-1}$) and alkalinity by titration (expressed in equivalents of $CaCO_3$). Sodium

bicarbonate was applied when alkalinity levels were below $120 \text{ mg L}^{-1} \text{ CaCO}_3$.

Bacteria

The probiotic concentration was confirmed by plating on TSA (Triplic Soy Agar). The probiotic was diluted until 10^{-8} following which aliquots of $1 \mu\text{L}$ of the 10^{-4} , 10^{-6} , and 10^{-8} dilutions were inoculated in plates, incubated at 32°C , and counted after 24 h. Samples of water were collected during the last four weeks of the culture to quantify *Vibrio*, total heterotrophic bacteria (THB), and total autotrophic bacteria (TAB). Water samples were diluted until 10^{-4} following which 1 mL of the last three dilutions were transferred to Petri plates containing culture media for heterotrophic bacteria (Plate Count Agar; PCA), chemoautotrophic bacteria (specific culture media), and *Vibrio* (Thiosulfate Citrate Bile Salt Sucrose; TCBS). The formula of the chemoautotrophic bacteria culture media is 0.5 g of $(\text{NH}_4)_2\text{SO}_4$; 0.5 g of NaHCO_3 ; 13.5 g of Na_2HPO_4 and K_2HPO_4 ; 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.014 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 0.18 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; and 1000 mL of water (Oliveira, 2003). Each analysis was performed in duplicate by the spread plate method and incubated at 30°C for 24-48 h, based on Silva *et al.* (1997). Plates with 25 and 250 colonies were used to calculate bacteria numbers (colony forming units; CFU/sample).

Phytoplankton

Before shrimp storage, all tanks were fertilized with urea, phosphoric acid, and sodium metasilicate to obtain a concentration of 2.8 mg L^{-1} nitrogen, 0.4 mg L^{-1} phosphorus, and 1.05 mg L^{-1} silica, respectively. The diatom *Chaetoceros calcitrans* was inoculated a day after fertilization at a concentration of $7 \times 10^4 \text{ cells mL}^{-1}$. Phytoplankton were collected three times a week using 50 mL plastic containers and immediately fixed with 4% formalin. A Neubauer chamber (cells mL^{-1}), slides, and coverglass were used for quantitative and qualitative analyses of phytoplankton under a binocular microscope (Coleman N-120-T, 40x magnification).

Statistical analyses

Data were analyzed by a Bartlett and Shapiro-Wilk tests to verify the homogeneity and normality of the variances, respectively. A one-way analysis of variance (ANOVA, $\alpha = 0.05$) was used to identify significant differences between treatments in water quality variables (temperature, DO, pH, salinity, TAN, nitrite-nitrogen, nitrate-nitrogen, silicate, orthophosphate, alkalinity, and settleable solids), bacterial densities (*Vibrio*, THB, and TAB), production variables (final weight, SGR, survival, yield, and FCR) and phytoplankton densities (Bacillariophyceae, Cyanophyceae,

Chlorophyceae, Haptophyceae, Dinophyceae, and Euglenophyceae). Percentage data of certain production variables were transformed using arcsine before processing by ANOVA and Tukey's test for comparison of means. The software Statistica version 7.0 (Statsoft Inc.) was used for all analyses.

RESULTS

Water quality

Water quality variables are presented in Table 1. No significant differences ($P \geq 0.05$) were found among treatments. The temporal variations of TAN, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$, and settleable solids are shown in Fig. 1.

The TAN levels did not exceed 2.4 mg L^{-1} in all treatments (Table 1, Fig. 1a). Nitrite-N levels remained below 1.5 mg L^{-1} , except during the eighth week in the P2.0 and P3.0 treatments, when values peaked to approximately 3.5 mg L^{-1} , and then dropped a week later (Fig. 1b). Nitrate-N levels increased steadily in a linear manner across the weeks in all treatments, but the means did not reach concentrations higher than 3.5 mg L^{-1} (Fig. 1c).

Settleable solids increased throughout the experiment and showed an overall mean of approximately 12 mL L^{-1} (Table 1). However, values between $20\text{-}30 \text{ mL L}^{-1}$ solids were identified during the last week of the current study (Fig. 1d). Orthophosphate showed an overall mean of approximately 4.6 mg L^{-1} (Table 1) and followed the same increasing trend as nitrate and settleable solids, reaching levels above 10 mg L^{-1} during the last week (data not shown).

