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The contribution of diatoms to bioflocs lipid content and the performance of juvenile *Litopenaeus vannamei* (Boone, 1931) in a BFT culture system

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Abstract

This study aimed to evaluate the contribution of three diatom species on the lipid content of bioflocs, their permanence on the bioflocs and influence on the growth performance of juvenile shrimps. Juveniles of *Litopenaeus vannamei* were reared (30 days; three replicates per treatment) in biofloc systems inoculated with diatoms *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Conticribra weissflogii* (W), or biofloc only (BF, chlorophycean rich). Water quality parameters were monitored daily and the microbiota on days 1, 10, 20 and 30. The lipid content and fatty acid profiles of bioflocs were analyzed at the end of the experiment. Shrimp survival rate (99%) at treatment A was significantly higher than at BF. The bioflocs in A treatment presented the highest lipid content, differing significantly from BF and W. The content of EPA (20:5) (n-3) was significantly higher in A and lower in BF, while linoleic acid (18:2) (n-6) was significantly higher in BF. The results indicate that high cell density of diatoms can be successfully maintained with silicate addition in biofloc systems and that the pennate *A. coffeaeformis* and the centric *C. weissflogii* are potentially better suited than the pennate *C. closterium* as food supplements for shrimp diets in biofloc nurseries system.

Keywords: microbiota, nursery, rearing, *Litopenaeus vannamei*

Introduction

Biofloc Technology Culture Systems (BFT) refer to the rearing of aquatic organism in high stocking densities without water renewal using strong aeration and predominantly aerobic and heterotrophic biota that form microbial flocs (bacteria, protozoa, rotifers and microalgae; Avnimelech 2007). In this kind of system, the water quality is maintained and an additional food source for the farmed organisms is provided (De Schryver, Crab, Defoirdt, Boon & Verstraete 2008). This system facilitates the production of aquatic animals at high stocking densities in a sustainable and bio-secure fashion (Vinatea, Gálvez, Venero, Leffler & Browdy 2009), reducing feed costs and improving shrimp growth rate (Wasielesky, Atwood, Stokes & Browdy 2006; Otoshi, Moss & Moss 2011). Little is known about how to maintain microalgae in these systems with high concentration of suspended material and reduced light, and with regard to their influence on the performance of farmed shrimp.

Microalgae are widely used in hatcheries to maintain water quality (Hargreaves 2006) and as a food source due to their nutritional value and ability to synthesize and accumulate polyunsaturated fatty acids (PUFAs) including omega-3 series. Shrimp prefer diatoms than other microalgae (Ju, Forster, Conquest, Dominy, Kuo & Horgen 2008; Ju, Forster & Dominy 2009). It is well known that the presence of diatoms (Bacillariophyceae) improves the growth of the shrimp *Litopenaeus vannamei* in intensive ponds (Moss & Pruder 1995) and

their juveniles can be fed with *Chaetoceros* spp., easily digested due to their low fibre content (Moss 1994); Moss 2000;. Shrimps produced with bioflocs and diatoms (*Conticribra weissflogii* and *Chaetoceros muelleri*) consumed less food, presented a more efficient feed conversion ratio and higher weight gain than shrimps grown in bioflocs (Godoy, Odebrecht, Ballester, Martins & Wasielesky 2012).

In nature, the habit of diatoms may be benthic, epiphytic or planktonic. Most benthic or epiphytic diatoms inhabit dissolved organic matter-rich environments, whereas planktonic species are also successful in environments with relatively low concentrations of dissolved organic matter (Round, Crawford & Mann 1990). In culture ponds with a high concentration of organic matter, pennate diatoms (Bacillariophycidae, Fragillariophycidae) of benthic habit like *Navicula* sp. often dominate (Moss 1994). However, the maintenance of high densities of diatoms in biofloc systems is hampered due to the competition with bacteria for nutrients, high level of suspended material (Godoy *et al.* 2012) and the reduced light conditions (Baloi, Arantes, Schveitzer, Magnotti & Vinatea 2013).

Depending on the species and culture conditions, benthic diatoms contain an average of 6.4% to 14.5% total lipids of the dry weight and fatty acids are 16% to 22% of lipids; 39% to 48% being polyunsaturated fatty acids (PUFA; Gordon, Neori, Shpigel, Lee & Harpaz 2006). However, Khatoon, Banerjee, Yusoff and Shariff (2009) found that *Amphora* sp., grown in a Conway culture medium contained 438 crude protein, 234 g of lipids and 184 g of carbohydrate per kilogram of dry matter, and the profile of PUFAs included 148 g EPA and 25 g DHA for each kilogram of total fatty acids.

The purpose of this study was to evaluate the contribution of three diatom species on the lipid content of bioflocs, their permanence on the bioflocs and the influence on the growth performance of *Litopenaeus vannamei* juveniles in BFT. The pennate diatoms *Amphora coffeaeformis* and *Cylindrotheca closterium* and the centric *Conticribra weissflogii* were compared to tanks only with bioflocs.

