Phytoplankton evolution during the creation of a biofloc system for shrimp culture.

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Abstract

Microalgae play a key role in the dynamics of biofloc technology aquaculture systems. Some phytoplankton groups, such as diatoms, are desired for their high nutritional value and contribution to water quality. Other groups, such as cyanobacteria, are undesired because of their low nutritional value and capacity of producing toxins. So, monitoring the phytoplankton community structure and succession is key for managing biofloc systems. However, research on phytoplankton in these systems is scarce and mostly done by microscopy. The primary objective of this research was to estimate phytoplankton community structure in shrimp biofloc system water samples, using high-performance liquid chromatography methods and CHEMTAX software. The major groups present in our system were diatoms, euglenophytes, cyanobacteria and chlorophytes, while dinoflagellates were only remarkable at the initial period. We observed a clear dominance of diatoms all along the 5 months that comprised a complete biofloc system culture. The characteristic succession of autotrophic processes by heterotrophs of the biofloc systems, was observed by the reduction of net primary production. Light intensity played a key role in determining the phytoplankton composition and abundance. Algal pigment analyses using high-performance liquid chromatography and subsequent CHEMTAX analysis in water samples was useful for estimating the phytoplankton community structure in the biofloc systems. However, we found some limitations when the biofloc system was in heterotrophic mode. Under these conditions, some dinoflagellates and cyanobacteria behaved as heterotrophs and lost or decreased their biomarkers pigments. So, further research is needed to increase knowledge on the accuracy of high-performance liquid chromatography /CHEMTAX under these conditions.

Keywords: CHEMTAX*;* high-performance liquid chromatography; *Litopenaeus vannamei*; pigments

Introduction

Biofloc technology (BFT) has been defined as an environmentally friendly aquaculture technique based on *in situ* microorganism production, and it is considered the "blue revolution" in aquaculture (Emerenciano et al. 2017). In BFT systems, physicochemical variables of the culture are modified to favor the proliferation of particular biotic communities, for both, improving the recirculation of nutrients (maintaining the water quality), and as direct food source for the cultured organisms (Avnimelech 2007). Biofloc systems are highly dynamic, and the physical, chemical, and biological interactions that occur into these systems are complex (Natrah et al. 2014; Emerenciano et al. 2017). Ju et al. (2008) pointed out that the relationship between environmental factors and the microbial community (bacteria and microalgae) present in the floc of aquatic culture ecosystems is one of the least understood areas of crustacean aquaculture. Knowledge of the abundance, composition and succession of the phytoplankton is a prerequisite for the successful management of BFT system (Lukwambe et al. 2015). Microalgae have an important nutritional value, that depends on its size and shape, digestibility, biochemical composition, and bioactive compounds as enzymes, vitamins, antioxidants, etc. (Emerenciano et al. 2017). We can classify phytoplankton into desired groups, because of their nutritional value and their positive effect on water quality, and not desired groups, because of their low nutritional value and bad effect on water quality (toxin production). In this sense, diatoms are one of the most desired groups, because they can enhance the contents of essential amino acids and highly unsaturated fatty acids in shrimp tissue, and increase shrimp production (Becerra-Dórame et al. 2011; Lukwambe et al. 2015; Brito et al. 2016). On the other side, cyanobacteria are generally considered an undesired group, which is favored by excessive concentrations of nitrogen and phosphorus. Cyanobacteria may produce uncontrolled blooms, that produce toxic compounds to aquatic animals, and can cause unpleasant flavors in cultured species (Sinden and Sinang 2016; Emerenciano et al. 2017). Their dominance in shrimp ponds has caused heavy economic losses (Ju et al. 2008).

Scarce studies on phytoplankton community structure have been reported on crustacean aquaculture in BFT (Casé et al. 2008; Ju et al. 2008; Becerra-Dórame et al. 2011; Schrader et al. 2011; Brito et al. 2016). Moreover, the majority focus on a short time period and do not study the full culture period. For instance, Lukwambe et al. (2015) studied the effect in shrimp production of the application of probiotics during one month; and both Becerra-Dórame et al. (2011) (microcosms experiment) and Brito et al.

(2016) (indoor trial) conducted experiments during 28 days to evaluate the productive response of the Pacific white shrimp (*Litopenaeus vannamei*). According to Ju et al. (2008), a major reason for this scarce information is the lack of rapid analytical techniques to monitor changes in the community structure of microorganisms. Almost all the BFT research used microscope methodology for studying phytoplankton composition and abundance. Only Ju et al. (2008) used an alternative methodology. They used high-performance liquid chromatography (HPLC) for detecting photopigments in samples, these pigments are algal biomarkers that allow to estimate the phytoplankton community structure. Diagnostic photopigment analyses are able to detect significant changes in phytoplankton and are routinely used for monitoring programs designed to observe trends in water quality in response to nutrient enrichment (Niemi et al. 2004; Sebastiá et al. 2012). Conventional microscope methodology has some disadvantages that can be overwhelmed using HPLC. Microscopy is time-consuming, labor intensive, potentially vulnerable to subjective judgements, and requires advanced taxonomic skills and expertise; while HPLC has proved to be rapid, reproducible, and cost-effective (Duarte et al. 1990; Schlüter et al. 2006; Ju et al. 2008; Schlüter et al. 2016). Moreover, algal groups with potentially harmful effects, such as cyanobacteria and dinoflagellates, could also be present at a low abundance in the phytoplankton community so microscope analysis could not detect them, while the high accuracy of the pigment method is able to detect all the functional groups present (Duarte et al. 1990; Schlüter et al. 2006; Schlüter et al. 2016). Ju et al. (2008) tested HPLC methodology during one week and used a multiple regression model to estimate the contribution of each phytoplankton group to total chlorophyll *a*. Multiple regression models were a common methodology during their research, but in recent years CHEMTAX software is mostly applied (Latasa et al. 2010; Garrido et al. 2011; Higgins et al. 2011; Ahmed et al. 2016) and it is recommended by the SCOR (Scientific Committee on Oceanic Research) (Roy et al. 2011).