Bacteria

Total heterotrophic bacteria (THB) density remained at high levels over the weeks (Fig. 2a). In the control treatment, heterotrophic bacteria decreased during the seventh week, re-establishing afterwards and reaching the maximum ($4 \times 10^8 \text{ CFU mL}^{-1}$) towards the end of culture. However, the inverse occurred in the probiotic treatments, with no significant difference. Total autotrophic bacteria (TAB) showed a high density during the sixth week, particularly in the treatments with intermediate probiotic concentration P1.0 and P2.0 (11×10^5 and $21 \times 10^5 \text{ CFU mL}^{-1}$, respectively) (Fig. 2b).

However, TAB decreased over time with mean densities below $5 \times 10^5 \text{ CFU mL}^{-1}$ (except for P2.0 and P3.0 during the eighth week). *Vibrio* counts showed low concentrations in all treatments as the culture progressed ($\sim 8 \times 10^2 \text{ CFU mL}^{-1}$), although the higher value found during the sixth week for treatment P0.5 had been an exception (Fig. 2c). However, no signifi-

Table 1. Mean values (\pm SE) and range (in parenthesis) of water quality variables monitored daily and weekly in an intensive culture of *Litopenaeus vannamei* using biofloc and a commercial probiotic. P0.5, P1.0, P2.0 and P3.0: probiotic concentrations (g m^{-3}), CTL: control treatment. In daily variables first column corresponds to the morning period and the second column, the afternoon period, for each treatment.

Variables	P0.5	P1.0	P2.0	P3.0	CTL
Daily variables					
Temperature ($^{\circ}\text{C}$)	25.9 \pm 0.21 28.6 \pm 0.25	25.9 \pm 0.21 28.7 \pm 0.25	25.9 \pm 0.21 29.1 \pm 0.24	26.0 \pm 0.21 29.1 \pm 0.25	25.9 \pm 0.21 29.0 \pm 0.23
DO (mg L^{-1})	6.3 \pm 0.16 5.4 \pm 0.20	6.4 \pm 0.16 5.4 \pm 0.19	6.4 \pm 0.14 5.5 \pm 0.18	6.3 \pm 0.15 5.3 \pm 0.18	6.3 \pm 0.15 5.4 \pm 0.19
pH	7.9 \pm 0.04 7.9 \pm 0.07	7.9 \pm 0.05 7.9 \pm 0.07	7.9 \pm 0.04 7.8 \pm 0.07	7.9 \pm 0.04 7.8 \pm 0.07	7.9 \pm 0.04 7.8 \pm 0.07
Weekly variables					
TAN (mg L^{-1})	1.60 \pm 0.14 (0.00-2.40)	1.55 \pm 0.14 (0.00-2.40)	1.58 \pm 0.14 (0.04-2.40)	1.60 \pm 0.13 (0.00-2.40)	1.68 \pm 0.13 (0.03-2.40)
$\text{NO}_2\text{-N}$ (mg L^{-1})	0.67 \pm 0.11 (0.008-2.33)	0.66 \pm 0.11 (0.007-2.62)	0.86 \pm 0.20 (0.008-8.29)	1.00 \pm 0.20 (0.008-6.46)	0.59 \pm 0.09 (0.009-1.82)
$\text{NO}_3\text{-N}$ (mg L^{-1})	1.66 \pm 0.37 (0.20-3.19)	1.67 \pm 0.39 (0.10-3.54)	1.97 \pm 0.43 (0.10-3.83)	1.48 \pm 0.42 (0.00-4.09)	1.69 \pm 0.43 (0.00-4.06)
SiO_2 (mg L^{-1})	20.67 \pm 3.21 (3.0-78.0)	16.85 \pm 2.40 (2.0-58.0)	21.10 \pm 3.06 (2.0-60.0)	20.54 \pm 2.64 (2.0-56.0)	18.21 \pm 2.43 (3.0-55.0)
$\text{PO}_4\text{-P}$ (mg L^{-1})	4.66 \pm 0.57 (0.9-14.5)	4.36 \pm 0.55 (1.1-13.9)	4.87 \pm 0.57 (1.0-15.1)	4.51 \pm 0.52 (1.1-16.2)	4.60 \pm 0.60 (1.2-17.5)
Alkalinity ($\text{mg CaCO}_3 \text{ L}^{-1}$)	155.25 \pm 5.93 (65.0-240.0)	150.29 \pm 6.35 (65.0-260.0)	144.15 \pm 4.79 (80.0-225.0)	147.60 \pm 4.63 (85.0-210.0)	153.19 \pm 6.0 (80.0-235.0)
Settleable solids (mL L^{-1})	13.11 \pm 1.86 (1.0-30.0)	12.55 \pm 1.62 (1.0-30.0)	12.36 \pm 1.80 (0.5-30.0)	12.34 \pm 1.60 (0.1-29.0)	10.80 \pm 1.41 (0.5-28.0)
Salinity	29.5 \pm 0.37 (26.5-35.0)	29.6 \pm 0.38 (26.5-35.0)	28.8 \pm 0.35 (25.0-34.0)	28.9 \pm 0.37 (26.0-35.0)	28.9 \pm 0.30 (26.0-33.0)

