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Effect of diatom supplementation during the nursery rearing of *Litopenaeus vannamei* (Boone, 1931) in a heterotrophic culture system

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Abstract Bio-floc shrimp culture systems have been investigated in an attempt to optimize water use and prevent the discharge of effluent into the environment. The importance of microalgae in maintaining water quality and nutrition of the shrimp is well known in conventional systems; however, its maintenance amid bio-flocs and its role in the shrimp performance in this system are still poorly understood. The aim of this study was to evaluate the contribution of diatoms in the performance of *Litopenaeus vannamei* reared during the nursery phase in intensive system with minimal water exchange. Shrimp (0.31 ± 0.10 g) were reared among diatoms, bio-flocs and the combination of the two forming the mixture medium. The survival of shrimp was high in all treatments (90–97%). However, the shrimp reared among diatoms showed higher weight gain ($P < 0.05$) and feed conversion ratio significantly more efficient, reaching a value of 0.47. The results indicate the importance of diatoms in bio-floc culture systems and points out to future research in an attempt to maintain a constant presence of these microalgae in culture medium without requiring successive inoculations.

Keywords Shrimp culture · Phytoplankton · Bio-floc · Microalgae · Sustainability

Introduction

The use of high rates of water exchange as a way to control water quality in shrimp farming has generated concern about the sustainability of culture systems (Sandifer et al.

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1991; Sandifer and Hopkins 1996; Browdy et al. 2001; Martínez-Córdova et al. 2009). Aspects of biosecurity and the pressure of environmental agencies due to the impact caused by effluents from shrimp farms led to the development of intensive farming systems where strong aeration without water exchange allows the formation of macro-aggregates (bio-flocs). The bio-flocs are formed predominantly by a biota of aerobic and heterotrophic bacteria, protozoa, microalgae, metazoan, exoskeletons, feces and remains of dead organisms (Schryver et al. 2008). This type of system reduces the risk of introduction and spread of diseases providing simultaneously also nutritional benefits to the organism cultured (McIntosh et al. 2000; Burford et al. 2003; Ballester et al. 2010). Bio-floc system allows the use of diets with lower crude protein (CP) content, which is supplied in part by the natural production associated with the formation of the bio-flocs. This results in reducing production costs, as well as lower environmental impact due to the reduction in the nitrogen input and consumption of fishmeal in the diet (Crab et al. 2007). Burford et al. (2004) reported that up to 29% of food consumed by the shrimp *Litopenaeus vannamei* (Boone, 1931) can be derived from the bio-flocs present in the heterotrophic medium.

With the intensification of the culture system increases the importance of bacteria in the nutrient cycle, although the phytoplankton has a major role in maintaining water quality through the process of photosynthesis (Hargreaves 2006). The high concentration of suspended matter that significantly reduces both light entrance and nutrient competition with heterotrophic bacteria seems to hinder the maintenance of high densities of diatoms in the midst of bio-flocs. However, due to the importance of these microalgae in maintaining water quality and nutrition of the shrimp be already well known in conventional systems (Patil et al. 2007), further studies need to be developed in order to assess its role in bio-floc culture systems.

The aim of this study was to evaluate the contribution of diatoms in the performance of *Litopenaeus vannamei* reared during the nursery phase in an intensive system with minimal water exchange.

Materials and methods

The experiment was carried out at the Aquaculture Marine Station of Federal University of Rio Grande (Estação Marinha de Aquacultura—EMA/FURG), located in Cassino Beach, Rio Grande, Southern Brazil (32°12′ S, 52°10′ W).

Preparation of culture media

To form the bio-floc medium (BM tank) it was used a circular tank (7,000 L; bottom area of 6.8 m^2) without water renewal, replacing only the volume lost by evaporation. The tank was fitted with strong aeration from a central blower with an air-lift system on the sides creating a counterclockwise movement to aid the resuspension of particulate matter. It was covered with an 80% black screen (Sombrite® 80% reduction of light intensity) to favor the heterotrophic community.

The uptake of water was from the Cassino Beach, filtered through a sand filter (1 mm) and kept in a reservoir. The tank was stocked with juveniles of *Litopenaeus vannamei* (1.97 ± 0.27 g) at a density of 100 shrimp m^{-2} to help in the formation of the bio-flocs (Ferreira 2008, unpublished results). The shrimp were fed with commercial diet (40% CP) at a ratio of 5% of the biomass twice a day. Organic fertilization was performed daily and included the addition of wheat bran (10 g), sugarcane molasses (210 g) and the commercial diet

offered to the shrimp, reaching a C/N ratio of approximately 17/1 (w/w). This ratio was measured and balanced according to the proximal composition of each ingredient.