The primary objective of this research was to estimate phytoplankton community structure in shrimp BFT water samples, using HPLC analysis and CHEMTAX software. The secondary objectives were to study the phytoplankton changes in the culture system during a complete biofloc culture cycle with high sampling frequency (weekly), and to analyze the environmental parameters that can affect the phytoplankton population. This research was developed in Gandia (València, Spain) from May to October 2016.

Materials and methods

Shrimp

Postlarvae white shrimp (PL) were purchased from a commercial laboratory (Shrimp Improvement Systems, SIS, Florida, USA), they were certificated as free of pathogen. PLs were moved to Universitat Politècnica de València (UPV) – Spain for BFT system experiment development. The *L. vannamei* shrimp were transferred to a nursery laboratory, where the PLs grow up to 0.0675 ± 0.0433 g, at which time the experiment started.

The shrimp were distributed in 9 square tanks filled with 2,250 L of water and with a surface of 3.2 m^2 each tank. Each tank was filled with disinfected seawater which had a salinity level of 22.5. The tanks were located in a greenhouse and constantly individually aerated. Shrimp density was 200 shrimp/m^2. The greenhouse system is very useful, in Mediterranean area, for shrimp culture during the cold and temperate seasons. During the hot season, the greenhouses need to be covered with different awnings to keep water temperature between 28 - 32ºC. Temperatures higher than 32ºC are critical for shrimp culture (Van Wyk and Scarpa 1999). In our study, water temperature was kept to optimum values with the following system: 1) at the beginning of the study period (May, 5) the greenhouse roof was covered with a white awning, 2) on day 59 (July, 8) the awning was substituted by a black one, and 3) on day 136 (September, 29) the black awning was removed, due to lower environmental temperature at the end of summer.

Every day the shrimp were fed with commercial feed (Le Gouessant) specifically designed for *L. vannamei*. Feed amount was calculated according to the shrimp biomass, according to Jory et al. (2001). Feeding was provided twice a day, 40% in the morning and 60% in the afternoon, and distributing the feed in feed trays.

Biofloc development was achieved following the methodology proposed by Avnimelech (1999) and Ebeling et al. (2006). The initial fertilization of the system was done with sucrose, with a theoretical 15:1 carbon/nitrogen ratio. During the experiment, sucrose was added when the ammonia reached a concentration greater than 1 mg/L, maintaining a carbon/nitrogen ratio of 6:1. Renewal of the water during the experiment was minimal and was performed when the nitrite level reached 8 mg/L. Higher nitrite levels could cause mortality in the shrimp *L. vannamei*, as indicated by Lin and Chen (2003).

Environmental parameters

Dissolved oxygen (DO), salinity and temperature were monitored in situ, using a multi-parameter probe (YSI ProODO and WTW Multi 340i respectively) twice a day. pH was measured once a day using pH-Meter BASIC 20^{+} the Crison.

Every two days an aliquot of water was collected to determine the concentration of total dissolved ammonia (N-TA mg/L) using the methodology described by Baumgarten et al. (2010), nitrites ($N\text{-}NO_2^-$ mg/L), using the methodology of Bendschneider and Robinson described in Baumgarten et al. (2010), the nitrates ($N\text{-}NO_3^-$ mg/L) were analyzed by means of the difference between nitrites plus nitrates using the methodology described by Grasshof (1976) and phosphates ($P\text{-}PO_4^{3-}$ mg/L) were analyzed following the colorimetric reaction described by Murphy and Riley (1962).

The biofloc volume (mL/L) and light intensity (lux) were measured weekly. The biofloc volume (BV) was determined by placing one litre of water in an Imhoff cone, following the methodology described by Avnimelech (2007). The light intensity was measured with a luxometer (Delta OHM HD9221).

Biological parameters

Samples for phytoplankton pigment analysis were filtered on GF/F fiberglass filters (25 mm diameter) once a week. Pigments were extracted using acetone (100% HPLC grade) and were measured using reverse-phase high-performance liquid chromatography (HPLC). The HPLC method employed was that proposed by Wright et al. (1991) slightly modified as per Hooker et al. (2001). The system was calibrated with external standards obtained commercially from the DHI Water and Environment Institute (Hørsholm, Denmark). Phytoplankton signature pigments analyses are able to detect significant changes in phytoplankton community composition over a broad range of time scales (Sebastiá et al. 2012).

In order to identify the phytoplankton groups present in the biofloc system, we observed a sample each tank in the microscopy some weeks, the aim of these samples is supplementing information on group presence for CHEMTAX. Utermohl (1985) was used for micro and macroplanktonic cell size. Phytoplankton samples were fixed with formaldehyde, concentrated according to UNE EN15204:2006, based on Utermohl (1985), and qualitatively examined under a LEICA DM IL inverted microscope.

Once the concentration of important photosynthetic pigments was determined, the phytoplankton community was studied using the CHEMTAX program (Mackey et al. 1996) version 1.95 (S. Wright, pers. comm.) to obtain the contribution to total chlorophyll *a* from the phytoplankton groups identified with microscopy. In order to identify groups of samples with similar characteristics, a cluster analysis was

performed using STATGRAPHICS Centurion XVI.I to group samples according to pigments concentration. City block distances were calculated and samples clustered according to Ward's method. Pigment samples were separated into two subsets because it is highly recommended to apply CHEMTAX to dataset where pigment ratios within the different groups do not change (Latasa et al. 2010). CHEMTAX was applied independently to each subset to obtain the contribution of eight phytoplankton groups to the Chl*a* stock: diatoms, dinoflagellates, euglenophytes, chlorophytes, cryptophytes, prymnesiophytes, prasinophytes, and cyanobacteria. The procedure was described in Latasa et al. (2010) and a complete description can be found in Sebastiá and Rodilla (2013). The final matrix used to estimate the contribution of the different groups to Chl*a* stock is presented in Table 1.

