Aquaculture 508 (2019) 60–65

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Managing input C/N ratio to reduce the risk of Acute Hepatopancreatic Necrosis Disease (AHPND) outbreaks in biofloc systems – A laboratory study

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ARTICLE INFO

Keywords:
Litopenaeus vannamei
Biofloc technology
Heterotrophic
Autotrophic
Probiotics
Acute Hepatopancreatic Necrosis Disease

ABSTRACT

Biofloc systems are microbial mature environments that are potentially less conducive disease outbreaks. We hypothesized that the way in which biofloc microbial communities are managed determines the level of disease protection. To investigate such hypothesis, *Litopenaeus vannamei* post-larvae were cultured for 21 days in biofloc environments created by different water management procedures. Five different types of bioflocs were created: autotrophic bioflocs without probiotics, autotrophic bioflocs with probiotics, heterotrophic bioflocs without probiotics, heterotrophic bioflocs with probiotics, and a flow-through system as a control. Heterotrophic bioflocs were obtained by daily addition of carbon (glucose) at an estimated C/N ratio of 18 throughout the experiment. For autotrophic bioflocs this input of carbon was applied only to start up the system and upon appearance of bioflocs (TSS $>$ 100 mg L^{-1}) and a drop in total ammonium nitrogen concentration below 0.05 mg L^{-1}, carbon dosing was stopped. Bioflocs cultured with addition of probiotics received a 0.5 ppm dose every 48 hours. After 21-d culture period, a 96 h challenge test was performed with a *Vibrio parahaemolyticus* strain known to cause AHPND. For each biofloc type, this challenge was performed in three different approaches: 1- Shrimp were taken out of their biofloc tanks and challenged by applying new seawater; 2-Shrimp from biofloc tanks were challenged in their respective biofloc suspensions; and 3- Non-experimental shrimp, randomly selected from a recirculation (RAS) system were challenged in the types of biofloc suspensions. Mortality was high when shrimp were challenged in new seawater, independent of treatment. When challenged in their respective biofloc suspensions shrimp survival was the highest in heterotrophic bioflocs with and without probiotic supplementation and the autotrophic bioflocs with probiotics, whereas shrimp survival in autotrophic bioflocs without probiotics was 50%. These results were similar when non-experimental shrimp originating from a RAS system were challenged in these biofloc suspensions. Taken together, results suggest that bioflocs as such can decrease the impact of a *Vibrio parahaemolyticus* challenge and that this protection depends on the operational parameters of the biofloc system. Moreover, probiotics can be used to complement the protective effect of bioflocs. This information reinforces the importance of microbial community management as a tool to reduce the risk of disease and establish highly biosecure systems.

1. Introduction

The high incidence of infectious diseases, resulting in mass mortalities, has become a real challenge for aquaculture industry, and the main cause of production and economic losses during last decades. It is the primary factor that negatively affects the future prospects of this sector (FAO, 2016). In many cases, mortalities caused by pathogenic bacteria cannot be attributed to specific pathogens, but rather to proliferation of opportunistic pathogenic bacteria (Defoirdt, 2016). Such bacteria are inherently present in the culture system and are highly effective in taking advantage of the continuous changes in the culture environment related to aquaculture-based use of the water (Skjermo and Vadstein, 1999). They are typically characterized as organisms that can become pathogenic following a perturbation in the environment

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https://doi.org/10.1016/j.aquaculture.2019.04.055
Received 6 June 2018; Received in revised form 19 April 2019; Accepted 21 April 2019
Available online 26 April 2019
0044-8486/ © 2019 Published by Elsevier B.V.

(Brown et al., 2012). Suboptimal rearing conditions such as extremely high stocking densities, associated with environmental parameters like low oxygen concentrations and variations in temperature can lead to opportunistic bacterial disease outbreaks. Vibriosis is the most widely known example (Kimes et al., 2012).