Materials and methods

Selection, isolation and cultivation of microalgae and formation of bioflocs

The pennate diatoms *Amphora coffeaeformis* (C. Agarth) Kützing (25–35 μm) and *Cylindrotheca closterium* (Ehrenberg) Reimann & J. Lewin (4–5 × 30–35 μm) were isolated using a capillary micropipette in successive dilutions from bioflocs produced in raceway at the Marine Aquaculture Station of the Federal University of Rio Grande-FURG, using water from Cassino Beach, Brazil (32°12 S; 51°50 W). Taxonomic identifications were performed using clean frustules mounted on slides (Christensen 1988). Specimens of the diatom *Conticribra weissflogii* (Grunow) K. Stachura-Suchoples & D. M. Williams (= Thalassiosira *weissflogii*) (12–15 μm) were obtained from the strains collection of the Microalgae and Microorganisms Laboratory, FURG. The diatoms were kept in a germination chamber (BOD type) in Guillard F/2 medium (Guillard 1975), which was prepared with previously filtered sea water (glass fibre filter Whatman GF/F pore size 0.45 μm; diam. 47 mm) and sterilized in an autoclave (120°C; 30 min). Three tanks (1000 L) containing filtered water (5 μm pore Cuno filter) were inoculated with the diatoms after they had reached the exponential growth phase to develop bioflocs.

The nominal C:N of the daily organic matter additions to the tank was approximately 20:1, considering the carbon and nitrogen concentrations in the commercial diet and organic fertilizers (Chamberlain, Avnimelech, McIntosh & Velasco 2001). Water was not exchanged during the experiment. Organic fertilization was added as sugar cane molasses (37.46% C; 0.57% N) when the concentration of total ammonium nitrogen was equal or higher than 0.5 mg L^{-1}. Avnimelech (1999) and Ebeling, Timmons and Bisogni (2006) determined that 6 g C is needed to convert 1 g total ammonium nitrogen (TA-N) into bacterial biomass. Silicate (SiO_2) concentration was measured every 4 days and added, if lower than 1 mg L^{-1}, in equal proportion to nitrogen (1N:1Si) (Brzezinski 1985). On the first day, the experimental units were inoculated with biofloc and diatoms (initial cell density of 3×10^4 cells L^{-1}) and shrimp were stocked to maintain biofloc medium.

The experimental system

Inside a greenhouse, 12 plastic rectangular tanks (0.5 m^2; 200 L) were equipped with aeration systems and airstones. The inocula was prepared with diatoms and biofloc and pumped with a submerged pump into their respective experimental units. The

treatment BF was prepared from biofloc mature system in a raceway (TA-N = 0.17 mg L^{-1}; NO_2 = 0.02 mg L^{-1}; NO_3 = 0.86 mg L^{-1}; $PO^3{}_4$ = 0.26 mg L^{-1}; alkalinity = 127 mg L^{-1}; SiO_2 = 0.4 mg L^{-1}; chlorophyll *a* = 6.3 μg L^{-1}; total suspended solids = 138 mg L^{-1}).

The experimental design was completely randomized with four treatments: *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Conticribra weissflogii* (W) and Biofloc (BF), all in triplicate. Diatom and microorganisms were fixed in Lugol's iodine solution (2%) and counted in sedimentation chambers on days 1, 10, 20 and 30. At least 10 random fields were analyzed using a Zeiss Axiovert microscope equipped with phase contrast at a 400 × final magnification (Utermöhl 1958).

Groups of 195 juveniles of *L. vannamei* (390 m^{-2}; initial weight 0.22 ± 0.03 g) from hatcheries of the Marine Aquaculture Station-FURG were stocked in the experimental units. Shrimp were fed twice a day (08:00; 17:00 hours) with a commercial feed Potimar Active 38 (38% CP, 1.6 mm, Guabi ®, Campinas, SP, Brazil) offered on feeding trays (∅= 15 cm, 5 mm mesh size, one per tank). The initial feeding rate was set at 10% of the total biomass and adjusted to 5% at the end of the 30 days experiment (Jory, Cabreras, Durwood, Fegan, Lee, Lawrence, Jackson, McIntosh & Castañeda 2001).

Thirty shrimp from each experimental unit were individually weighed to adjust the feeding rate and returned to the tanks on days 1, 10 and 20; all shrimp were measured at the end of the experiment. The growth performance was evaluated according to the shrimps survival S (%) = [(initial n – final n)/initial n × 100], where n = number of shrimp; final weight FW (g) = ∑ final weight of live shrimp/total shrimp; weight gain WG (g) = ∑ FW−initial weight (g); specific growth rate SGR (%/day) = [(ln final weight (g)−ln initial weight (g))/time in days) × 100]; final biomass FB (g) = ∑ FW of live shrimp; feed conversion ratio FCR (%) = {[total feed consumed (g)/WG (g)] × 100 [assuming that all feed offered was consumed]}.