During all the experiment net primary production of the water column (mgO_2 / (L·h)) was measured once a week, using the equation (1) proposed by Strickland (1960). Three transparent bottles were filled with water culture, and were left dangling to 3 cm under water surface. In each tank, average net primary productivity was calculated 8 hours after. Net primary productivity informs about the trophic state of the biofloc system, a positive net primary production indicates that the system is autotrophic, while a negative one indicates that the system is heterotrophic.

$$\text{Net primary production } \left(\frac{\text{mg}\,O_2}{\text{L}\cdot\text{h}}\right) = \left(\frac{\text{final}\,O_2 \text{ light bottle} - \text{initial } O_2 \text{ light bottle}}{\text{time}}\right) \quad (1)$$

Table 1. Matrices of pigment to Chl*a* ratios obtained from CHEMTAX for the samples of both clusters. Per correspond to peridinin, 19'But to 19'butanoyloxyfucoxanthin , Fuc to fucoxanthin, 19'Hex to 19'hexanoyloxyfucoxanthin, Neo to neoxanthin, Pras to prasinoxanthin, Viol to violaxanthin, Allo to alloxanthin, Lut to lutein, Zea to zeaxanthin and Chl*b* to chlorophyll *b* .

Class / Pigment	**Per**	**19'But**	**Fuc**	**19'Hex**	**Neo**	**Pras**	**Viol**	**Allo**	**Lut**	**Zea**	**Chl*b***
Diatoms											
Cluster 1	-	-	0.290	-	0.001	-	-	-	-	-	-
Cluster 2	-	-	0.387	-	0.001	-	-	-	-	-	-
Dinoflagellates											
Cluster 1	0.569	-	-	0.018	-	-	-	-	-	-	-
Cluster 2	0.333	-	-	0.025	-	-	-	-	-	-	-
Euglenophytes											
Cluster 1	-	-	-	-	0.017	-	-	-	-	-	0.427
Cluster 2	-	-	-	-	0.030	-	-	-	-	-	0.587
Chlorophytes											
Cluster 1	-	-	-	-	0.021	-	0.018	-	0.087	0.040	0.183
Cluster 2	-	-	-	-	0.050	-	0.046	-	0.022	0.067	0.272
Cryptophytes											
Cluster 1	-	-	-	-	-	-	-	0.121	-	-	-
Cluster 2	-	-	-	-	-	-	-	0.127	-	-	-
Prasinophytes											
Cluster 1	-	-	-	-	0.065	0.017	0.109	0.000	0.018	0.082	0.421
Cluster 2	-	-	-	-	0.047	0.305	0.053	0.000	0.021	0.072	0.236
Prymnesiophytes											
Cluster 1	-	0.011	0.236	0.278	-	-	-	-	-	-	-
Cluster 2	-	0.012	0.243	0.257	-	-	-	-	-	-	-
Cyanobacteria											
Cluster 1	-	-	-	-	-	-	-	-	-	0.592	-
Cluster 2	-	-	-	-	-	-	-	-	-	0.260	-

Statistical analysis

Previously to statistical analysis, we calculated weekly average of environmental parameters to be able to compare with phytoplankton pigments data, collected weekly. Normality and homoscedasticity of all variables were tested before multivariate analysis. As all the variables were not normally distributed, a non-parametric one-way analysis of variance (Kruskal–Wallis) was performed to statistically assess variations in the median fraction of all monitored variables within the experimental tanks. This analysis was also used for comparing chemical parameters, biofloc volume and phytoplankton absolute composition in different lighting conditions (white awning, black awning and no awning). Spearman rank correlation analyses were performed on environmental parameters (pH, temperature, DO, N-TA, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and biofloc volume) and phytoplankton groups, Chl*a* and net primary production in order to examine significant relationship.

Complementarily, the redundancy analysis (RDA) was selected from among the different multivariate ordination methods available (Braak and Smilauer 2002). Phytoplankton pigments, shrimp weight and net primary production were included in CANOCO 4.5 as dependent variables and environmental variables were included as independent variables. The statistical significance of the relationships was evaluated using Monte Carlo permutation tests with a manual forward selection procedure, under 499 permutations (Seoane et al. 2011).

Results

During the experiment no statistically significant differences were observed on physicochemical parameters within the experimental tanks according to Kruskal–Wallis analysis results ($P > 0.05$). The average recorded pH was 7.79 ± 0.37, whereas the average temperature and dissolved oxygen in the water were 27.6 ± 1.8 ºC and 5.96 ± 0.40 mg/L, respectively. The salinity was stable during all the experiment with an average of 22.5 ± 0.0. The values of light intensity, chemical parameters and biofloc volume, varied along the study period, showing statistically significant differences. The mean, standard deviation (SD) and Kruskal-Wallis analysis P-value are presented in Table 2, for three periods: white awning (beginning), black awning (middle) and no awning (end). $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and biofloc volume showed a clear increasing trend.

Table 2 Average of light intensity, chemical parameters and biofloc volume for the three periods studied: white awning (beginning), black awning (middle) and no awning (end). Kruskal-Wallis analysis significance results are shown in P-value column.