Vibriosis is caused by Gram negative bacteria of the genus *Vibrio*, and can be considered as one of the most prevalent diseases that affects cultured aquaculture organisms worldwide (Chatterjee and Haldar, 2012). Among *Vibrio* spp. that are able to cause disease special attention has recently been given to *Vibrio parahaemolyticus*, which has been reported to be one of the the main etiological agent that causes the Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) (Tran et al., 2013). This disease has caused mass mortality of penaeid shrimp in China, Vietnam, Malaysia, Thailand, Mexico and the Philippines (Hirono et al., 2016), and typically appears during the first 20 to 30 days of culture.

Considering the substantial impact resulting from the prevalence of AHPND causing *Vibrio*, farmers in many cases have adopted use of antibiotics and/or disinfectants to control this bacterial disease. The indiscriminate use of antibiotics, especially in situations when there is no apparent disease, evidently results in a major problem of resistance development by bacterial aquaculture pathogens (Defoirdt et al., 2007; Crab et al., 2010; Mohapatra et al., 2013). Moreover, the random use of disinfectants has also proven ineffective in treating diseases caused by Vibrio species and in some cases has even been suggested to contribute to disease risk (Attramadal et al., 2012; De Schryver et al., 2014).

In this context, there is an urgent need to find non-antibiotic based and environmental friendly alternatives to prevent AHPND from occurring (Sha et al., 2016). Defoirdt et al. (2011) and Defoirdt (2016) suggest that an approach which simultaneously considers pathogen, host and environment will probably be the most effective solution in the long term to prevent pathogenic diseases. Manipulations of man-based aquaculture systems to manage the microbial community based on ecological selection principles are the key to minimizing the risk of vibriosis outbreaks including those causing AHPND (De Schryver and Vadstein, 2014; De Schryver et al., 2014).

Biofloc Technology (BFT) is a fish and shrimp culture technique based on stimulating and establishing a microbial community to achieve nutrient recycling, mainly nitrogen waste, and to maintain water quality (McIntosh et al., 2001; Wasielesky et al., 2006; Avnimelech, 2007).

In addition, other benefits such as improved growth, survival and disease resistance can also be expected (Wang, 2016; Xu et al., 2016; Crab et al., 2010). The method of water management in biofloc systems determines nitrogen conversion processes as well as influences biofloc production. A bacterial autotrophic biofloc system is mainly based on nitrifying bacteria that oxidize ammonium to nitrite and nitrite to nitrate, fixing inorganic carbon in the process (Kuhn et al., 2010). A predominantly autotrophic biofloc system can be achieved by providing inputs with a low C/N ratio (considerably < 10). Such systems are characterized by a slow accumulation of suspended solids (Ebeling et al., 2006; Ray and Lotz, 2014), because of low biomass yield per unit of ammonium nitrogen oxidized. For instance, for every gram of total ammonium nitrogen (TAN) converted to nitrate nitrogen (NO_3-N), 4.18 g of oxygen is consumed and 0.20 g of microbial biomass is produced (Ebeling et al., 2006).

A predominantly heterotrophic biofloc system can be achieved by providing inputs (feed and a supplemental organic carbon source) with a collective C/N ratio of at least 15. Heterotrophic bacteria assimilate ammonium nitrogen into their bacterial biomass by utilizing organic carbon as a source of energy (De Schryver et al., 2008) and for cellular growth. In this way, nitrogen is removed from the water phase. Heterotrophic nitrogen removal leads to a higher accumulation of solids in the water compared to bacterial autotrophic nitrogen removal. For each gram of TAN assimilated by the heterotrophic bacteria 8.07 g of microbial biomass is produced and 4.71 g of oxygen is consumed (Ebeling et al., 2006).

The C/N operational parameter of biofloc systems directly influences the composition of the microbial community in the bioflocs. Suita et al. (2015) quantified the microbial community in autotrophic biofloc with limited carbon supplementation (C/N ratio of 6) and found higher abundance of free coccoid bacteria compared to adhered bacteria.