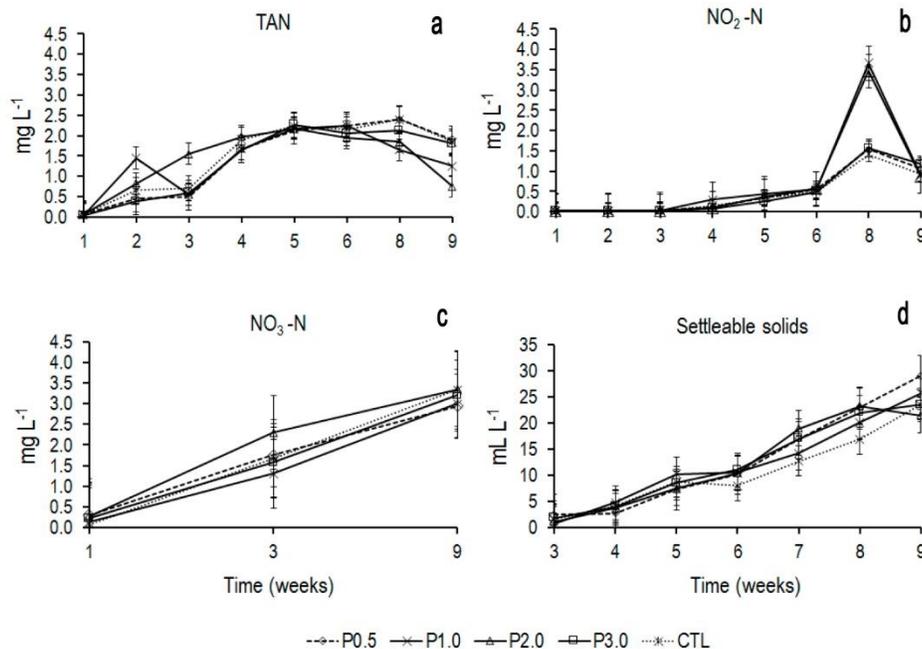


Figure 1. a) Temporal variation of total ammonia nitrogen, b) nitrite-nitrogen, c) nitrate-nitrogen, and d) settleable solids in an intensive culture of *Litopenaeus vannamei* using biofloc and a commercial probiotic. Data points symbolize treatment means.