Variables	White awning	Black awning	No awning	P- value
Light intensity (lux)	3,106 ± 932	1,477 ± 1546	4,346 ± 1787	0.000
N-TA (mg/L)	0.24 ± 0.45	0.07 ± 0.06	0.17 ± 0.05	$7.7x10^{-11}$
$N\text{-}NO_2^-$ (mg/L)	3.55 ± 4.88	10.31 ± 7.58	0.26 ± 0.13	0.000
$N\text{-}NO_3^-$ (mg/L)	0.35 ± 0.27	12.32 ± 14.33	54.02 ± 9.52	0.000
$P\text{-}PO_4^{3-}$ (mg/L)	0.48 ± 0.59	2.83 ± 2.12	9.57 ± 2.81	0.000
BV (mL/L)	1.9 ± 3.0	9.6 ± 7.3	15.8 ±6.8	0.000

The following signature pigments were detected in water samples: peridinin, fucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, diadinoxanthin, alloxanthin, lutein, zeaxanthin and chlorophyll *b*. According to pigment analysis and microscope observations these phytoplankton groups were present: diatoms, dinoflagellates, chlorophytes, cryptophytes, euglenophytes, prasinophytes, prymnesiophytes and cyanobacteria. A Kruskal-Wallis analysis was performed to detect statistically significant differences in phytoplankton groups abundance (absolute contribution to chlorophyll *a*, µg/L) between tanks (statistically significant differences P-value < 0.01), the analyses are presented in Table 3. No statistically significant differences were found in any group abundance between experimental tanks, except for diatoms ($P = 1 \cdot 10^{-8}$). Diatom absolute abundance was significantly higher in tanks 1, 2 and 3. The contribution to Chl*a* of each one of the observed phytoplankton groups is represented in Fig. 1 along the study period. But, due to the observed differences, we calculated the mean concentration for tanks 1, 2 and 3 (n = 3), Fig. 1a, and, for the rest of tanks (n = 6), Fig. 1b.

A progressive Chl*a* increase is observed during the first weeks (day 1 to day 52) (Fig. 1). The first two weeks Chl*a* concentration was below the detection limit due to the initial disinfection process. On day 52 (week 8) an absolute maximum concentration of 496 ± 236 µg/L was observed. After day 59 Chl*a* concentration started to decrease, and remained below 200 µg/L until day 143. Later the Chl*a* concentration increased until 411 µg/L. The Kruskal-Wallis analysis showed statistically significant differences among periods (P-value), with significantly lower light intensity during black awning coverage. Thus, the temporal variation in Chl*a* concentration could be related to lighting conditions.

Table 3 Average of phytoplankton groups abundance (µg/L), chlorophyll *a* (µg/L) and net primary production (mgO_2 / (L·h)) and standard deviation for the three periods studied: white awning (beginning), black awning (middle) and no awning (end). Kruskal-Wallis analysis significance results are shown in P-value column.

Variables	White awning	Black awning	No awning	P- value
Diatoms	70.20 ± 64.54	91.48 ± 76.79	99.32 ± 73.69	0.087
Dinoflagellates	15.03 ± 29.50	0.04 ± 0.15	0.10 ± 0.14	$2.8x10^{-11}$
Euglenophytes	32.86 ± 35.82	21.82 ± 22.25	84.16 ± 47.14	$3.3x10^{-11}$
Chlorophytes	67.38 ± 93.00	3.68 ± 14.77	29.04 ± 40.69	$3.7x10^{-10}$
Cryptophytes	1.29 ± 1.94	0.59 ± 1.98	0.89 ± 1.27	0.001
Prasinophytes	4.49 ± 6.37	4.14 ± 3.54	1.39 ± 2.43	$2.8x10^{-4}$
Prymnesiophytes	0.12 ± 0.25	0.03 ± 0.09	0.17 ± 0.24	0.003
Cyanobacteria	99.94 ± 142.45	9.43 ± 22.16	75.55 ± 65.55	$8.4x10^{-7}$
Chlorophyll *a*	4.49 ± 6.37	4.14 ± 3.54	1.39 ± 2.44	$2.8x10^{-4}$
Net photosynthesis	0.32± 0.36	-0.05± 0.34	-0.29 ± 0.10	0.000