Bioflocs can protect aquatic animals from microbial disease in several cases. Survival of white shrimp grown in bioflocs was improved when shrimp were challenged with myonecrosis virus (IMNV) (Ekasari et al., 2014). Crab et al. (2010) used *Artemia franciscana* as a model system to verify the effects of bioflocs, and found higher survival when the brine shrimp were challenged with *V. harveyi*. The authors suggested that bioflocs protect against bacterial diseases. However, only little is known about the mode of action.

The application of probiotic bacteria has become another successful alternative for the control of diseases (Gram et al., 1999; Gildberg et al., 1997). By definition, probiotics are live microorganisms that provide health benefits to the host when administered in adequate levels. They can be applied via water or feed (Moriarty, 1998; Skjermo and Vadstein, 1999), either single or as a combination of strains (Villaseñor et al., 2015). Among the different bacterial species with probiotic properties, genus *Bacillus* has been most widely applied in penaeid culture (Decamp and Moriarty, 2006; Villaseñor et al., 2013; Sapcharoen and Rengpipat, 2013; Fyzul et al., 2014). Nimrat et al. (2012) observed that the use of *Bacillus* as a potential probiotic enhanced the number of beneficial bacteria in shrimp and in seawater. Similarly, Sha et al. (2016) verified antibacterial activity and increased immune-related gene expression when probiotics were administered. Li et al. (2009) reported that *Bacillus* had positive effects on shrimp immune responses and disease resistance, with reduction in the number of Vibrio. Zokaeifar et al. (2014) observed a significantly higher survival of L. *vannamei* supplemented with *Bacillus subtilis* after a challenge with *V. harveyi*.

The above-mentioned results were obtained in flow-through systems. However, positive effects of probiotic application are also reported in biofloc systems. For instance, Krummenauer et al. (2014) observed higher survival of shrimp during an outbreak of *V. parahaemolyticus* when reared in bioflocs supplemented with a multi-strain probiotic, including *Bacillus*. Ferreira et al. (2015) isolated a *Bacillus licheniformis* strain from biofloc suspension which was positively confirmed for its antagonistic activity against *V. alginolyticus*. Aguillera-Rivera et al. (2014) suggested that the addition of a commercial probiotic mixture contributed to the prevention of opportunistic pathogenic bacteria outbreaks in a BFT system and in clear water without bioflocs.

The possible combined activity of bioflocs and probiotics to provide protection for shrimp against bacterial pathogens requires further investigation. Based on the theory of De Schryver and Vadstein (2014) and De Schryver et al. (2014), we therefore hypothesized that biofloc operational parameters (more particularly the input C/N ratio) influence microbial community composition, which can result in production of bioflocs with differential capacity to control disease. This hypothesis was verified using a *V. parahaemolyticus* strain that causes AHPND as a pathogen. In addition, the effect of Bacillus-based probiotics on control of AHPND independently of input C/N ratio was determined.

2. Material and methods

2.1. Growing of biofloc suspensions in preparation of the experimental trial

To prepare bioflocs for use in the experiments, *L. vannamei* juveniles of about 5 g obtained from Shrimp Improvement Systems (SIS, Miami, USA) were stocked for 70 days in 12 tanks of 40 L (35 L effective seawater volume) at a biomass of 100 g $tank^{-1}$. Seawater was maintained at a salinity of 32 ppt and temperature of 27.9 ± 0.6 °C in a climate controlled room. Shrimp were fed a commercial diet (Crevetec grower®

2 × 4 mm, 40% crude protein) initially at 8% of wet body weight day^{-1} and then gradually decreased to 5%.

Six tanks were operated to grow autotrophic biofloccs, three tanks with and three tanks without application of probiotics. Six tanks were operated to grow heterotrophic biofloccs, three tanks with and three tanks without application of probiotics. To promote initial growth and establishment of the biofloccs in all biofloc tanks, a glucose (D+ glucose - VWR®) solution was added as carbon source continuously throughout the day to each tank with a peristaltic pump (Multi channel cassette pump 205 CA – Watson-Marlon®) to provide an estimated daily input C/N ratio of 18. The amount of glucose to be added was calculated based on theoretical daily nitrogen excretion by the shrimp, considering daily feeding ratio, protein content of feed and shrimp stocking density. The calculations were done by following methods described by Ebeling et al. (2006) and Avinimelech (1999).