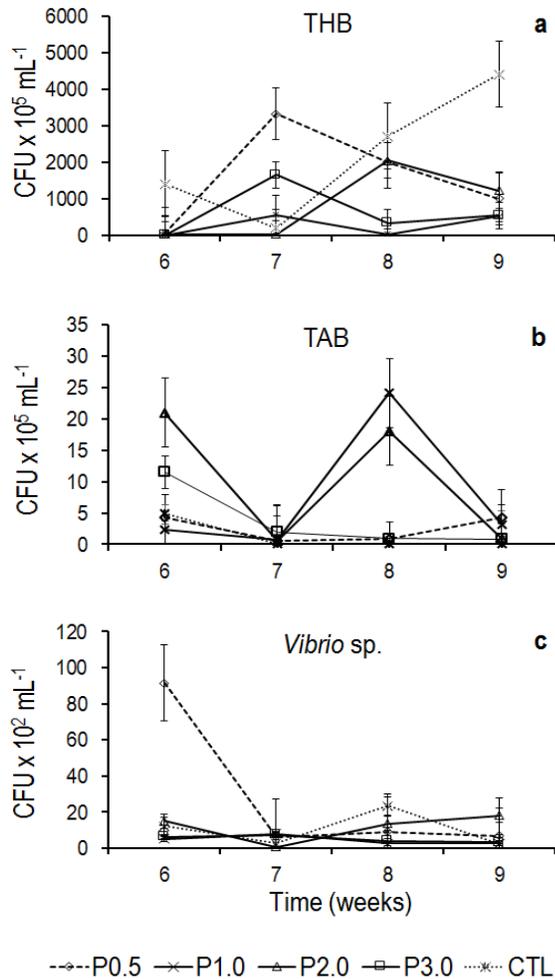


Figure 2. Weekly variation of a) Total Heterotrophic Bacteria: THB, b) Total Autotrophic Bacteria, (TAB) and c) *Vibrio* in an intensive culture of *Litopenaeus vannamei* using biofloc and a commercial probiotic. Data points symbolize treatment means.

cant differences were found between treatments ($P \geq 0.05$).

Post-larvae performance

Performance variables of *L. vannamei* are shown in Table 2 as final weight, specific growth rate, survival rate, yield, and feed conversion ratio. After 61 culture days, survival ranged from 68.8% to 89.9%, SGR from 8.9% to 9.4% day⁻¹, and FCR showed values ranging from 1.2 to 1.6. Hypothesis tests were applied to estimations of each variable, and the results indicated that there were no significant differences between treatments ($P \geq 0.05$).

Phytoplankton

Phytoplankton were represented by the classes Cyanophyceae (49.1%), Chlorophyceae (38.0%), Haptophy-

ceae (6.3%), Bacillariophyceae (3.2%), Dinophyceae (2.3%), and Euglenophyceae (1.1%), and no significant difference was observed among treatments ($P \geq 0.05$). Total densities of all phytoplankton between treatments were 121×10^4 , 127×10^4 , 162×10^4 , 163×10^4 , and 182×10^4 cell mL⁻¹, in P0.5, P1.0, CTL, P2.0, and P3.0 treatments, respectively (data not shown).

Phytoplankton showed several blooms. Initially, a dominance of Bacillariophyceae was observed due to fertilization and inoculation of the diatom *C. calcitrans*. This group showed a marked reduction during the following week and remained at low concentrations throughout the culture. A peak during the sixth week in P2.0 was an exception. From the second week, Cyanophyceae and Chlorophyceae succeeded Bacillariophyceae as the concentration of nutrients increased over the culture period and were the most dominant and constant groups (Figs. 3a-3c). However, short duration peaks of Dinophyceae (2-4th week), Euglenophyceae (6-8th week), and Haptophyceae (6-9th week) were present during certain periods of the culture (Figs. 3d-3f).

DISCUSSION

TAN concentrations remained below levels cited by Lin & Chen (2001) that recommended a safety threshold for *L. vannamei* juveniles of between 3.55-3.95 mg L⁻¹ TAN (salinity of 25-35) after acute toxicity tests. According to Samocha *et al.* (2007), molasses addition is an effective tool to maintain low levels of TAN. Thus, the control of C/N is essential to facilitate the removal of this form of nitrogen by bacteria. In relation to nitrite-N, concentrations found in the present study were below safe levels indicated for *L. vannamei* juveniles of between 15.2 and 25.7 mg N L⁻¹, at salinity of 25-35 (Lin & Chen, 2003). The data suggest that the shrimp were comfortable even in the highest concentrations found (~3.5 mg L⁻¹).

On the other hand, it has been shown that nitrate accumulation is quite common as a culture period progresses; however, nitrate concentration can change with the types of management adopted. This can be observed both in Kuhn *et al.* (2010) with 220 mg NO₃-N L⁻¹ at salinity of 11, and in Correia *et al.* (2014) with a mean of 95 mg NO₃-N L⁻¹ at salinity of 31, who identified much higher values; however, an adverse effect on the shrimp was not identified. The general trends over time of ammonia, nitrite, and nitrate concentrations found in the present study were expected, as the culture was initiated using clear chlorinated water, and because both assimilation and nitrification processes were controlled and stabilized until complete bacterial establishment in biofloc (ninth

Table 2. Mean (\pm standard error) of survival and growth performance variables of an intensive culture of *Litopenaeus vannamei* (~2 mg) using biofloc and a commercial probiotic. P0.5, P1.0, P2.0 and P3.0 - probiotic concentrations. CTL: control treatment, Wf: final weight, SGR: specific growth rate, FCR: feed conversion ratio, S: survival, Yd: yield.