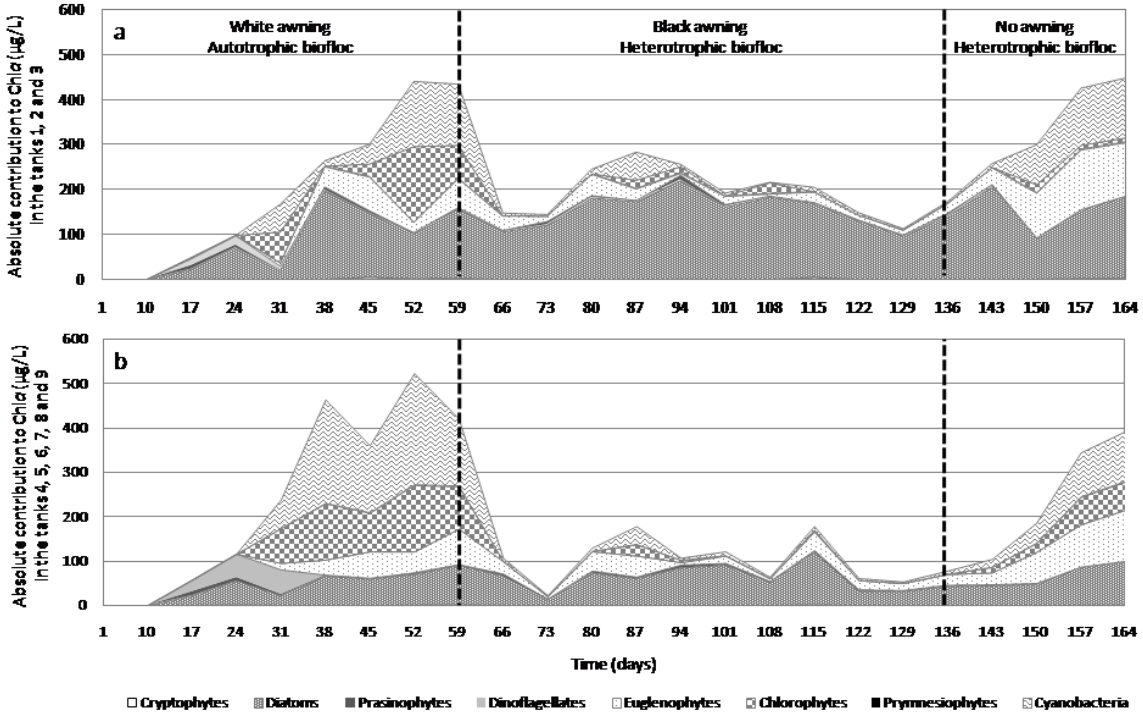

Fig. 1 Phytoplankton groups mean contribution to chlorophyll a concentration (µg/L) temporal evolution. a) Tanks 1, 2 and 3 b) Tanks 4, 5, 6 and 7.

According to net primary production values, autotrophic processes predominate during the first weeks, a period characterized by the absence of nitrifying bacteria and high levels of T-NA. This period coincides with the presence of white awning (Fig. 1). Subsequently, nitrifying bacteria develop, and net photosynthesis decreases until reaching negative values when heterotrophy predominates in the system.

The same temporal trend was observed in all the tanks for diatoms, however, concentration values differ (Fig. 1a). Tanks 1, 2 and 3 showed the higher diatoms abundance along all the study period, the average value was 138 ± 78 µg/L Chl*a*. The other tanks showed an average value of 60 ± 54 µg/L Chl*a*. Dinoflagellates were present only from day 17 to day 31 with an average value of 35 ± 37 µg/L Chl*a*. The rest of the study period their presence were minimal (<1.8 µg/L Chl*a*). Chlorophytes were mainly abundant during day 31 to day 59 with an average value of 94 ± 98 µg/L Chl*a*. They were also present from day 87 to 94, and from day 143 to 164, but their abundance was lower and average value was 29 ± 41 µg/L Chl*a*. Euglenophytes temporal trend was similar to that observed for Chl*a* (phytoplankton biomass) with two peaks. Their average abundance was 37 ± 40 µg/L Chl*a*. Cyanobacteria also show a similar trend to Chl*a* characterized by two peaks, however, they reduced their abundance to minimums from day 66 to 136. Their average abundance was 100 ± 142 µg/L Chl*a* during the first peak, and 76 ± 66 µg/L Chl*a* during the second peak. Prasinophytes were a low abundant group, that appears on day 17 and has an average of 4 ± 5 µg/L Chl*a*. Cryptophytes and prymnesiophytes are the groups less abundant with average values of 0.87 ± 1.88 µg/L Chl*a* and 0.08 ± 0.20 µg/L Chl*a* respectively.

Spearman rank correlation analyses were performed on environmental parameters (pH, temperature, DO, N-TA, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and biofloc volume) and phytoplankton groups, Chl*a* and net primary production in order to examine significant relationship. Table 4 shows the correlation results.

Table 4 Rank correlation matrix (Spearman's) between environmental (T^a –temperature, DO – dissolved oxygen, N-TA - total dissolved ammonia, $N\text{-}NO_2^-$ -nitrites, $N\text{-}NO_3^-$ - nitrates, $P\text{-}PO_4^{3-}$ - phosphates and BV – biofloc volume) and biological variables (phytoplankton groups).

	T^a	DO	pH	N-TA	$N\text{-}NO_2^-$	$N\text{-}NO_3^-$	$P\text{-}PO_4^{3-}$	BV
Diatoms	**0.210[a]**	**-0.321[a]**	**-0.329[a]**	-0.078	**0.282[a]**	0.119	**0.348[a]**	**0.299[a]**
Dinoflagellates	**0.192[a]**	**0.191[a]**	**0.212[a]**	0.132	-0.032	-0.034	**-0.135[b]**	-0.115
Euglenophytes	-0.006	**-0.510[a]**	**-0.492[a]**	**0.191[a]**	0.132	**0.185[a]**	**0.549[a]**	**0.509[a]**
Chlorophytes	**0.219[a]**	-0.114	-0.049	**0.186[a]**	0.077	0.009	0.115	**0.150[b]**
Cryptophytes	0.126	-0.053	-0.026	-0.064	0.118	0.018	0.084	0.121
Prasinophytes	0.131	-0.110	-0.043	-0.101	**0.258[a]**	-0.089	0.054	0.055
Prymnesiophytes	-0.064	**-0.194[a]**	**-0.260[a]**	-0.091	**0.151[b]**	**0.223[a]**	**0.345[a]**	**0.386[a]**
Cyanobacteria	**0.179[a]**	**-0.427[a]**	**-0.351[a]**	**0.190[a]**	**0.239[a]**	0.086	**0.433[a]**	**0.445[a]**
Chlorophyll *a*	**0.302[a]**	**-0.295[a]**	**-0.235[a]**	**0.159[b]**	**0.208[a]**	0.052	**0.311[a]**	**0.288[a]**
Net Photosynthesis	**0.547[a]**	**0.532[a]**	**0.735[a]**	0.032	0.008	**-0.504[a]**	**-0.743[a]**	**-0.650[a]**

