Asian Fisheries Science ***31S*** (2018): 210–225

ISSN 0116-6514
E-ISSN 2071-3720
https://doi.org/10.33997/j.afs.2018.31.S1.015

Asian Fisheries Science
The Journal of the Asian Fisheries Society

Effect of Biofloc on the Survival of Whiteleg Shrimp, *Penaeus vannamei* Boone 1931, When Challenged with a Pathogenic Strain of *Vibrio parahaemolyticus*, the Causative Agent of Acute Hepatopancreatic Necrosis Disease (AHPND)

A.P. SHINN[1,2,3,*], J. JIRAVANICHPAISAL[1], D. GRIFFITHS[1], A. POKHARATSIRI[1], P. BURANA[1], T. SUMON[1], C. TONGMEE[1], O. DECAMP[4] and L. GALLI[1]

[1]Fish Vet Group Asia Limited, Chonburi, Thailand
[2]Benchmark Animal Health, Edinburgh Technopole, United Kingdom
[3]Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand
[4]INVE Aquaculture, Bangkok, Thailand

Abstract

To understand better the effect of different water conditions on the survival of whiteleg shrimp, *Penaeus vannamei*, when challenged with a pathogenic strain of *Vibrio parahaemolyticus* (VP_{AHPND}), a series of challenge trials with biofloc were conducted. Challenges investigated the effect of holding individual shrimp for short periods (5–10 days) in either biofloc or in clear water prior to exposure to VP_{AHPND} in either biofloc or in filtered biofloc. Shrimp reared and challenged in unfiltered biofloc had the lowest mortality rates (0 and 6.7 %; $P < 0.05$), followed by those held in clear water for 10 days and challenged in clear water (33.3 and 20 %; $P < 0.05$). Shrimp reared in unfiltered biofloc and but challenged in clear water had the highest rates of mortality (80 and 60 %). A second validation trial, included the use of filtered biofloc. Shrimp reared and challenged in unfiltered biofloc had the lowest rate of mortality (13.3 %), followed by those reared in biofloc but challenged in 2 µm-filtered biofloc (20 %). The highest mortality was in shrimp reared in biofloc but challenged in clear water (73.3 %; $P < 0.01$). The results demonstrate that biofloc can protect whiteleg shrimp from VP_{AHPND} and that the management of biofloc in aquaculture ponds can assist in controlling bacterial infections.

Keywords: acute hepatopancreatic necrosis disease**,** bacterial community, biofloc, quorum quenching, shrimp disease, *Penaeus vannamei, Vibrio parahaemolyticus*

[*]Corresponding author. E-mail address: andy.shinn@fishvetgroup.com

Introduction

Acute hepatopancreatic necrosis disease (AHPND) of whiteleg shrimp (*Penaeus vannamei* Boone 1931) and giant tiger prawn (*P. monodon* Fabricius 1798) has had devastating impacts on shrimp production in the People's Republic of China (NACA-FAO 2011; Panakorn 2012), Malaysia (Lightner et al. 2012; NACA, 2012; Kua et al. 2016), Mexico (Nunan et al. 2014; Soto-Rodríguez et al. 2015), Thailand (Flegel 2012; Leaño and Mohan 2012; Joshi et al. 2014; Chonsin et al. 2016; Songsangjinda and Polchana 2016), Viet Nam (Lightner et al. 2012; Tran et al. 2013; Hien et al. 2016), and most recently from the Philippines (Dabu et al. 2015; dela Peña et al. 2015) and India (Ananda Raja et al. 2017), where it has been a major cause of economic loss (Shinn et al. 2018). Elsewhere, AHPND infections are reported from Costa Rica and Honduras (see Jun et al. 2015), while mortalities of *P. monodon* with manifestations of an AHPND-like condition in Cambodian ponds that were reported throughout 2011–2013 were not verified by diagnostic testing (Lang and Sothea 2016). Infections of *Vibrio parahaemolyticus* associated with the mass mortality of Chinese white shrimp, *Fenneropenaeus chinensis* (Osbeck 1765) have also been reported (Zhang et al. 2014).

Since its advent, there has been a concerted research effort to better understand the causative agent of AHPND – the bacterium *Vibrio parahaemolyticus* with a toxin gene-bearing plasmid (Han et al. 2015a; Lee et al. 2015; Hirono et al. 2016), its mode of action and host pathology (Lightner et al. 2012; Flegel 2012; Tran et al. 2013; Lai et al. 2015; Soonthornchai et al. 2016; Tinwongger et al. 2016), field surveys and conditions under which mortality has occurred (Sriurairatana et al. 2014; Soto-Rodríguez et al. 2015; Chonsin et al. 2016; Hastuti and Haryadi 2016), and investigations into practices that either mitigate against infection, curb its spread or affect its treatment (Panakorn 2012; Han et al. 2015b; Bondad-Reantaso 2016; Jun et al. 2016; Zheng et al. 2016).