To develop the diatom medium (DM tank), we used a circular tank (5,000 L) equipped with the same aeration system described above. This tank was inoculated by two diatom species, *Thalassiosira weissflogii* and *Chaetoceros muelleri*, maintaining an average concentration of 637 and 3,512 $\times$ 10^5 cells mL^{-1}, respectively. At every 5 days, the volume of the tank was completely renovated and a new inoculation of the two species of diatoms was performed with the cells in the exponential growth phase, to ensure that the density, physiological state and a high level of quality were constant. Furthermore, the renewal prevented the development of heterotrophic organisms (mainly protists) in the culture medium.

The mixture medium (MM) consisted of a tank (800 L) equipped with the same aeration system already described (named MM matrix tank), which was formed by water from BM and DM matrix tanks in equal proportion (1:1), maintaining a working volume of 600 L. The diatoms were added in this treatment by the same manner as in the DM treatment, and the volume of the tank was completely renovated at every 3 days.

Experimental design

The experimental design was completely randomized with four replicates for each one of three treatments (BM, DM and MM). Each experimental unit consisted of a fiberglass tank (80 L) with 0.33 m^2 of bottom area, equipped with a circular hose ($^1/_2$ inch) with four porous stones set at the bottom to keep intense aeration. The water from each matrix tank (BM, DM and MM) was pumped via submersible pumps to their respective experimental units (four fiberglass tanks), and returned by gravity. This strategy was used to maintain constant the characteristics of the culture media evaluated. According to Decamp et al. (2003; 2007), the development of different microorganism groups may occur in isolated units of the same treatment in intensive systems without water renewal, influencing the results of the experiment. The total volume of each experimental unit was recirculated on average 43 times a day.

Shrimp culture system and performance

The experiment started when the total suspended solids (TSS) in the medium for development of the bio-flocs reached a concentration of 250 mg L^{-1}, value within the recommended standards for bio-floc systems (Samocha et al. 2007). Each experimental unit was stocked with 100 juveniles of *L. vannamei* (0.31 $\pm$ 0.10 g), reaching a stocking density of 300 shrimp m^{-2}. The shrimps were fed with commercial diet (40% CP) offered three times a day in feed trays. The supply of formulated feed was adjusted daily according to the consumption of the shrimp. Every morning the food not consumed was separated from other wastes present in the trays and oven dried at 60°C (until constant weight) to evaluate the dry weight. After 30 days, it was determined the weight gain and survival ratio of the shrimp. To determine the feed conversion rate (FCR), the natural food was not taken in account, being calculated as follows:

$$\mathrm{FCR} = \text{formulated food consumed (dry weight)}/\text{weight gain}$$

Analysis of physical, chemical and biological water parameters

Temperature, pH, salinity, dissolved oxygen (DO) and water transparency (Secchi disc) were monitored every morning (8 h) in each tank using a multiparameter instrument

(YSI® Model 556 MPS—USA). At every 3 days, water samples were collected to quantify the concentration of total ammonia nitrogen ($N–NH_3 + NH_4$; UNESCO 1983), dissolved inorganic nitrite ($N–NO_2$) and phosphate ($P–PO_4$) according to Aminot and Chaussepied (1983), and silicate (SiO_2) according to Strickland and Parsons (1972). The concentration of chlorophyll *a* (Chl *a*) and total suspended solids (TSS) were evaluated in triplicate with the same frequency by fluorescence (Welschmeyer 1994) and by the differential weight of the glass fiber filters (GF/F 47 mm) before and after the filtration of suspended matter (Strickland and Parsons 1972), respectively.

Statistical analysis

One-way analysis of variance (one-way ANOVA, $\alpha = 0.05$) was used to detect differences between treatments. Before performing the analysis of shrimp survival, the data were transformed by arcsine square-root. All tests were conducted after the confirmation of homogeneity of variances (Levene test) and normality of the data distribution (Kolmogorov–Smirnov test). The abiotic parameters of water quality, concentration of Chl *a* and TSS were analyzed by nonparametric Kruskal–Wallis test.