Water quality

Temperature, pH, salinity and dissolved oxygen were measured twice daily (08:00; 17:00 hours) in each experimental unit (YSI model ® 556 MPS-USA). Water samples were collected filtrated in glass-fibre filters (GF 50-A), to quantify: total ammonia nitrogen (N-TA: NH_3 + NH_4; UNESCO 1983) and nitrite (N-NO_2) were measured every 2 days, nitrate (N-NO_3; Aminot & Chaussepied 1983) and phosphate (P-PO_4; Aminot & Chaussepied 1983) were measured once a week, and the concentration of silicate (SiO_2; Strickland & Parsons 1972) was measured every 4 days using a digital Micronal B342 II spectrophotometer. The concentration of total suspended solids (TSS) was measured every 2 days by filtering water samples (50 mL) through glass-fibre filters (Whatman GF/F pore size 0.45 μm; diameter 47 mm) and estimating the difference between final and initial weight of each filter (Association of Official Analytical Chemists 2000) using a Sartorius balance (0.001 g). The concentration of chlorophyll *a* (Chla) was measured every 2 days using a calibrated Turner Design fluorimeter, following the method proposed by Welschmeyer (1994). Alkalinity was monitored weekly by titration with hydrochloric acid to the methyl orange endpoint (American Public Health Association 1998).

Lipid content and fatty acid profile of biofloc

At the end of the experiment, bioflocs samples were collected from each treatment, filtered through strainer (50 μm), washed with 20 mL (0.5 M ammonium formate) (Zhu & Lee 1997) concentrated and dried in an oven (60°C) to constant weight.

A total of 1 g biofloc and 6 mL chloroform: methanol (2:1 v/v) were added to a test tube at room temperature (20°C), subjected to magnetic stirring and afterwards centrifuged for 5 min. The organic phase was carefully collected, and the solvent was evaporated under reduced pressure. The lipid fraction was dried to a constant weight in an oven at 60°C. The total lipid fraction was calculated by determining the difference between the weight of the original and final flasks. (Zhu, Zhou & Yu 2002; D'Oca, Viêgas, Lemões, Miyasaki, Morón-Villarreyes, Primel & Abreu 2011). All the procedures were performed in triplicate.

Fatty acid methyl esters (FAMEs) were prepared by esterification with BF_3 10% in methanol (Metcalfe & Schmitz 1961). Briefly, the sample containing the lipid fraction (300 mg) was placed in a test tube to which 3 mL boron trifluoride/methanol was added. The mixture was heated in a water bath at 70°C for 20 min. The derivative mixture was washed in a separatory funnel with

15 mL hexane and 20 mL distilled water. The organic and aqueous phases were then separated. The organic phase containing the fatty esters was dried, and the solvent was evaporated at 50°C. Afterward, the fatty acid profile was determined using a GCMS-QP2010Plus chromatographic system (Shimadzu, Kyoto, Japan) equipped with a split/split less injector coupled with a mass detector. The detector operation temperatures were as follows: interface, 280°C and source, 230°C. The detection was set to the full-scan mode to scan 30 to 500 m/z in 0.2 s. The ionization mode used was electron impact at 70 eV. The operating conditions of the chromatograph were as follows: injector, 250°C; column, 80°C (initial temperature, 0 min0, followed by a gradient of 10°C min^{-1} up to 180°C and then 7°C min^{-1} to a final temperature of 330°C; He gas flow, 1.3 mL min^{-1}; pressure, 88.5 kPa; average linear velocity, 42 cm s^{-1}; injection volume, 1 ml with a split ratio of 1:100. The column used was 5% Crossbond diphenil/95% dimethyl polysiloxane (30 m × 0.25 mm × 0.25 μm, Restek, Bellefonte, PA, USA). The compounds were identified by their retention times and confirmed by mass spectrometry.

Statistical analysis

The shrimp growth performance was evaluated using a one-way ANOVA ($\alpha = 0.05$) after verification of the homoscedasticity (Levene's test) and normality of the data (Kolmogorov–Smirnov test). The specific growth rate, survival and fatty acid concentrations were arcsine transformed before analysis. Differences among the treatments were tested with Tukey's multi-comparison test (Sokal & Rohlf 1969). The abiotic parameters of water quality, concentration of Chl *a* and TSS were analyzed by nonparametric Kruskal–Wallis test.

Results

Water quality parameters

Water quality values are shown in Table 1. The mean values of temperature, dissolved oxygen, pH, salinity, alkalinity, total suspended solids (TSS), Chl *a*, nitrate and phosphate exhibited no significant differences between treatments. However, Total ammonia nitrogen (TA-N) showed significant differences between treatments, with higher values in W and C and lower in BF and A. The values of silicate were significantly higher in A, C and W and lower in the BF tanks. Nitrite concentrations were significantly lower in treatments W and BF and higher in the C tanks.