[a] p<0.01, [b] p<0.05

Complementarily, the redundancy analysis (RDA) performed with CANOCO 4.5 is showed in Fig. 2. For a detailed interpretation of the graphs, see Ter Braak (1994). The RDA retained five variables: DO, N-TA, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$and biofloc volume. These variables together explained 39% of the variance in the biological variables (phytoplankton composition, phytoplankton biomass (Chl*a*) and net primary production). The biological variables were classified according to their association with the environmental variables. Distance between sample points symbols in the diagram approximates the dissimilarity of their pigment composition, measured by their Euclidean distance (Ter Braak 1994). We observed that samples taken from day 1 to day 38 are grouped by days in the triplot chart (Fig. 2), where the samples of the same day are located very close. That shows that phytoplankton composition is very similar in all the tanks for the same day, while between different days is more diverse. Samples from the rest of the study period are not grouped daily. Environmental variable arrows point in the expected direction of the steepest increase of values of environmental variable. Axis 1 shows a gradient of the variables: DO increasing to the right side, and an opposite gradient of $P\text{-}PO_4^{3-}$ and biofloc volume. The acute angle between variable arrows indicates high correlations between individual environmental

variables. For example, P-PO_4^{3-} and biofloc volume show a positive correlation, which is confirmed by the Spearman rank analysis. High values of these variables are highly correlated with euglenophytes and cyanobacteria. In the opposite side, high DO is strongly correlated with dinoflagellates, and to a minor extent to net primary production (see also Spearman Rank Table 4). Axis 2 shows a gradient of N-TA and N-NO_3^-. High concentration of N-TA is strongly correlated with chlorophytes and prasinophytes abundance (see also Spearman Rank Table 4). High concentration of N-NO_3^- is strongly correlated with prymnesiophytes abundance (see also Spearman Rank, Table 4). The sample symbols can be projected perpendicularly onto the line overlaying the arrow of particular environmental variable. The sample points are in the order of predicted increase of values of the particular environmental variable, so sample points projecting onto the coordinate origin are predicted to correspond to samples with an average value of that environmental variable. For instance, at the beginning of the study period, samples from day 1 to day 31, we have high DO levels. Samples are grouped temporally in three groups. In the first group, we find samples from day 1 to day 10. This first two weeks all pigments concentration was below the detection limit. In the second group, we find sample from day 17 to 31. These samples are characterized by high increasing N-TA concentrations. On day 31 the nitrification processes started and N-NO_2^- was detected. From day 1 to day 24 the highest DO were observed. In the third group, we include all other samples, these samples do not show a temporal gradient. During this period a point cloud is observed in the left side of the Fig. 2, Canoco graph.