Once biofloccs appeared (TSS > 100 mg L^{-1}) and total ammonium nitrogen (TAN) concentrations dropped below 0.05 mg L^{-1}, usually within 15 days, carbon dosing was stopped in the autotrophic biofloc tanks to allow a shift towards a nitrifying community. In the heterotrophic biofloc tanks, glucose supplementation was sustained during the 70 days of biofloc preparation period. Total suspended solids content was kept below 500 mg L^{-1}.

In the tanks with probiotic supplementation, the probiotic Sanolife ProW® (INVE Aquaculture, Belgium) consisting of a mixture of *Bacillus subtilis* and *Bacillus licheniformis* strains, in a total bacterial concentration of 5 × 10^{10} CFU g^{-1}, was added to the culture water every 48 h, at a concentration of 0.5 ppm according to the manufacturer's recommendations.

2.2. Biofloc experimental trial

2.2.1. Culturing of shrimp in different biofloc suspensions

Experimental post-larvae of whiteleg shrimp L. *vannamei* were obtained from Shrimp Improvement Systems (SIS, Miami, USA) at the age of 10 days (PL_{10}) and maintained in the larval raceway system of the Laboratory of Aquaculture & *Artemia* Reference Center (Ghent University, Belgium) until use at PL_{22}.

Shrimp PL_{22} (0.025 ± 0.01 g) were cultured at 30 shrimp per tank for 21 days in 15 rectangular transparent acrylic tanks with a bottom area of 50 cm^2 and 10 L effective volume, allocated to a temperature controlled room. Temperature in all tanks was maintained between 27.8 and 28.8 °C for the experimental period. Salinity was maintained between 34 and 35 ppt. Aeration was provided by an air blower connected to diffusers placed in each aquarium and the light regime was set at 12 h light/12 h dark.

The experiment consisted of five treatments with three replicate tanks each:

- Shrimp cultured in autotrophic biofloccs without probiotics application (A-BF).
- Shrimp cultured in autotrophic biofloccs with probiotics application (A-BF + ProW).
- Shrimp cultured in hetetotrophic biofloccs without probiotics application (H-BF).
- Shrimp cultured in hetetotrophic biofloccs with probiotics application (H-BF + ProW).
- Shrimp cultured in a water flow-through system (FT).

The biofloccs experimental tanks were filled with 5 L of biofloc suspension previously prepared in an autotrophic or heterotrophic way (see Section 2.1), and supplemented with 5 L natural seawater. During the 21-d culture period, biofloccs were maintained as described under 2.1. For the control tanks operated as flow-through system, tanks were filled with 10 L natural seawater and a water exchange of 80% was done every 48 h. Natural pre-heated seawater was used for the renewal procedures. In all tanks, freshwater was used to compensate for evaporative losses and to maintain salinity throughout the experimental period.

Shrimp were fed a 40% crude protein (CP) commercial diet (Crevetec grower®) twice daily (0900 and 1800 h) for the 21-d culture period. Feeding rate was adapted according to the methodology adapted from Jory et al. (2001). Dissolved oxygen (DO) and pH were measured daily (0900 and 1800 h) using a portable DO meter (Field LabOxi – Oxical SL - WTW®), and a pH meter (pH Control- JBL®), respectively. Dissolved inorganic nitrogen (TAN, NO_2-N, and NO_3-N), and total suspended solids (TSS) were determined weekly (APHA, 1999).

2.3. V. parahaemolyticus challenge

2.3.1. Preparation of AHPND pathogen

Pathogenic *Vibrio parahaemolyticus* strain PV1 was originally isolated from diseased shrimp in Thailand. Presence of toxic genes in the bacterial strains was previously confirmed using specific primers AP2 and AP3. The strain was preserved at −80 °C in Marine Broth (40.1 g L^{-1}, Carl Roth) containing 20% sterile glycerol.