Results

Water quality (Table 1)

The water temperature of culture media showed no significant difference ($P > 0.05$) between treatments (average 24.04°C). DO concentration was significantly higher in DM ($P < 0.05$) when compared to other media (Fig. 1a). The pH and water transparency values were significantly different ($P < 0.05$) among the three treatments; higher in the DM, followed by MM and BM (Table 1). The MM showed higher salinity compared to DM and

Table 1 Water quality parameters in the culture of *L. vannamei* juveniles under intensive nursery conditions in diatom medium, bio-floc medium and mixture medium

Parameters	Diatom	Bio-floc	Mixture
Temperature (°C)	24.90 ± 2.73^{a}	23.53 ± 2.34^{a}	23.71 ± 2.87^{a}
Dissolved oxygen (mg L^{-1})	13.78 ± 3.12^{a}	8.82 ± 2.30^{c}	11.32 ± 1.96^{b}
pH	8.59 ± 0.42^{a}	7.50 ± 0.16^{c}	8.02 ± 0.13^{b}
Transparency Secchi (cm)	30.13 ± 7.86^{a}	11.70 ± 1.91^{c}	22.63 ± 6.94^{b}
Salinity (g L^{-1})	37.55 ± 1.08^{b}	37.23 ± 2.76^{b}	39.86 ± 3.08^{a}
Chl *a* (μg L^{-1})	553.80 ± 176.51^{a}	343.30 ± 184.97^{ab}	247.90 ± 228.32^{b}
TSS (mg L^{-1})	132.70 ± 103.25^{b}	496.60 ± 132.04^{a}	100.50 ± 84.90^{b}
TAN (mg L^{-1})	0.28 ± 0.51^{a}	0.02 ± 0.02^{bc}	0.19 ± 0.37^{ab}
NO_2–N (mg L^{-1})	0.55 ± 0.50^{a}	0.02 ± 0.03^{bc}	0.05 ± 0.08^{b}
PO_4–P (mg L^{-1})	0.04 ± 0.03^{a}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}
SiO_2 (mg L^{-1})	0.28 ± 0.63^{a}	0.12 ± 0.20^{a}	0.08 ± 0.10^{a}

The data are represented as average ± standard deviation. Equal letters in the same line indicate that the averages do not differ significantly ($P > 0.05$)

Chl a Chlorophyll *a*, *TSS* total suspended solids, *TAN* total ammonium nitrogen, *NO_2–N* dissolved inorganic nitrite, *PO_4–P* phosphate, *SiO_2* silicate

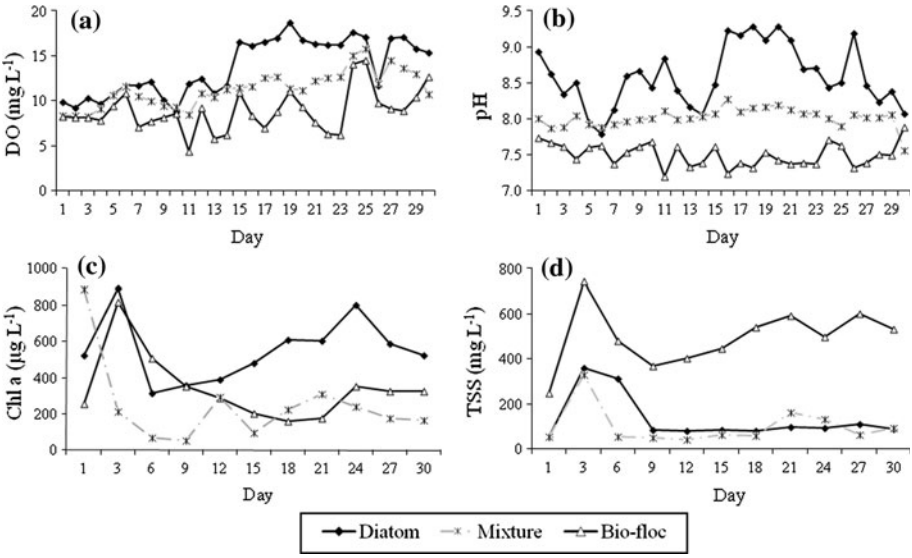

Fig. 1 Water quality parameters during the culture of *Litopenaeus vannamei* under intensive nursery conditions in diatom medium, bio-floc medium and mixture medium. **a** Dissolved oxygen; **b** pH; **c** chlorophyll *a* and **d** total suspended solids

BM. The concentration of Chl *a* was higher in DM (553.80 µg L^{-1}) although not significantly different ($P > 0.05$) from BM (343.30 µg L^{-1}; Fig. 1c), and TSS concentrations were significantly higher in BM (Fig. 1d).