Microbiota

The microbiota included organisms of various trophic levels, among them, diatoms, chlorophyceae, cyanobacteria, heterotrophic protists and unidentified cells (Table 2). Diatoms remained high throughout the experiment except for BF, where centric (5–8 μm; 3.4×10^5 cells L^{-1}) and pennate (15–20 μm; 1×10^7 cells L^{-1}) diatoms were only found on the first day of experiment.

In our experiment, the three diatom species added to the culture system were successfully maintained. The pennate diatom *A. coffeaeformis* remained at high density (10^7–10^8 cells L^{-1}) throughout the experiment and the cells mostly formed aggregates, were attached to other households, epiphyton, or were found being preyed upon by other organisms. The centric diatom *C. weissflogii* showed similar cell density (10^7–10^8 cells L^{-1}) but the vast majority of their cells grew free, not forming bioflocs. The pennate diatom *C. closterium*, presented lower cell concentration (10^6–10^7 cells L^{-1}) and was also mainly found unattached to bioflocs.

The larger number of protozoans present in the treatments with *A. coffeaeformis* and *C. weissflogii* may be due to the extracellular polymeric substance (EPS) produced by these diatoms.

Cyanobacteria were present in all treatments: coccoid cyanobacteria were more abundant in treatment A (2.87×10^4 indiv L^{-1}) and less abundant in W (0.10×10^4 indiv L^{-1}), and filamentous cyanobacteria (Pseudanabaenaceae) were more abundant in treatment W (52.96×10^4 indiv L^{-1}) and less so in C (8.14×10^4 indiv L^{-1}). The chlorophytes *Planctonema* sp. (17.8×10^7 indiv L^{-1}) and *Ocystis* sp. (0.3×10^7 indiv L^{-1}) were denser in treatment BF.

Among heterotrophic protists, ciliates in three size ranges (C1 < 25 μm; C2 25–50 μm; C3 > 50 μm), flagellates (F1 < 10 μm; F2 10–20 μm) and dinoflagellates (D1 < 15 μm; D2 > 25 μm) were present. In treatment A, ciliates and dinoflagellates of all size ranges, flagellates F1 and spherical unidentified cells (UN A 5–7 μm; UN B 17–22 μm) were observed. Treatment

Table 1 Mean values ± standard deviation of the water quality parameters in the rearing of juvenile *L. vannamei* in treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Conticribra weissflogii* (W) and biofloc (BF)

	Treatments			
Parameters	**A**	**C**	**W**	**BF**
Temperature a.m. (°C)	25.99 ± 1.45	25.89 ± 1.43	25.89 ± 1.46	25.64 ± 1.35
Temperature p.m. (°C)	28.67 ± 2.59	28.76 ± 2.36	28.46 ± 2.39	28.91 ± 2.76
DO a.m. (mg L^{-1})	5.80 ± 1.18	5.84 ± 1.25	5.67 ± 1.26	5.76 ± 1.55
DO p.m. (mg L^{-1})	5.09 ± 1.61	5.17 ± 1.56	5.08 ± 1.58	5.03 ± 1.594
pH a.m.	8.08 ± 0.16	8.08 ± 0.19	8.06 ± 0.13	8.05 ± 0.20
pH p.m.	8.17 ± 0.23	8.14 ± 0.24	8.12 ± 0.22	8.22 ± 0.34
Salinity (g L^{-1})	37.20 ± 1.36	37.61 ± 1.01	37.36 ± 1.28	37.00 ± 1.34
Chl *a* (μg L^{-1})	219.9 ± 120.76	175.0 ± 137.82	199.5 ± 84.31	282.5 ± 297.99
Alkalinity (mg L^{-1} $CaCO_3$)	191.5 ± 58.83	172.5 ± 45.57	221.6 ± 72.37	189.9 ± 41.70
TSS (mg L^{-1})	260.1 ± 183.95	261.7 ± 197.28	270.4 ± 205.63	245.2 ± 152.76
TA-N (mg L^{-1})	1.1 ± 1.18[a]	1.2 ± 1.04[b]	2.0 ± 2.64[b]	0.8 ± 1.01[a]
NO_2- N (mg L^{-1})	1.3 ± 2.97[a]	3.4 ± 5.47[b]	0.6 ± 2.01[a]	1.3 ± 2.98[a]
NO_3- N (mg L^{-1})	4.2 ± 6.65	3.4 ± 4.98	1.5 ± 2.37	2.5 ± 4.84
PO^3_4 – P (mg L^{-1})	1.0 ± 0.69	1.1 ± 0.71	1.0 ± 0.65	1.0 ± 0.73
Silicate (mg L^{-1})	0.5 ± 0.30[a]	0.6 ± 0.71[a]	0.4 ± 0.40[a]	0.2 ± 0.28[b]

DO, dissolved oxygen; TSS, total suspended solids; Chl *a*, chlorophyll *a*; TA-N, total ammonium nitrogen; NO_2-N, dissolved inorganic nitrite; NO_3-N, dissolved inorganic nitrate; PO_4^3-P, phosphate.
Different letters in the same row indicate significant differences ($P < 0.05$).