Prior to use, the bacterial strain was grown overnight at 28 °C on agar bacteriological grade (20 g L^{-1}, Biokar diagnostics) and then subcultured to log phase in marine broth (40.1 g L^{-1}, Carl Roth) at 28 °C with continuous shaking. Bacterial cell numbers were subsequently determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France) and set to an optical density of 1.0, corresponding to approximately 1.2 × 10^9 cells mL^{-1}.

This suspension of *V. parahaemolyticus* was then used in the AHPND challenge as mentioned below (Section 2.3.2), and added to water at a final concentration of 10^7 CFU mL^{-1} 24 h prior to stocking of shrimp in the challenge units.

2.3.2. AHPND challenge test

At the end of the 21-d culture period, a *V. parahaemolyticus* (PV1; AHPND strain) challenge test was performed.

The challenge test aimed to investigate protective effect of the different biofloc types against *V. parahaemolyticus* infection, and was designed to verify whether protection was related to the suspension of the biofloccs as such (hypothesis 1, H1), or whether it was related to the influence that biofloccs might have on shrimp upon long term exposure (through immunomodulation) (hypothesis 2, H2). For that, the challenge was performed by applying three different approaches, as follows:

1. Five experimental PL_{43} were randomly selected from each experimental biofloc tank (see Section 2.2.1), and challenged in new seawater (BF to SW) to test H2.
2. Five experimental PL_{43} were randomly selected from each experimental biofloc tank (see Section 2.2.1) and transferred together with 1 L of biofloc suspension from the same tank (see Section 2.2.1). The shrimp were challenged in their respective biofloc suspensions (BF to BF) to test H1.
3. Five non-experimental PL_{43} were randomly selected from a recirculation (RAS) system (and thus had no previous contact with biofloccs), and were transferred together with 1 L of each biofloc suspension (see Section 2.2.1). Then the shrimp were challenged in these biofloc suspensions (SW to BF) to test H1.

The challenge tanks had a volume of 1 L and each challenge test consisted of a negative (non-challenged) control (also five shrimp) in addition to challenged treatments. The challenge test was run for 96 h and shrimp mortality was determined daily.

2.4. Statistical analyses

All survival data were transformed in arcsin in order to fulfill the requirements of homoscedasticity and normality of ANOVA. However,

Table 1
Mean (± standard deviation) values of water quality parameters during 21-d culture of L. *vannamei* culture in autotrophic (A-BF) and heterotrophic based (H-BF) bioflocs with and without supplementation of probiotic and in a flow-through (FT) system.

Parameters	A-BF	A-BF + ProW	H-BF	H-BF + ProW	FT
DO (mg L^{-1})	5.88 ± 0.47[a]	5.90 ± 0.41[a]	5.97 ± 0.39[a]	6.01 ± 0.34[a]	5.98 ± 0.37[a]
pH	7.73 ± 0.09[a]	7.78 ± 0.08[a]	7.80 ± 0.04[a]	7.86 ± 0.06[a]	7.88 ± 0.09[a]
Salinity (ppt)	34 ± 2[a]	35 ± 2[a]	35 ± 1[a]	34 ± 1[a]	35 ± 1[a]

Different superscript letters indicate significant differences for a specific parameter ($p < 0.05$).

Table 2
Mean (± standard deviation) values of nitrogen compounds (Total Ammonium Nitrogen – TAN, Nitrite – NO_2-N and Nitrate - NO_3-N) and total suspended solids (TSS) during 21-d culture of *L. vannamei* culture in autotrophic (A-BF) and heterotrophic based (H-BF) bioflocs with and without supplementation of probiotic and in a flow-through (FT) system.