The concentration of total ammonia nitrogen (TAN) was higher in DM, however there was no significant differences ($P > 0.05$) when compared to other media. The nitrite results were significantly higher ($P < 0.05$) in DM (Table 1). The average concentration of dissolved inorganic phosphate was similar in all treatments ($P > 0.05$), and the average concentration of silicate was two to three times higher in DM compared to other treatments.

Shrimp performance

The survival of *L. vannamei* juveniles was high in all treatments, ranging from 90 to 97% without statistical difference ($P > 0.05$; Fig. 2a). However, the shrimp in the DM had significantly higher weight gain ($P < 0.05$) compared to the shrimp reared in MM and BM (Fig. 2b), which did not differ between them ($P > 0.05$). Likewise, the feed conversion ratio was significantly more effective in DM ($P < 0.05$) reaching a value of 0.47. This rate was also low in MM and BM (0.76 and 0.80), but no significant differences were observed between these two treatments (Fig. 2c).

Discussion

Water quality

Temperature is one of the most important factors that control the growth of marine shrimp. Wyban et al. (1995) reported that juveniles of *L. vannamei* (3.9 g) had reduced growth at

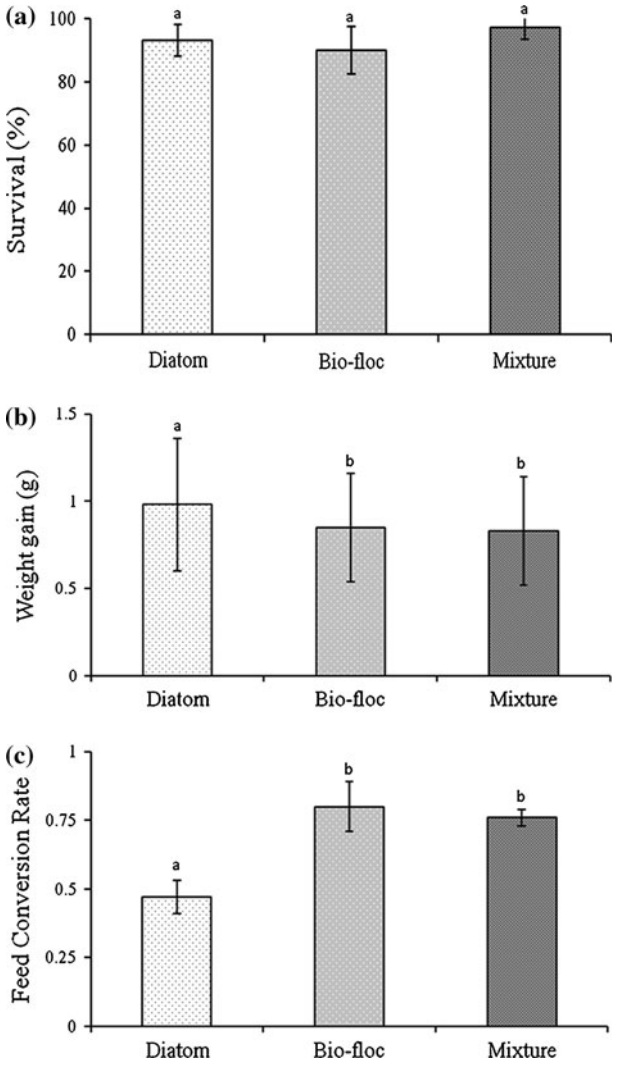

Fig. 2 Performance of *Litopenaeus vannamei* juveniles cultured under intensive nursery conditions in diatom medium, bio-floc medium and mixture medium. **a** Survival; **b** weight gain and **c** feed conversion ratio. *Different letters above* each *bar* indicate a significant difference between treatments ($P < 0.05$). Data are represented as average ± standard deviation

23°C when compared to the same size class grown at 27 and 30°C. In the present experiment, the average water temperature (24.05°C) remained slightly below the optimum range for growth of *L. vannamei*. According to Decamp et al. (2003), the salinity of the three different media was suitable for the culture of *L. vannamei*. A slight salinity increase in all treatments throughout the experiment due to the water evaporation process in the tanks was found.