Variables	P0.5	P1.0	P2.0	P3.0	CTL
Wf (g)	0.54 \pm 0.03	0.60 \pm 0.02	0.62 \pm 0.10	0.62 \pm 0.09	0.46 \pm 0.02
SGR (% day ⁻¹)	9.19 \pm 0.08	9.34 \pm 0.07	9.35 \pm 0.25	9.38 \pm 0.21	8.93 \pm 0.08
S (%)	81.4 \pm 2.03	77.6 \pm 5.66	74.4 \pm 15.78	68.8 \pm 17.85	89.9 \pm 8.35
Yd (kg m ⁻³)	1.15 \pm 0.07	1.21 \pm 0.06	1.08 \pm 0.13	1.01 \pm 0.21	1.01 \pm 0.13
FCR	1.4 \pm 0.08	1.3 \pm 0.08	1.2 \pm 0.04	1.2 \pm 0.01	1.6 \pm 0.20

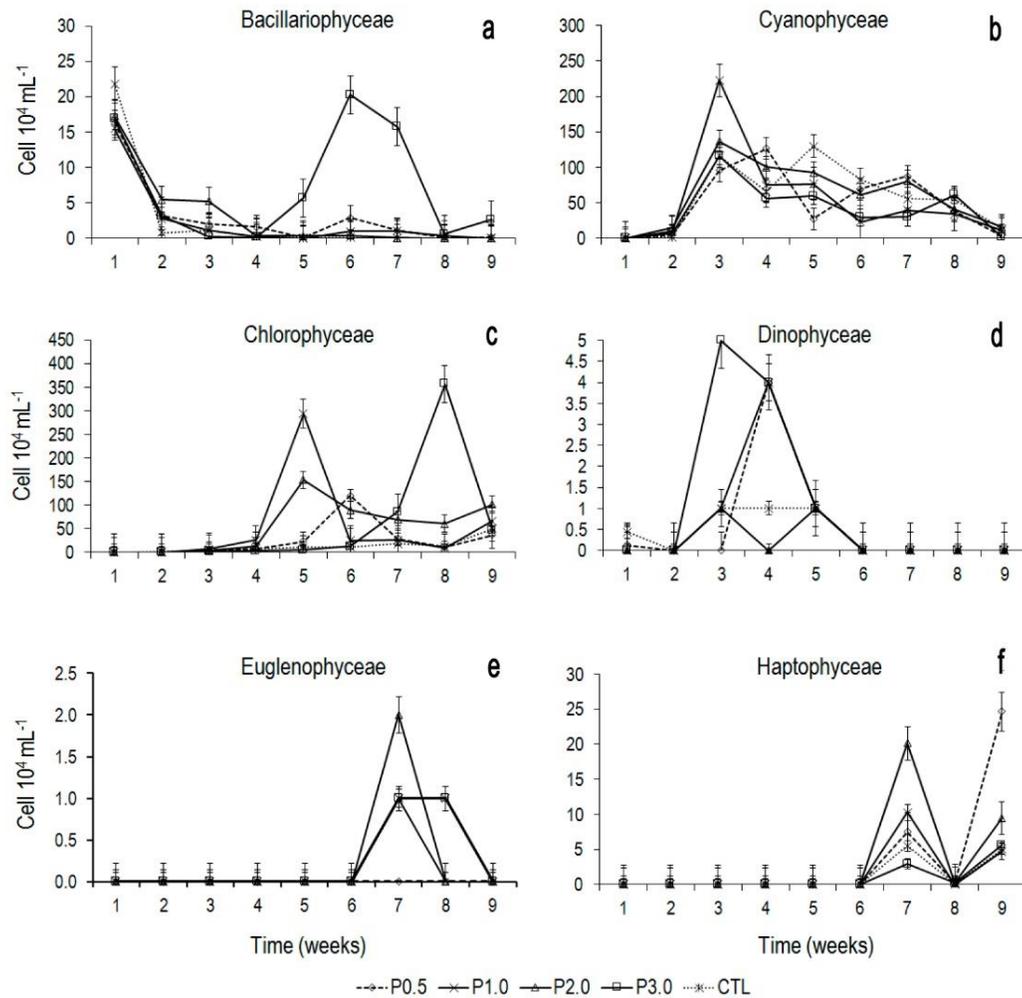


Figure 3. Phytoplankton classes succession: a) Bacillariophyceae, b) Cyanophyceae, c) Chlorophyceae, d) Dinophyceae, e) Euglenophyceae, and f) Haptophyceae in an intensive culture of *Litopenaeus vannamei* using biofloc and a commercial probiotic.