With respect to the latter, biosecurity, management of the culture environment, husbandry practices and the application of appropriate probiotics have been central issues (De Schryver et al. 2014). The beneficial effects of biofloc culture (i.e. floculated aggregations of protein-rich organic material consisting of bacterial biomass, microalgae, faecal material, protozoa etc.) on the growth rate, robustness and immune parameters of shrimp are well documented (Wasielesky et al. 2006; Crab et al. 2012; Xu et al. 2013; Ekasari et al. 2014; Suita et al. 2015, 2016a, b). The use of biofloc in intensive closed culture systems is favoured, as organic material can be recycled by microorganisms into microalgae and bacterial biomass which consequentially aggregates to form flocculated material. This biofloc stimulates the growth of heterotrophic and autotrophic microorganisms to process organic residues, converting carbon and nitrogenous waste (e.g. ammonia) into new bacterial biomass (Avnimelech 1999; Luis-Villaseñor et al. 2015). The need for water exchange in the grow-out phase of commercial marine shrimp production, therefore, is reduced (Suita et al. 2016a).

A reduction in the abundance of *Vibrio* has been reported in biofloc systems (De Souza et al., 2012), while the application of selected probiotics on the nutritional profile of biofloc in the culture of a range of shrimp species, including whiteleg shrimp, has been studied (De Souza et al. 2012; Silva et al. 2012). These changes in the microbial community and in the shrimp status have led to better growth and survival rates (Crab et al. 2010; Xu et al. 2013; Aguilera-Rivera et al. 2014). The use of disinfectant without the proper preconditioning of ponds prior to stocking shrimp can lead to impoverished microbial communities, creating conditions that may facilitate the proliferation and domination of certain bacterial species like *V. parahaemolyticus* (De Schryver et al. 2014). The current study, therefore, set out to investigate the potential protective effects of biofloc and shrimp culture conditions prior to and during infection with a pathogenic strain of *V. parahaemolyticus* (VP_{AHPND}).

Materials and Methods

Culture of Experimental Animals

The effect of biofloc on the survival of whiteleg shrimp when exposed to VP_{AHPND} was determined. A single cohort of postlarvae (PL) stage 12 *P. vannamei* was obtained from a specific pathogen free (SPF) facility (details withheld for confidentiality) within Thailand and transferred to the quarantine facility within Fish Vet Group Asia Limited's (FVGAL) research aquarium in Chonburi. On receipt, the PL were disinfected in 100 ppm povidone iodine, stress tested and then held under quarantine while a subsample of 150 PL were tested for the microsporidian *Enterocytozoon hepatopenaei* (EHP), for AHPND and for five viral agents: infectious hypodermal and haematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), white-spot syndrome virus (WSSV) and yellow head virus (YHV) following World Organisation for Animal Health (OIE) approved methodologies (10 batches of 15 PL samples tested in 70 separate polymerase chain reaction (PCR) reactions). Once the PL had been demonstrated to be free of these diseases, they were stocked into 400 L tanks containing a 2-week-old culture of biofloc in 15 ppt pretreated seawater held at ambient conditions (28–29 °C). The biofloc was started using a regime of feeding tanks with rice bran, commercial shrimp feed and sugar that was adjusted daily over the 14 days prior to the receipt of the shrimp. A system of inverted air pipes was used within each tank to provide a moderate level of water movement such that dissolved oxygen levels did not fall below 5.5 $mg.L^{-1}$. The shrimp were initially stocked at 100 $PL.L^{-1}$ and then graded as they grew.

Trial One

Experimental groups for assessment

When the shrimp were approximately 1 ± 0.05 g, 60 shrimp were transferred to a 200 L tank containing 15 ppt pretreated, non-biofloc, seawater. The tanks contained a mature biofilter that was preconditioned on ground shrimp feed and sugar only for 10 days prior to the receipt of shrimp.

The shrimp were maintained at 5 % body weight per day on Charoen Pokphand (CP) Starbird 5093 S feed for 10 days; approximately 10 % of the water in each tank was exchanged daily. A second 200 L tank was stocked with 60 shrimp five days after the transfer of the first tank from the same cohort of shrimp; tank conditioning, maintenance and husbandry followed that applied for the first tank. A challenge with a pathogenic strain of VP_{AHPND} (isolate FVG0001) was conducted 10 days after the stocking of the first tank and five days after the second. For the challenge, a total of 180 1.5 L glass vessels were used and set up in a preconditioned temperature-controlled room within the challenge facility. The temperature was 27.48 ± 0.32 °C (average ± 1 S.D.; range = 26.68–28.36 °C) and monitored for three days prior to the start of the trial and then monitored throughout via the use of two Onset HOBO® UA-001-64 (Bourne, MA, USA) data loggers in each room which recorded the temperature every 15 min.