The highest DO concentration (supersaturation) in DM was the result of intense photosynthetic activity of the diatoms. The lowest DO concentration in BM was probably due

to a higher respiration rate of the heterotrophic microbial community. However, the DO concentration was kept within the optimum range for *L. vannamei* growth in all treatments. Similar results were found by Burford et al. (2003) in a nycthemeral monitoring (every 30 min for 24 h) of the DO concentration in a super-intensive culture system of *L. vannamei* in ponds without water exchange, in which DO concentration was high (10 mg L^{-1}) especially in the afternoon as a result of microalgae photosynthesis. The high pH observed in the DM is also due to the photosynthesis process conducted by the microalgae, with the removal of CO_2 from the medium. The pH variation was low in the BM throughout the experiment and remained close to neutrality. Even with significant differences between treatments the pH remained in a range considered adequate for the proper performance of penaeid shrimp (Cohen et al. 2005). Wasielesky et al. (2006) evaluated the effect of natural production in a super-intensive culture of *L. vannamei* juveniles in bio-floc systems and have reported pH of 7.65 in the medium water, similar to that observed in our experiment for the BM treatment.

Cyanobacteria and pennate diatoms were the main autotrophic organisms present in the BM, probably originated from the Cassino Beach (local uptake water) that have developed significantly in an environment rich in nutrients, and were responsible for the high concentration of Chl *a*. High concentrations of Chl *a* (134–509 μg L^{-1}) were also observed by other authors in shrimp ponds operated in bio-floc systems (Burford et al. 2003; Decamp et al. 2007).

BM had the lowest Secchi transparency (11.70 cm), evidencing a direct relationship with the highest concentration of TSS in the medium. TSS values close to that found in this experiment were observed by Avnimelech (2007) in tilapia ponds (*Oreochromis mossambicus*) in the presence of bio-flocs.

TAN and nitrite concentrations suffered greater changes in DM, probably due to the water exchange of the tank at every 5 days, with the inoculation of diatoms and the input of nitrogen compound residues used in the preparation of the culture media of microalgae (Jensen et al. 2004). However, in all treatments the concentrations of nitrite and TAN remained below the safety levels recommended for juveniles of *L. vannamei* (Lin and Chen 2001; 2003). The low concentration of nitrite and TAN in BM is due to the microbial community that uses these sources of nitrogen to form their biomass (microbial protein). Moreover, there is a high nitrification rate in bio-floc systems due to the nitrifying bacteria that ensure the rapid oxidation of ammonia and toxic nitrite to nitrate, relatively harmless to shrimp (Holl et al. 2006; Boyd 2007a). Promising results were also obtained by Kuhn et al. (2008) in an experiment where the effluent from a commercial farm of tilapia received biological treatment and the bio-floc produced was supplied as food supplement for the cultivation of marine shrimp. Water of excellent quality, with low concentrations of TAN (0.05 mg L^{-1}) and nitrite (0.02 mg L^{-1}) resulted in higher survival and growth of shrimp.

The concentration of phosphate remained low in all treatments when compared to other studies carried out in heterotrophic media (McIntosh et al. 2000; Burford et al. 2003; Casillas-Hernández et al. 2007). Regular applications of phosphate fertilizers in aquaculture ponds are used to stimulate the growth of phytoplankton and increase the availability of natural food for the organisms cultured. In excavated ponds, the phosphate ions are rapidly sequestered by the sediment and the concentration in the water column is low (Boyd 2007b), but in bottom-covered tanks and closed recirculation systems it is expected an accumulation of this ion throughout the period of cultivation. The low phosphate concentrations observed in this study probably resulted from an active uptake by bacteria and microalgae present in the culture medium. Heterotrophic bacteria are responsible for a

large fraction of the orthophosphate absorption (P_i) in the oceans and in freshwater, estimated at around 60% (Kirchman 1994). However, studies corrected for the phytoplankton absorption resulted in a smaller fraction (24–46%) of P_i absorption attributed to the heterotrophic bacteria (Kirchman 2000).

Shrimp performance

The high survival observed for the *L. vannamei* juveniles in the present study shows that the conditions were favorable for cultivation in all media tested. High survival rates have been reported in intensive and super-intensive systems (Gómez-Jiménez et al. 2005; Wasielesky et al. 2006; Kuhn et al. 2008; Ballester et al. 2010), indicating that the shrimp growth is enhanced when cultured in the presence of bio-flocs (Avnimelech 1999; Hari et al. 2006; Samocha et al. 2007).

The greatest weight gain and more efficient feed conversion in DM confirm that diatoms participated significantly to the better performance of the shrimp because they consumed less food (79% of provided commercial diet) than those reared in treatments MM and BM (87% of provided commercial diet).