week). Furthermore, pH values remained below 8, temperature between 25 and 29°C and salinity of 30, which favors the speed of nitrification reactions and promote reduction of ammonia toxicity (Frías-Espicueta & Páez-Osuna, 2001; Lin & Chen, 2001; Vinatea, 2010; Isnansetyo *et al.*, 2014).

Settleable solids results were consistent with other studies that recommend values between 10-15 mL L⁻¹ for shrimp (Samocha *et al.*, 2007; Taw, 2010). Although higher values (20-30 mL L⁻¹) were identified during the last week of the current study, settling tanks or foam fractionators were not used for the removal of

solids as no adverse effect on either water quality or shrimp was perceived. Furthermore, settleable solids results were much smaller than in Gaona *et al.* (2016) that evaluated different water flows for solids removal in culture with biofloc and found volumes until 50 mL L⁻¹ and above 100 mL L⁻¹ for treatments with and without solids removal, respectively, without significant differences in nitrogen compounds and growth of *L. vannamei*, and survival more than 90% for all treatments. A tendency of accumulation also occurred for orthophosphate (maximum levels above 10 mg L⁻¹). According to Correia *et al.* (2014), who found similar results, phosphorus accumulation is additionally expected in biofloc systems; however, high concentrations can be reduced with the use of settling tanks or foam fractionators. This accumulation occurs mainly due to the frequent addition of feed and no water exchange. In our study, the removal of solids to reduce orthophosphate was not conducted; however, as previously reported for settleable solids, no damage was detected to the system.

Under the condition of the current study, water quality results indicated that the commercial probiotic had no bioremediation effect. Hence, application of a probiotic to maintain water quality in an intensively controlled biofloc system appears unnecessary. This assertion is additionally supported by Zhou *et al.* (2009) who found no difference in water quality of *L. vannamei* larvae and post-larvae culture with different probiotic (*B. coagulans*) concentrations. Moreover, McIntosh *et al.* (2000) compared the effects of a commercial probiotic containing *Bacillus subtilis*, *B. megaterium*, and *B. polymyxa* with another probiotic containing *B. licheniformis* and reported that phosphorus accumulated over time and that ammonia, nitrite, and nitrate were maintained at low levels by the native microbial population of the tanks and not due to probiotic addition.

Bacteria results indicated a predominance of heterotrophic bacteria in the bioremediation of water quality, independent of the use of probiotic. Devaraja *et al.* (2002), studying changes in microbial populations in ponds treated with and without commercial probiotics, similarly found high density of heterotrophic bacteria in all treatments. The same authors emphasize that *Bacillus* spp. were the dominant species in all water and sediment samples, which was additionally confirmed by Ferreira *et al.* (2015), that isolated *Bacillus* spp. from the super-intensive marine shrimp system with biofloc, added cells in culture tanks, and did not observe significant differences in heterotrophic bacteria count neither in water quality parameters.

The *Vibrio* load is one of the most important factors affecting crop performance, and according to Luis-Villasenor *et al.* (2013), several *Vibrio* species present in biofloc are not beneficial to shrimp. In our study, bacteria from biofloc were the main factor responsible for *Vibrio* control and not the commercial probiotic added in the current experiment. This assertion may be supported by the predominance of heterotrophic and autotrophic bacteria in relation to *Vibrio* in all treatments, which certainly used their antagonisms to control the abundance of *Vibrio* in culture tanks.