Parameters	A-BF	A.BF + ProW	H-BF	H. BF + ProW	FT
TAN (mg L^{-1})	0.07 ± 0.03[a]	0.05 ± 0.01[a]	0.02 ± 0.04[a]	0.01 ± 0.01[a]	0.02 ± 0.01[a]
NO_2-N (mg L^{-1})	0.28 ± 0.07[a]	0.32 ± 0.04[a]	0.20 ± 0.05[a]	0.26 ± 0.05[a]	0.22 ± 0.05[a]
NO_3-N (mg L^{-1})	24 ± 2[b]	24 ± 4[b]	0.20 ± 0.25[a]	0.22 ± 0.10[a]	0.20 ± 0.02[a]
TSS (mg L^{-1})	118 ± 38[a]	110 ± 11[a]	334 ± 113[b]	408 ± 72[b]	–

Different superscript letters for a specific parameter indicate significant differences.

variances of this variable were not equal and normally distributed therefore a non-parametric test (Kruskal–Wallis test) was applied ($P > 0.05$).

3. Results

3.1. Water quality

Water quality parameters during the 21-d culture period in the different biofloc types are given in Tables 1 and 2. No differences were observed among the treatments in terms of DO, pH and salinity (Table 1). The levels of TAN and NO_2-N were equal among treatments (Table 2). NO_3-N concentrations were significantly higher in A-BF treatments (A-BF and A-BF + ProW). Total suspended solids were measured only in BFT treatments, since in FT system there was no accumulation of significant amounts of particulates. This parameter was highest in the H-BF treatments (H-BF and H-BF + ProW).

3.2. Survival in V. parahaemolyticus challenge test

3.2.1. Experimental shrimp cultured in bioflocs, and challenged in new seawater (BF to SW)

No mortality was observed in the negative control (without pathogen supplementation) during 96 h of experiment. After 96 h, survival values were significantly lower in all experimental treatments as compared to the negative control (Table 3).

3.2.2. Experimental shrimp cultured in bioflocs, and challenged in biofloc suspension (BF to BF)

No mortality was observed in the negative control during 96 h of experiment. At 96 h after immersion, 100% mortality was found in FT treatment, which was significantly different from the A-BF treatment. Lower survival was also observed for this latter treatment comparing with A-BF + ProW and the heterotrophic bioflocs with and without probiotics (Table 3).

3.2.3. Non-experimental shrimp challenged in biofloc suspension (RAS to BF)

No mortality was observed in the negative control during the 96 h of the experiment. At the end of the challenge trial, survival was higher for all treatments, except for FT and A-BF (Table 3).

4. Discussion

The most common method used by farmers to keep water quality parameters within the adequate range for growth and survival of farmed animals is continuous replacement of culture water with external water (Hopkins et al., 1993). The use of biofloc technology offers the possibility to maintain good water quality with minimal or no water exchange (De Schryver et al., 2008). Despite the difference in operational parameters among treatments in this study, water quality levels were kept within the recommended range for L. *vannamei* (Lin and Chen, 2001; Lin and Chen, 2003; Kuhn et al., 2010). In flow-through treatment, levels were maintained within the optimal range for L. *vannamei* through water exchanges performed every 48 h.

The levels of TAN and nitrite-N did not differ significantly among treatments during the 21-d culture period. Nitrate-N levels, however, were significantly higher in A-BF systems, which is indicative of nitrification. Ray and Lotz (2014) observed similar results when monitoring inorganic nitrogen levels in a chemoautotrophic-based and three H-BF systems. Removal of ammonium nitrogen in A-BF occurs mainly by nitrification (Hargreaves, 2006) which occurs especially at low C/N ratio (typically below 5).

Nitrogen conversion occurs differently in heterotrophic-based

Table 3
Mean values ± standard deviation of survival (%) of Pacific white shrimp cultured for 21 days in autotrophic or heterotrophic bioflocs with or without probiotic supplementation, or in a flow-through system and then challenged with AHPND causing *V. parahaemolyticus* in new seawater (BFT to SW) in their respective bioflocs (BFT to BFT), and cultured in a RAS system and then challenged with *V. parahaemolyticus* causing AHPND in the respective bioflocs (RAS to BFT).