W also included flagellates, ciliates, unidentified cells of both sizes and dinoflagellates D1. Flagellates, unidentified UNA and UNB, ciliates C1 and C2 and dinoflagellates D1 were observed in treatment C. Ciliates, unidentified of all size classes, flagellates F1 and dinoflagellates D1 were present in the treatment BF tanks.

Lipid content and fatty acid profile of biofloc

The lipid content varied from 2.64 (± 0.15) to 5.11 (± 1.02) (Table 3). Treatment A exhibited the highest lipid content of biofloc (5.11%), differing significantly from treatments BF and W. The concentration of the fatty acids considered essential for farmed organisms eicosapentanoic acid (EPA; 20:5) (n-3) was significantly higher in treatment A (4%) and lower in BF (1%), while the linoleic acid (18:2) (n-6) was significantly higher in treatment BF (12.6%).

Growth performance of shrimp

The mean survival rate of the shrimp (88–99%) exhibited differences among treatments, being significantly higher in A and lowest in BF (Table 4). The shrimp consumed less feed in treatment A, but the apparent FCR did not differ significantly among treatments. The lowest mean values of final weight, weight gain and the poor FCR were recorded for treatment C, although there were no significant differences in each index.

Discussion

Water quality

All the water quality parameters in the culture systems remained within the recommended range for successful growth of *Litopenaeus vannamei* (Ponce-, Martinez-Palacios & Ross 1997; Van Wyk & Scarpa 1999). The adequate levels of N-TA, N-NO_2 and N-NO_3 throughout the trial probably resulted from the removal of these compounds by the microbial community (Van Wyk & Scarpa 1999; Lin & Chen 2001, 2003).

In biofloc systems, sudden changes of N-TA and N-NO_2 can occur as well as the accumulation of N-NO_3, due to variations in the microbial biomass during the culture period (Cohen, Samocha, Fox, Gandy & Lawrence 2005), even with a higher C:N ratio (15–20:1) (Gao, Shan, Zhang & Bao 2012b). Khatoon *et al.* (2009) observed higher N-TA and N-NO_2 concentrations in the control than in the groups with diatoms addition during the culture of *Penaeus monodon*, and Sanchéz, Fox, Gatlin &

Table 2 Mean, minimum and maximum density of the microbiota in treatments *Amphora coffeaeformis* (A), *Cylintrotheca closterium* (C), *Conticribra weissflogii* (W) and biofloc (BF)

	Treatments			
Taxon	**A**	**C**	**W**	**BF**
Diatoms (10^7 cells L^{-1})				
A. coffeaeformis (25–35 μm)	5.4 (2.2 – 13.2)	*	*	*
C. closterium [4–5(30–35 μm)]	*	2.2 (0.3 – 3.5)	*	*
C. weissflogii (12–15 μm)	*	*	5.3 (1.6 – 14.2)	*
Centric (5–8 μm)	*	*	*	<0.034
Pennate (15–20 μm)	*	*	*	<1.0
Chlorophyceae (10^7 indiv L^{-1})				
Planctonema sp	*	*	*	17.8 (6.5 – 24.1)
Ocystis sp.	*	*	*	0.3 (0 – 1.0)
Cyanobacteria (10^4 indiv L^{-1})				
Coccoid colonies	2.87	0.44	0.10	0.51
Trichomes Pseudanabaenoidae (25–100 μm)	12.20	8.14	52.96	41.59
Heterotrophic protists (10^6 cells L^{-1})				
Ciliates I (<25 μm)	2.3 (0.9 – 6.3)	0.8 (0 – 2.5)	1.6 (0 – 8.2)	0.6 (0 – 0.9)
Ciliates II (25–50 μm)	95.2 (0.4 – 1.1)	0.1 (0 – 0.4)	0.5 (0 – 2.0)	0.1 (0 – 0.1)
Ciliates III (>50 μm)	0.5 (0 – 1.5)	*	0.1 (0 – 2.8)	1.6 (0.2 – 4.4)
Flagellates I (<10 μm)	184.8 (0 – 290)	14.2 (0 – 30.7)	54.4 (0 – 68.5)	11.9 (2.0 – 22.6)
Flagellates II (10–20 μm)	*	1.5 (0 – 2.2)	1.6 (0 – 2.5)	*
Dinoflagellates I (<15 μm)	3.6 (0.2 – 6.4)	1.0 (0 – 2.3)	3.6 (0 – 6.1)	5.2 (0.1 – 16.8)
Dinoflagellates II (>25 μm)	0.8 (0.6 – 1.2)	*	*	*
Unidentified (10^6 cells L^{-1})				
UN UNA (<10 μm)	4.4 (2.0 – 7.7)	8.5 (0 – 19.8)	0.7 (0 – 1.1)	10.3 (4.5 – 16.1)
UN B (>10 μm)	6.4 (0.2 – 22.4)	2.6 (0 – 11.5)	13.3 (0 – 17.8)	4.2 (0.9 – 11.7)

*Cell density lower than 10^5 cells L^{-1}.