Thus, despite the numerous benefits of commercial probiotics in various cultures cited in the literature, such as in Janeo *et al.* (2009), that observed a significant reduction of the *Vibrio* spp. in water with weekly applications of *Bacillus* sp. and *Nitrobacter* sp. in a semi-intensive system, for intensive biofloc cultures, the use of these products remains controversial, as the main bacteria found are additionally present in biofloc (Ferreira *et al.*, 2015). Therefore, the use of probiotics would be interesting only in case of system disturbances as dominance of undesirable or pathogenic microorganisms, disease outbreaks, or any other damage to the shrimp or water quality, where dominance of beneficial native bacteria preferably isolated from the digestive tract of shrimp seems to be an alternative to the environmental conditions reestablishment (Leyva-Madrigal *et al.*, 2011; Hao *et al.*, 2014). On the other hand, in balanced systems, which maintains rigorous control of its parameters, the probiotic is unnecessary due to the presence of biofloc, which by itself provide protection and resistance to the animal (Avnimelech, 2009; Ekasari *et al.*, 2014).

The bioflocs were further investigated for their effect on performance variables and phytoplankton communities. Performance variables of *L. vannamei* were partially conflicting with results found by Aguilera-Rivera *et al.* (2014), as those authors found significant differences in survival and health status when a probiotic was added to biofloc. However, both studies are in agreement concerning growth performance, which was not clearly affected by addition of probiotic in a BFT system. Moreover, our results are in full agreement with the study by McIntosh *et al.* (2000) who reported no significant differences in survival (83%-98%), final weight (10.93-12.79 g), and FCR (1.99-2.39). A similar low influence of the effect of probiotic on the performance in shrimp farming systems with zero or limited water exchange was observed by Devaraja *et al.* (2002) and Patnaik *et al.* (2007). This indicates that performance improvements with or without the addition of probiotic in biofloc systems may change according to the culture conditions.

Phytoplankton communities are continuously sensitive to changes in water quality, mainly oxygen, nutrients, and contaminants, and hence are excellent environmental indicators (Casé *et al.*, 2008). The presence of Cyanophyceae, Chlorophyceae, Haptophyceae, Bacillariophyceae, Dinophyceae, and Euglenophyceae in the current study were shown by Yusoff *et al.* (2002) as representatives of phytoplankton commonly found in aquaculture ponds. However, much lower microalgae biomass (10^3 to 11×10^3 cell mL⁻¹) were found by these authors, studying phytoplankton succession and a probiotic with minimal water exchange (*Bacillus* sp. and *Saccharomyces* sp.). Our results are comparable with Samocha *et al.* (2007), who found values ranging between 128×10^4 and 258×10^4 cell mL⁻¹ with limited water exchange but without probiotics, indicating that probiotic addition may be unnecessary in controlled systems, since bacteria and phytoplankton from biofloc additionally have the same bioremediation and biocontrol functions.

Phytoplankton showed blooms of short duration in which a species was quickly replaced by others of different groups as reported in Burford *et al.* (2003). Blooms occurred due to the cumulative effect of factors favoring bloom development, including high levels of nitrogen and phosphorus, no water exchange, high oxygen supply, and constant water column mixing, allowing phytoplankton access to nutrients and light (Funge-Smith & Briggs, 1998; Burford *et al.*, 2003). In intensive systems, high feeding rates and stocking density produce large amounts of nutrients and wastes, causing eutrophication (Funge-Smith & Briggs, 1998). Over culture time, with increased eutrophication, turbidity, and temperature, besides reduction of silica levels and luminosity, diatom dominance can be replaced by cyanobacteria and dinoflagellates blooms (Yusoff *et al.*, 2002; Casé *et al.*, 2008). However, on one hand, Cyanophyceae is undesirable but commonly found in marine shrimp farming (Casé *et al.*, 2008; Vinatea *et al.*, 2010; Maia *et al.*, 2013), whereas on the other hand, the participation of Chlorophyceae and Bacillariophyceae positively contribute to shrimp farming as important sources of food.

In summary, water quality in biofloc cultures may not deteriorate even with high nutrients levels in intensive crops, because intense mechanical aeration favors degradation of toxic nitrogen and other compounds by the microbial community that additionally serve as a food supplement for shrimp (Avnimelech, 2009). BFT is an efficient alternative against pathogens, maintains water quality at optimal levels, and enhances survival and growth of shrimp in no water exchanges systems (Crab *et al.*, 2010, 2012; Xu *et al.*, 2013).

CONCLUSION

The addition of a probiotic in the selected concentrations under culture conditions using a biofloc system did not affect water quality, bacteria, phytoplankton, and *L. vannamei* performance, during an intensive nursery culture without water exchange. Biofloc was sufficient to maintain the system balance independent of the use of a probiotic and was shown to exert excellent bioremediation and biocontrol when culture management was properly implemented.

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