Survival (%) after 96 h	A-BF	A.BF + ProW	H-BF	H. BF + ProW	FT	Neg. Control
BF to SW	15 ± 21.6[a]	3.33 ± 3.3[a]	3.0 ± 3.55[a]	33.3 ± 41.6[a]	1.60 ± 0.94[a]	100 ± 0.0[b]
BF to BF	46.6 ± 30.5[b]	100 ± 0.0[c]	100 ± 0.0[c]	93.3 ± 11.5[c]	0.0 ± 0.0[a]	100 ± 0.0[c]
RAS to BF	20 ± 20.0[a]	100 ± 0.0[b]	100 ± 0.0[b]	100 ± 0.0[b]	0.0 ± 0.0[a]	100 ± 0.0[b]

Different letters indicate statistical differences ($P < 0.05$).

bioflocs, as was observed in the present study. The continuous addition of glucose as an organic carbon source increases the C/N ratio and stimulates the production of microbial biomass in presence of oxygen, while assimilating the nitrogen (Avinimelech, 1999; Ebeling et al., 2006). The microbial community is managed in such a way that nitrifiers are outcompeted (Ebeling et al., 2006). Investigating effects of different C/N ratios in a L. *vannamei* biofloc culturing system, Xu et al. (2016) affirm that the microbial community can shift from chemoautotrophic bacteria (nitrification) to heterotrophic bacteria when carbon input increases or vice versa.

The stimulation of heterotrophic bacterial growth through continuous carbon addition also leads to an increase in total suspended solids (TSS) levels as compared to autotrophic growth. In this sense, TSS was significantly higher in heterotrophic treatments during the experimental period than in all other treatments, which corroborates with the observations of Ray and Lotz (2014) who compared chemoautotrophic and heterotrophic based bioflocs. Higher bacterial densities (hence TSS levels) indicate higher bacterial density in the system, being one of the possible reasons for the results of survival obtained in different systems in the present study.

The experimental period of 21 days of culturing was followed by a *V. parahaemolyticus* challenge test to induce AHPND. Hargreaves (2013) emphasized the need to understand the role of bioflocs in controlling or encouraging pathogenic bacteria, especially vibrios. In the present study, the presence of bioflocs clearly affected the resistance of L. *vannamei* to the AHPND-causing pathogen, although this effect depended on the type of bioflocs used.

When shrimp originating from autotrophic or heterotrophic biofloc systems were challenged with the pathogen in new seawater, there was no significantly increased survival as compared to shrimp that originated from the flow-through system. Thus, despite the fact that immunostimulatory effects have been attributed to bioflocs in earlier studies (Ekasari et al., 2014; Bossier et al., 2016; Ahmad et al., 2017), this kind of activity could not provide a significant protection against the pathogenic challenge for the shrimp in the current study. This suggests that bioflocs grown in this study did not work by means of immunomodulation of shrimp upon long term exposure (H2), and that the protective effect induced by bioflocs should have another mode of action besides a direct stimulatory effect of the shrimp.

When non-experimental shrimp (that had not been previously exposed to bioflocs) were challenged in different types of biofloc suspensions from the 21-d culture period, clear differences in survival could be observed. A similar pattern could be observed for the shrimp that were cultured in different biofloc systems for 21 days and then were challenged in their respective suspensions. These observations confirm hypothesis 1 that the protection is related to the biofloc suspension as such.

An explanation for the differences in survival between biofloc types may be found in the differences in microbial ecology according to the type of suspension. De Schryver and Vadstein (2014) suggested use of the ecological theory of r/K selection to manage microbial communities in aquaculture. According to this concept, an environment rich in nutrients per microbial cell, low in competition and with frequent perturbations selects for microorganisms with a high capacity to quickly exploit nutrients and increase rapidly in population size, termed fast-growing opportunistic r-strategists (De Schryver and Vadstein, 2014). Since a flow-through system cannot sustain high levels of micro-organisms due to continuous wash-out, this typically represents an environment that favors fast-growing r-strategists and as such, in theory, provides a low level of competition and control when a fast-growing pathogen such as the *V. parahaemolyticus*, is introduced (Attramadal et al., 2014). In correspondence with this theory, water from the flow-through system could not provide protection to the shrimp and hence a high mortality of shrimp was observed.