Table 3 Mean percentage ± standard deviation of lipid content in relation to the dry biomass and fatty acids of the biofloc in treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Conticribra weissflogii* (W) and biofloc (BF)

	Treatments			
	A	**C**	**W**	**BF**
Lipid content (%)	5.11 ± 1.02^{a}	4.49 ± 0.70^{ab}	2.64 ± 0.15^{c}	3.79 ± 0.29^{b}
Fatty acid profile				
14: 0	6.50 ± 4.24^{a}	7.6 ± 0.14^{b}	8.95 ± 2.47^{b}	3.10 ± 0.85^{a}
15: 0	0.95 ± 0.21^{a}	1.1 ± 0.00^{a}	1.55 ± 0.35^{b}	0.85 ± 0.07^{a}
16: 0	33.90 ± 5.65	39.5 ± 1.41	36.60 ± 3.81	35.50 ± 1.48
17: 0	0.30 ± 0.10	0.7 ± 0.30	0.65 ± 0.07	0.60 ± 1.20
18: 0	6.65 ± 5.44	6.05 ± 0.77	6.35 ± 2.05	4.25 ± 0.21
24: 0	0.30 ± 0.03	0.6 ± 0.04	0.65 ± 0.07	0.70 ± 0.00
16: 1	17.76 ± 5.18	19.15 ± 0.42	18.35 ± 3.04	7.80 ± 0.14
18: 1C	18.95 ± 8.69^{a}	7.4 ± 0.84^{b}	8.55 ± 3.60^{b}	21.40 ± 0.84^{a}
18: 1T	5.00 ± 2.12^{a}	6.05 ± 1.30^{b}	7.35 ± 1.20^{b}	7.80 ± 0.28^{b}
18: 2	4.35 ± 0.49^{b}	3.5 ± 0.14^{b}	4.60 ± 2.68^{b}	12.60 ± 0.56^{a}
20: 5	4.00 ± 2.40^{a}	2.45 ± 0.07^{b}	1.85 ± 0.49^{b}	1.00 ± 0.28^{c}

Different letters in the same row indicate significant differences ($P < 0.05$).

Lawrence (2012) also observed significant differences in N-NO_2 concentrations in tanks with and without the addition of diatoms in *L. vannamei* culture. When comparing tanks receiving biofloc, tanks with diatoms and mixed tanks (biofloc and diatoms), Godoy *et al.* (2012) noted significant differences in water quality variables. The diatoms probably absorbed a significant part of the nutri-

Table 4 Mean values ± standard deviation of the performance parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Conticribra weissflogii* (W) and biofloc (BF)

	Treatments			
Parameters	**A**	**C**	**W**	**BF**
Initial mean weight (g)	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03
Final mean weight (g)	2.43 ± 0.89	1.75 ± 0.45	2.58 ± 0.92	2.15 ± 0.59
Survival (%)	99.5 ± 0.89^{a}	92.8 ± 4.2^{ab}	94.8 ± 1.4^{ab}	88.0 ± 11.4^{b}
Weight gain (g)	2.23 ± 0.89	1.55 ± 0.45	2.38 ± 0.92	1.95 ± 0.59
Final mean biomass (g m^{-2})	944.1 ± 349.7	643.2 ± 187.9	955.8 ± 348.6	744.2 ± 263.0
Specific growth rate (%)	5.15 ± 0.01	5.14 ± 0.01	5.15 ± 0.01	5.15 ± 0.01
Feed Conversion Ratio (%)	0.84 ± 0.37	1.08 ± 0.54	0.96 ± 0.35	1.03 ± 0.63

Different letters in the same row indicate significant differences ($P < 0.05$).

ents in the autotrophic systems, but less in the presence of biofloc due to the high concentration of suspended material reducing the light penetration, which in turn likely reduces the nutrients absorption rates of the diatoms.

Silicate, required by diatoms for building their siliceous frustules, remained relatively high in treatments where this nutrient was added to avoid limitation (A, C and W). In the BF treatment without silicate addition, this nutrient was close to minimum required for diatom growth (0.1 mg L^{-1}, Reynolds 2006). We may conclude that silicate addition was essential for the growth and maintenance of high diatom cell density in the biofloc system, and that the lower silicate concentration in BF caused the drastic diatom reduction in this treatment. It is generally believed that abundance of diatoms is promoted by fertilization with nitrate and silicate (Boyd 2009) but to our knowledge no systematic studies were conducted showing the silicate limitation and influence of silicate addition in culture tanks of biofloc systems.