The feed conversion rate is an extremely important index in the aquaculture activity, since the cost of food generally represents up to 60% of the total cost of the production (Wasielesky et al. 2006). A strict technical control is necessary so that the feed supplied to the cultured organism is efficiently converted into biomass. Shrimp reared in DM had feed conversion ratio of 0.47 and its weight gain was significantly higher (17%) while consuming less commercial diet when compared to the MM and BM treatments. Wasielesky et al. (2006) reported FCR close to 1 for *L. vannamei* juveniles reared in bio-floc systems, and Moss and Moss (2004), in an intensive nursery culture of *L. vannamei* juveniles with the use of artificial substrates, reached a FCR of 0.73, which was attributed to the natural food associated with the substrates (attached bacteria, protozoa and microalgae).

There are some studies in the literature assessing the nutritional content of bio-flocs (McIntosh et al. 2000; Chamberlain et al. 2001; Tacon et al. 2002; Wasielesky et al. 2006; Azim et al. 2008). The protein content from different bio-flocs assessed by Ju et al. (2008) ranged from 26 to 42%. The authors found that the essential amino acid (EAA) levels in the floc samples were approximately half of the total amino acids, suggesting that these materials may be good supplemental sources of EAA for shrimp. The EAA structures of the bio-floc were similar to those of the formulated feeds used in that study. In general, the nutritional content of the bio-flocs seems to be directly related to the microbial communities that inhabit these aggregates.

A great efficiency in our experiment occurred in the presence of diatoms *T. weissflogii* and *C. muelleri* as food source for *L. vannamei* juveniles. The diatoms were the only component that differed in presence and quantity in relation to the other treatments (statement supported by the characterization and quantification of the microbial community by microscopy).

Microalgae are widely used in hatcheries as food source and to maintain water quality in farm ponds. Its importance as a source of polyunsaturated fatty acids (PUFAs) was assessed by Patil and Gislerød (2006). Microalgae have higher lipid stability in comparison with traditional PUFAs because they are naturally rich in vitamins and antioxidant carotenoids, and because their lipids are bio-encapsulated by the cell wall. They are an important source of food for aquaculture due to its nutritional value and the ability to synthesize and accumulate large amounts of omega-3 PUFAs.

Chaetoceros muelleri is a microalgae rich in essential nutrients for the larval stage of the shrimp and, together with *Thalassiosira weissflogii*, are commonly used in hatcheries (Brown et al. 1997). Analyses of the proximal composition show that *C. muelleri* has protein and total lipid content of 43.11 and 21.48%, respectively (Jaime-Ceballos et al. 2006).

Moss and Pruder (1995) compared the effects of pond water with selective removal of particles on the growth of *L. vannamei* juveniles, where the particles were removed by passing water through a series of mechanical filters and activated carbon. The shrimp has increased the growth rate in 53% in the presence of suspended particles between 0.5 and 5 μm compared to the value in clear water, and particles larger than 5 μm promoted 36% of further growth. Nearly half of the particulate organic carbon in pond water was in the form of centric and pennate diatoms, and they were an important item in the diet of shrimp because the shrimp feces containing large numbers of empty frustules. However, it was unclear whether they were eaten alive or as components of the aggregates. Diatoms are easily digestible by shrimp due to its low fiber content (Moss 2000); the post-larval shrimp digestibility (in vitro) of *C. muelleri* protein reaches 94% (Jaime-Ceballos et al. 2006).

The absence of response to diatoms in the mixed treatment may be due to better harvesting of single diatoms than bioflocs by the juveniles. The diatoms in the MM treatment became an integral part of the bio-flocs forming large aggregates, and maybe due to its different particle sizes the grazing pressure exerted by the small shrimp has been compromised.

Conclusions

Natural food has been of great importance for the performance of the shrimp in all treatments, but the availability of diatoms contributed to a better performance of *L. vannamei* juveniles in the treatment DM. Our results suggest the importance of the presence of these microalgae in culture medium during the nursery phase. However, the high concentration of suspended matter significantly reduces the light entrances into the system and consequently intervenes on photosynthesis and on the growth of microalgae, thus this is an important point that needs to be taken in account. Additional researches in this field concerning the microalgae management and its dynamics in intensive aquaculture systems are needed, more specifically the effect of other diatom/bio-floc ratios, testing other microalgae species, and in an attempt to maintain a constant presence of diatoms in the culture medium with minimal water exchange, without requiring successive inoculations. Also microbiological aspects need further investigation, particularly a detailed microbiological characterization of the bio-flocs, possible manipulation of the microbial community and the interactions between autotrophic and heterotrophic organisms.

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