Biofloc suspensions theoretically represent a microbial environment with a lower amount of available nutrients per microbial cell and with less environmental perturbations. Such systems select for more slow-growing K-strategist bacteria and result in a more stable community composition. Such an environment shows a higher level of control for the proliferation and activity of opportunistic fast-growing micro-organisms (Skjermo and Vadstein, 1999). This controlling effect of the bioflocs towards the opportunistic *V. parahaemolyticus* pathogen could indeed be assumed based on the higher survival of shrimp in these treatments. Nonetheless, there was a difference in the level of protection that autotrophic and heterotrophic bioflocs could provide, which again may be explained with the differences in microbial ecology between these two types of bioflocs.

According to theory of De Schryver and Vadstein (2014), heterotrophic bioflocs continuously supplemented with carbon show the presence of a highly abundant yet stable microbial community mainly dominated by heterotrophic microorganisms of the K-strategist type.

Upon introduction in these biofloc suspensions of fast-growing heterotrophic pathogens that are driven by carbon – like the *V. parahaemolyticus* in this study - these are faced by a highly competitive environment resulting in low chance for proliferation.

The autotrophic bioflocs also represent a stable microbial ecosystem that theoretically is dominated by K-strategists that do not thrive on the carbon added continuously to the system. As a result, the level of competition and control upon introduction of a heterotrophic fast-growing pathogen is expected to be lower, which was confirmed by a lower survival of the shrimp in the challenge test as compared to the heterotrophic bioflocs.

An additional effect for both the autotrophic and heterotrophic bioflocs may be the attachment of the pathogen to microbial aggregates present in the system. De Schryver et al. (2008) suggested that extracellular polymeric substances (EPS) that can be found within bioflocs contribute to encapsulation of microbial cells, binding components to the floc. More et al. (2014) affirm that EPS have an important role in floc formation and aggregation of different organic/inorganic compounds with adsorption abilities. Such adsorption of the pathogen by the bioflocs, in combination with the highly competitive environment, is likely the explanation for the (partial) inactivation and/or reduction of the infective pressure of the *V. parahaemolyticus* observed in this study, especially in both heterotrophic bioflocs. Again in this case, a positive correlation can be made between survival after challenge and bacterial biomass (evidenced by TSS levels), which were both higher in heterotrophic-based bioflocs than in autotrophic-based bioflocs.

Associated with that, biofloc may have an effect on quorum sensing regulation of Vibrio, decreasing its activity and virulence towards the host (Crab et al., 2010). This hypotheses put needs to be verified.

To compensate for cases of a lower protective characteristic of bioflocs, probiotics were applied to increase the protection level of shrimp against AHPND. Some researchers have observed that the use of probiotics, most of them *Bacillus* spp., promoted resistance against *Vibrio* infections (Balcazár et al., 2007; Villaseñor et al., 2015; Sha et al., 2016). From the results in this study, it is clear that Sanolife PRO—W™ was indeed able to increase the protective capacity of the autotrophic biofloc system in the case of *V. parahaemolyticus* exposure. Similarly, Krummenauer et al. (2014) verified that the use of a multistrain probiotic could contribute to increasing the survival rates in biofloc systems at event of a vibriosis outbreak. Aguillera-Rivera et al. (2014) also obtained higher survival in a biofloc system supplemented with probiotics, with a low level of lesions in shrimp tissues. As protection was already high in case of heterotrophic bioflocs in the present study, no additional protection was provided by probiotics.

In conclusion, the results of this study illustrate the clear protective potential of biofloc systems in case an AHNPD-causing pathogen is introduced in the water, evidenced by enhanced survival values. Further studies that include an analysis of the microbial community composition in the bioflocs and the changes therein during shrimp culture should be performed to unravel the protective effects as described above. In addition, this study clearly showed the potential of using

probiotics in case biofloc would not be able to provide full protection. Overall, the observations from this study clearly show the importance of microbial management in aquaculture systems and more specifically the influence of operational parameters of biofloc systems to minimize disease risk.

Acknowledgements

The authors are grateful for the financial support provided by the Coordination for the Improvement of Higher Level Personnel (CAPES).

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