Bioflocs: microbiota, lipid content and fatty acid profile

Several groups of microalgae are important as food source for zooplankton in aquaculture environments, transferring their nutrients to higher levels of the food chain (Ray, Seaborn, Leffler, Wilde, Lawson & Browdy 2010).

Among the microalgae, diatoms present excellent nutritional value contributing with essential amino acids and polyunsaturated fatty acids (PUFAs) from the omega-3 series (Ju *et al.* 2008). The nutritional value of diatoms is provided to a great extent by the fatty acid composition, the high lipid content (13–19.7%; Renaud, Thinh & Parry 1999) and levels of PUFAs (26.1–47.2%), especially the essential ones (20:5) (n-3) **(**Brown, Jeffrey, Volkman & Dunstan 1997; Renaud *et al.* 1999**),** while chlorophyceans are poor in these fatty acids (0–3%) (Brown *et al.* 1997). Apart from this, diatoms digestion by shrimp is facilitated by their low fibre content (Moss 2000) and are preferred when compared to other microalgae (Ju *et al.* 2008, 2009).

In our experiment, the three diatom species added to the culture system were successfully maintained. The pennate diatom *A. coffeaeformis* remained at high density (10^{7}–10^{8} cells L^{-1}) throughout the experiment and the cells mostly formed aggregates, were attached to other households, epiphyton, or were found being preyed upon by other organisms. The centric diatom *C. weissflogii* showed similar cell density (10^{7}–10^{8} cells L^{-1}) but the vast majority of their cells grew free, not forming bioflocs. The pennate diatom *C. closterium*, presented lower cell concentration (10^{6} –10^{7} cells L^{-1}) and was mainly found unattached to bioflocs. The larger number of protozoans present in the treatments with *A. coffeaeformis* and *C. weissflogii* may be due to the extracellular polymeric substance (EPS) produced by these diatoms.

The EPS matrix provides a stable microenvironment and optimal conditions for cell growth (Decho 2000). It is important for the adhesion to substrates (Daniel, Chamberlain & Jones 1987), locomotion (Edgar & Pickett– Heaps 1984) and provides resistance to toxins (Decho 1990). The benthic diatoms produce different types of EPS, which vary in structure and composition of carbon

hydrates, according to irradiance, nutrients availability and the vertical migration rhythm associated with photosynthesis and cell growth phase (Smith & Underwood 2000; Staats, Stal, DeWinder & Mur 2000; De Brouwer & Stal 2002). It was shown that benthic diatoms produce higher EPS concentration when nutrients are limiting (Underwood, Boulcott, Raines & Waldron 2004).

The composition and abundance of phytoplankton in shrimp farming systems are extremely variable, but cyanobacteria and chlorophyceae, commonly found in marine and brackish biofloc systems, were present in this study. Ray *et al.* (2010) and Vinatea, Gálvez, Browdy, Stokes, Venero, Haveman, Lewis, Lawson, Shuler and Leffler (2010) also found a predominance of cyanobacteria and chlorophyceans in relation to other plankton groups in biofloc systems. The prevalence of the cyanobacteria in shrimp culture is probably related to the accumulation of phosphorus and the eutrophication of the culture environment, as documented by Emerenciano, Ballester, Cavalli & Wasielesky 2011).

In biofloc systems, heterotrophic protists make part of the microbial aggregate (Ray *et al.* 2010). However, factors such as the addition of feed and diatoms appear not to influence the development of these organisms and their composition was similar in most treatments. The ciliates play an important role in energy flow in aquatic ecosystems as predators of microalgae, bacteria and fungi and as a food source for metazoans, fish and shrimp larvae (Nagano & Decamp 2004). The abundance and diversity of ciliates are good indicators of water quality and ecosystem dynamics (Decamp, Warren & Sánchez 1999). The ciliates stand out as bioindicators in external filter systems (Decamp, Cody, Conquest, Delanoy & Tacon 2003), as they predate bacteria and increase the absorption of particles by the biofilm (Eisenmann, Letsiou, Feuchtinger, Beisker, Mannweiler, Hutzler & Arnz 2001). These authors observed a succession of ecological groups in the BFT system with a tendency towards dominance by free-living ciliates in the early stages and a transition to scrapers after the second week of production. The flagellates are taxonomically diverse including autotrophic and heterotrophic species. Flagellates are source of highly unsaturated fatty acids (HUFAs) and sterols (Decamp & Nagano 2001) and their importance together with ciliates in the diet of *Farfantepenaeus paulensis* larvae was demonstrated (Thompson, Abreu & Cavalli 1999).