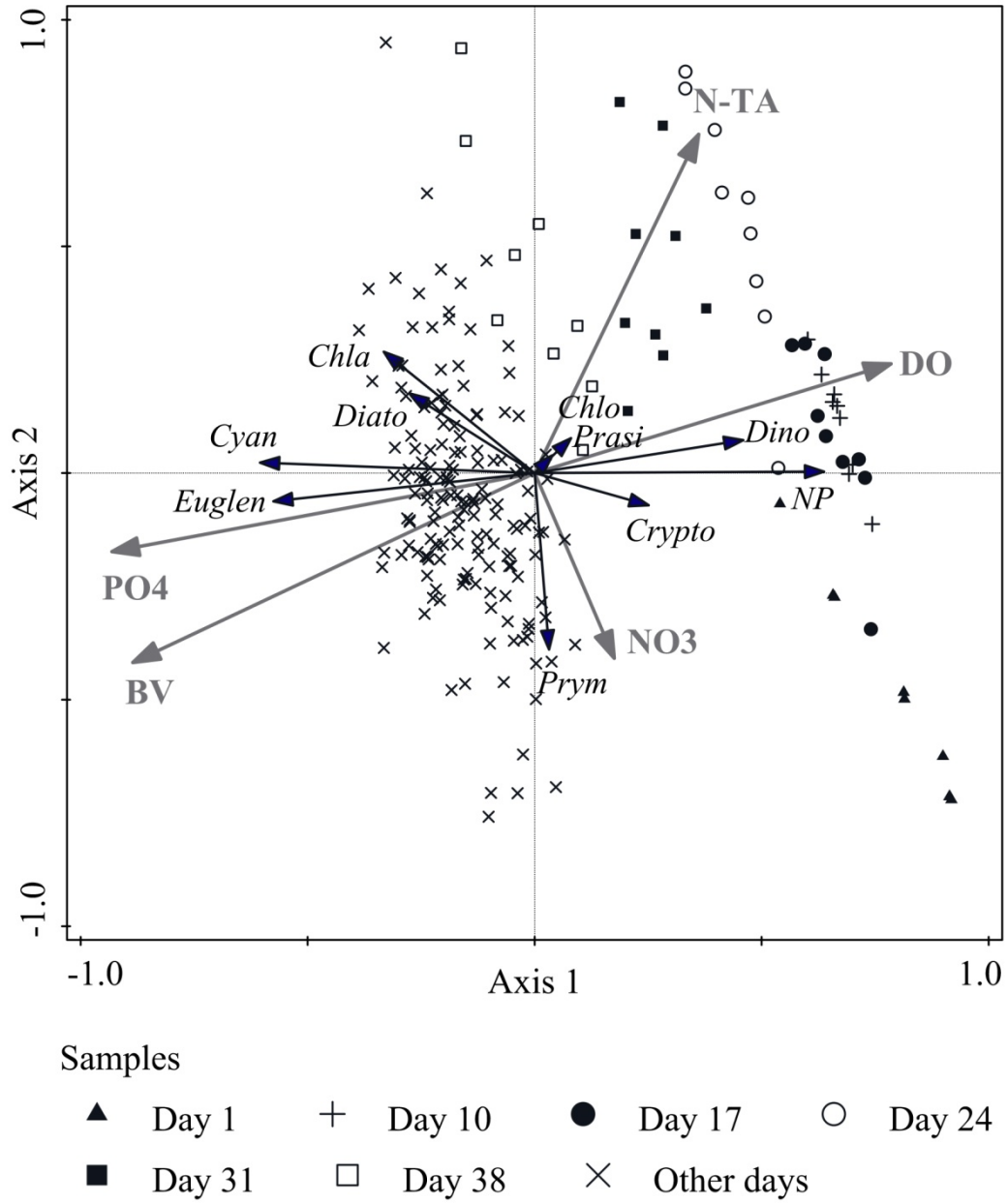

Fig. 2 Correlation plots of the RDA, on the relationship between the environmental variables (gray arrows), the biomass of the phytoplankton groups, the net primary production and total chlorophyll *a* (black arrows) and samples. Sample symbol corresponds to the sampling day detailed in the legend. Labels in black arrows mean: Diato - diatoms, dino - dinoflagellates, chloro - chlorophytes, crypto - cryptophytes, euglen - euglenophytes, prasi - prasinophytes, prym - prymnesiophytes, cyan - cyanobacteria, NP - net primary production and Chla - Chlorophyll *a*. Labels in gray arrows mean: DO – dissolved oxygen, BV –biofloc volum, PO4 – phosphates, NO3 – nitrates, N-TA – total dissolved ammonia.

Discussion

All the water quality parameters in the tanks remained within the recommended rate suitable for growing *L. vannamei*, specially the values of pH, temperature, dissolved oxygen and salinity (Van Wyk and Scarpa 1999). The biofloc systems are characterized by a peak of N-TA, followed by peak of $N\text{-}NO_2^-$ and finally an accumulation of $N\text{-}NO_3^-$ in the system, what coincides with the observed evolution of these variables (Table 2) (Azim and Little 2008; Avnimelech 2009). Also, in all biofloc system we can observe an accumulation of PO_4^{3-} and biofloc volume (Ray et al. 2011; Correia et al. 2014). The levels of ammonia and nitrites can be toxic, but, during the experiment, these levels were maintained within the limits of safety determined by Lin and Chen (2001) and (2003). The accumulation of $P\text{-}PO_4^{3-}$ and biofloc volume started from the beginning of experiment and followed during all the time. $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ do not have any negative effect on shrimp. The biofloc volume values were lower than maximum recommended by Avnimelech (2009). The succession of autotrophic processes by heterotrophs, observed by the reduction of net primary production (Table 3 and Fig. 1), is characteristic of the biofloc systems (Vinatea et al. 2010; Marinho et al. 2016). The significant inverse correlation of net primary production with BV and $P\text{-}PO_4^{3-}$ (Table 4 and Fig. 2), is explained because these variables values increase during heterotrophic culture phase (Ray et al. 2011; Correia et al. 2014).

Chlorophyll *a* (Chl*a*) is commonly used as a proxy of phytoplankton biomass, also in BFT systems (Gaona et al. 2011). The range of Chl*a* concentration measured is similar to the one observed in other *L. vannamei* BFT systems (Gaona et al. 2011; Baloi et al. 2013; Martins et al. 2016). At the beginning of the study period, from day 1 to 10, no pigments were detected. This is due to the process of initial chlorination of the seawater to eliminate bacteria, which also reduces the amount of phytoplankton (Yusoff et al. 2002). The temporal variation in Chl*a* concentration is related to lighting conditions, as revealed by Kruskal-Wallis analysis (Table 3), so it is the evolution of all the phytoplankton groups except diatoms (Table 3). Other authors have observed a direct relationship between reduced light intensity and phytoplankton decrease (Gaona et al. 2011; Baloi et al. 2013; Martins et al. 2016). All phytoplankton groups presented significantly lower abundances (µg/L Chl*a*) with black awning, while diatoms abundance showed no statistically significant differences (Fig. 1).

Four major groups of phytoplankton, including diatoms, dinoflagellates, cyanobacteria and chlorophytes are usually observed in *L. vannamei* biofloc systems (Lukwambe et al. 2015; Martins et al. 2016). In our

study, the major groups were diatoms, euglenophytes, cyanobacteria and chlorophytes, similar to the observed by Schrader et al. (2011) in other biofloc systems, while dinoflagellates were only remarkable at the initial period. The most abundant phytoplankton group was diatoms all along the study period (Fig. 1). The diatom predominance is commonly observed in BFT (Schrader et al. 2011 (in some tanks); Godoy et al. 2012; Martins et al. 2016). However, other authors remark an abundance decrease at the end of their studies, and a replacement by undesired cyanobacteria (Yussoff et al. 2002; Schrader et al. 2011 (in some tanks)). This decrease has been related to a silica limitation and to a phosphorus enrichment. Martins et al. (2016) observed that silica addition was essential for the growth and maintenance of high diatom cell density in the biofloc system. Coastal waters used to fill the aquaculture tanks in our study are characterized by high silica levels. This is due to groundwater discharges rich in silica (Sospedra et al. 2017), because of the lixiviation of biogenic silica from the wetland species of Gramineae, which are characterized by high silica content (Sebastiá et al. 2012; Sebastiá and Rodilla 2013). This high initial concentration of silica can explain the maintenance of diatom levels all time long. The predominance of diatoms is highly desired because of their nutritional properties, they can enhance the contents of essential amino acids and highly unsaturated fatty acids in shrimp tissue, and their consumption improve shrimp growth (Godoy et al. 2012; Brito et al. 2016; Martins et al. 2016). On the contrary, cyanobacteria are undesired because their nutritional value is low, are commonly responsible of noxious blooms, impart unpleasant flavors to cultured animals and negatively affect water quality (Paerl and Tucker 1995; Yusoff et al. 2002; Ju et al. 2008; Schrader et al. 2011).