The recommended lipid level for crustaceans is less than 10% (D'Abramo 1997). The nutritional value of microalgae as a feed is influenced to a great extent by the fatty acid composition of their lipids. In diatoms, the lipid content (13–19.7%; Renaud *et al.* 1999) offer high levels of PUFAs (range 26. 1–47. 2%), especially the essential 20:5 (n-3) **(**Brown *et al.* 1997; Renaud *et al.* 1999**).** In the present work, the lipid content of the biofloc was within the recommended level (A, 5.1%; C, 4.5%; BF, 3.8%; W, 2.6%). Other studies have found lower values (0.6%, Tacon, Cody, Conquest, Diyakaran, Forster & Decamp 2002; 0.5%, Wasielesky *et al.* 2006; 1.2–2.3; %, Ju *et al.* 2008), 2.1–3.6%, Maicá, Borba & Wasielesky 2011), but also higher ones (12.5%, McIntosh, Samocha, Jones, Lawrence, Mckee, Horowitz & Horowitz 2000).

Among the essential fatty acids, the concentration of eicosapentanoic acid (EPA) (20:5) (n-3) was significantly higher in treatment A (4%), and the concentration of linoleic acid (18:2) (n-6) was significantly higher in treatment BF (12.6%). Penaeid shrimps lack synthesis of (n-3) and (n-6) fatty acids and exhibit a low rate of phospholipid biosynthesis, thus requiring these nutrients in a supplemented diet (Tacon 1987). In this experiment, fatty acids (20:5) (n-3) were supplied in the biofloc, especially in treatment A.

According to Gao, Yu, Liang and Gao (2012a), the lipid content in *A. coffeaeformis* from tidal mud are 26.1% dry weight. Khatoon *et al.* (2009) found *Amphora* sp. contained the highest amount of PUFA, EPA and DHA when compared with *Navicula* sp. and *Cymbella* sp.

Growth performance of shrimp

The natural productivity in biofloc systems provides supplemental food resources, reducing feed costs and improving shrimp growth rate (Wasielesky *et al.* 2006; Otoshi *et al.* 2011). The ability of *L. vannamei* to utilize natural productivity in BFT systems is well documented (Burford, Thompson, McIntosh, Bauman & Pearson 2003; Wasielesky *et al.* 2006; Decamp, Conquest, Cody & Forster 2007; Otoshi *et al.* 2011; Godoy *et al.* 2012).

Our results show the beneficial effects of the addition and permanence of the diatoms *A. coffaeformis* and *C. weissflogii* in the biofloc system on nutrition quality and growth of juvenile shrimp. Improved growth of *L. vannamei* in intensive

systems with pennate and centric diatoms was also observed by Moss and Pruder (1995). Khatoon *et al.* (2009) found that the use of diatoms increased the survival rate and growth of postlarvae, as indicated by the higher protein, lipids, PUFA, EPA and DHA content of the shrimp raised in tanks with substrates coated with mixed of *Amphora*, *Navicula* and *Cymbella* than those reared in control tanks. Also in semi-intensive systems, high concentration of diatoms (*Navicula* sp.) coincided with higher growth percentage (22–390%; Otoshi *et al.* 2011).

The presence of microalgae in the microbial floc plays a key role in improving shrimp growth rate (Ju *et al.* 2009). The survival rate was highest in the A (99%) and W (95%) indicating that shrimp juveniles are favoured when commercial feed and diatoms are provided. Also, in heterotrophic microbial-based systems the addition of diatoms provided higher survival rates (90-97%; Godoy *et al.* 2012).

The lower FCR (0.84 and 0.96) in A and W treatments indicates that the addition of *Amphora coffeaeformis* and *Conticribra weissflogii* resulted in optimal food sources for juveniles shrimp. Sánchez, Fox, Gatlin and Lawrence (2012) reported that microalgae present in the culture system significantly improved weight gain and FCR of shrimp, thus potentially reducing the feed cost associated with shrimp production. It is known that *L. vannamei* has a good ability to utilize the microbial community present in biofloc systems as a food source (Kent, Browdy & Leffler 2011; Xu, Pan, Zhao & Huang 2012). However, the availability of these microbial aggregates alone is insufficient for the satisfactory growth of shrimp.

In intensive systems, beneficial microbial community should be developed and sustained (Ray *et al.* 2010). In general, it is difficult to maintain high densities of diatoms in biofloc systems, because of competition with bacteria for nutrients, high levels of suspended matter and light reduction (Godoy *et al.* 2012). Our results showed that when adding diatoms *A. coffeaeformis* and *C. weissflogii* the control of silicate concentration is necessary in the management strategy of the BFT.

Conclusions

The results highlight the great potential of diatoms *Amphora coffeaeformis* and *Conticribra weissflogii* as food supplement for shrimp diet in biofloc nurseries system. The presence of *A. coffeaeformis* showed highest lipid content of the flocs, mainly EPA. Significant higher survival rate, best weight gain, average final biomass and FCR of *L. vannamei* juveniles were observed in the treatments with the diatoms *A. coffeaeformis* and *C. weissflogii* compared to *C. closterium* and biofloc only. The successful maintenance of high cell density of the diatoms in the biofloc culture system required the addition of silicate, which otherwise was reduced to limiting levels for the growth of diatoms.

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