The Kruskal-Wallis analysis results revealed that cyanobacteria biomass was lower during low light intensity conditions (black awning). However, cyanobacteria are myxotroph organisms that can take advantage of different environmental conditions, by changing their trophic mode. Thus, in low light conditions, they can adopt heterotrophy mode and reduce their pigment content (Chl*a* and zeaxanthin) (Yu et al. 2009; Lohscheider et al. 2011; Gris et al. 2017). These can explain the lower pigment concentrations measured by HPLC, and the lower cyanobacteria biomass estimated by CHEMTAX. But, microscope controls demonstrated high abundance of filamentous cyanobacteria also during low light conditions. Positive correlation between cyanobacteria and phosphate and biofloc volume (Table 4 and Fig. 2) has also been observed in other BFT studies (Yussoff et al. 2002; Green et al. 2014). Green et al. (2014) explained that filamentous cyanobacteria help to cohesion the different components of the floc, obtaining larger aggregates.

Dinoflagellates are usually one of the dominant phytoplankton groups in biofloc cultures (Ju et al. 2008; Ballester et al. 2010; Manan et al. 2016; Marinho et al. 2016), which sometimes persist throughout the study period (Yusoff et al. 2002). In our study, peridinin, which is the signature pigment of dinoflagellates, was only present from day 17 to 31, when the biofloc system was not mature. This period was characterized by autotrophic processes, and dinoflagellates showed significant positive correlation with net primary production (Table 4). Although no peridinin was detected in later stages, dinoflagellates were observed under the microscope. Dinoflagellates are mixotrophic organisms, capable of feeding on various prey species, including bacteria, flagellates, diatoms, heterotrophic protists and metazoans (Ismael 2003; Jeong et al. 2010). At the same time, they are able to perform photosynthesis, thus increasing their rate of growth (Li et al. 1999). It is possible that, while the system was autotrophic, the dominant dinoflagellates in the water were autotrophic or mixotrophic dinoflagellates, with a high rate of peridinin (Jeffrey et al. 1975). As the biofloc system matures, the light and net primary production decrease, so the dinoflagellates present show a heterotrophic behavior. This would cause a decrease in the synthesis of peridinin, which is no longer detected by HPLC (Li et al. 1996). Note that, depending on the species, dinoflagellates presence can adversely affect the immune system of shrimp, due to production of toxins (Pérez-Linares et al. 2008; Campa-Córdova et al. 2009; Pérez-Morales et al. 2017). Although not all species are harmful, as some are used as a nutrient source in carcinoculture (Ge et al. 2016).

Chlorophytes are usually present in biofloc systems (Yusoff et al. 2002; Manan et al. 2011; Schrader et al. 2011). They are a desirable group as they remove ammonium (Chen 2001), and improve shrimp yield and survival (Ge et al. 2016). Maicá et al. (2012) observed, in their microscopic counts, that chlorophytes dominated biofloc systems at low salinities (2-4), but were replaced by diatoms at salinities of 25. Ju et al. (2008) also observed a predominance of lutein, signature pigment of chlorophytes (Schlüter et al. 2006), in samples with a low salinity (5-18), and an increase in fucoxanthin, signature pigment of diatoms (Schlüter et al. 2006), with increasing salinity. Only Martins et al. (2016) observed that chlorophytes dominated their biofloc culture at high salinity (37) and absence of diatoms. Our tanks have an intermediate salinity (22.5) in which the two groups coexist, although the diatoms are more abundant as shown in Fig. 1.

Euglenophytes are one of the major groups in most biofloc systems, although they are not the most abundant (Green et al. 2014; El-Dahhar et al. 2015; Marinho et al. 2016). This group is able to adapt to waters with a wide spectrum of salinity (Figueroa et al. 1998), but they have been found in greater

quantity in studies with freshwaters (Schrader et al. 2011) or low salinity waters (Ju et al. 2008). In our study, their abundance is highly correlated with biofloc volume (Fig. 2), a relationship already observed by Green et al (2014), and with phosphates (Horabun 1997). Kingston (1999) attributed this relation to their inhibition by high light intensities, showing greater abundances in highly turbid environments, as the one characteristic of a mature biofloc system.

Conclusion

Algal pigment analyses using HPLC and subsequent CHEMTAX analysis in water samples can provide useful information for estimating the phytoplankton community structure in the BFT systems. This technique is very useful for monitoring the abundance variation of beneficial as well as potentially harmful algae. However, we found some limitations when the BFT systems are in heterotrophic mode. Under these conditions, some dinoflagellates and cyanobacteria behave as heterotrophs and lose or decrease their biomarkers pigments. The HPLC/CHEMTAX methodology is widely applied for monitoring nutrient enriched waters, in both continental and marine ecosystems. But, little research has been developed in heterotrophic systems. So, further research is needed to increase knowledge on the accuracy of HPLC/CHEMTAX under these conditions. The analysis of phytoplankton evolution allowed us to observe the key role played by light intensity on abundance and composition of phytoplankton during the creation of a biofloc system. In general, a major light intensity caused an increase in phytoplankton biomass as indicated by Chl*a* concentration. This increase is mainly due to higher abundances of euglenophytes, chlorophytes and cyanobacteria. However, diatoms and net primary production were not significantly affected by different light intensity. Diatoms abundance was constant all along the study period, while primary production followed the normal trend in biofloc system. Coastal waters used to fill the aquaculture tanks in our study were characterized by high silica levels, that allowed to maintain diatom population. According to our results, and in agreement with other authors, light intensity and dissolved silica concentration are key parameters for controlling phytoplankton composition and abundance.

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