Bacterial challenge with Vibrio parahaemolyticus

For the bacterial challenges, four experimental groups were set up: 1) shrimp reared in biofloc and then maintained in clear water for 5 days before challenge; 2) shrimp reared in biofloc and then maintained for 10 days in clear water before challenge; 3) shrimp reared in biofloc then challenged in biofloc; and 4) shrimp reared in biofloc and then immediately transferred to clear water (< 4 h) before challenge. Each test vessel was filled with 400 mL of the relevant water, then stocked with a single shrimp; the shrimp were transferred approximately four hours prior to challenge. Fifteen shrimp were individually challenged in each group; the experiment was conducted using two doses of VP_{AHPND} (i.e. a total of 120 shrimp). The challenged groups were run against a corresponding set of controls with a total of 60 shrimp.

To prepare the bacterial inoculum, the VP_{AHPND} was cultured in tryptone soya broth (TSB) supplemented with 2 % NaCl and incubated under shaking conditions (i.e. 250 rpm) at 28 °C for 12 h. Thereafter, the bacteria were precipitated by centrifugation at 900 × g for 10 min at 10 °C and the resultant bacterial pellet subsequently resuspended in sterile 15 ppt brackish water. The bacterial cell number of the resultant VP_{AHPND} medium was estimated by measuring the optical density at 600 nm (OD_{600}), where for VP_{AHPND}, an OD value of 1.0 corresponded to approximately 1.0×10^8 cfu.mL^{-1}. The bacterial cell number was then adjusted and verified by viable plate counts following standard methods.

For the challenge, each shrimp was held in a glass jar containing 400 mL of 15 ppt (either biofloc or in pretreated clear) aerated seawater at 27.5 °C and then given either a 1.2 mL.vessel^{-1} (dose 1) or a 1.8 mL.vessel^{-1} (dose 2) of VP_{AHPND} (isolate FVG0001; OD_{600} = 1.012; initial concentration of the inoculum was 1.28×10^8 cfu.mL^{-1}). The bacterial doses used were determined from pretests conducted with three doses of VP_{AHPND} using three shrimp per dose held under the same experimental conditions as those used for the main challenge. Shrimp from the same population as those used for the main challenge were used for the pretests; the pretests were conducted 48 h before the main challenge. Approximately five minutes after the shrimp had been challenged, 1 mL water samples were taken from a minimum of three test vessels in each experimental group.

The average bacterial dose per vessel was determined by taking 10 µL of a 10-fold dilution of the water sample and dropping this onto a TCBS plate, incubating it at 28 °C overnight and then making manual colony counts. The shrimp were maintained for 24 h, after which a further 400 mL of the liquid medium appropriate to each test condition was added (i.e. clear water or biofloc). After 48 h post-challenge, 50 % of the water in each test vessel was replaced with fresh medium. Each shrimp was given a daily ration of 5 feed pellets. The shrimp in each jar were evaluated every 3 h post-challenge and any mortalities were noted. The trial was terminated 96 h post-challenge after the frequency of mortalities had stabilized.

Trial Two

To verify the results, a second trial was conducted using similar experimental conditions to those used for trial one. Five challenge conditions were used; shrimp that were reared in biofloc to an average weight of 1.762 ± 0.085 g (mean ± S.D.) were either: 1) maintained in clear water for 7 d before challenging in clear water; 2) maintained in clear water for 7 d before challenging in biofloc; 3) maintained in biofloc and challenged in biofloc; 4) maintained in biofloc and challenged in biofloc filtered through a < 2 µm felt bag used as a standard for water filtration on commercial shrimp farms; or 5) maintained in biofloc but challenged in clear water. For each experimental condition, a total of 15 individually held shrimp were used and assessed against non-VP_{AHPND} unchallenged shrimp; temperature in the challenge room was 28.25 ± 0.66 °C (average ± 1 S.D.; range = 25.90–29.25 °C). For the challenge, only a single dose was explored, i.e. 4 mL VP_{AHPND} inoculum in each vessel (isolate FVG0001; OD_{600} = 1.046; initial concentration of the inoculum was 1.28×10^8 cfu.mL^{-1}). The challenge dose was determined following a series of pretests on shrimp from the same population, 48 h prior to the main challenge. The total suspended solids in the biofloc medium was determined to be approximately 670 mg.L^{-1} by filtering 1 L of the shrimp culture water through preweighed No. 93 Whatman filter paper and then drying the residue for 24 h before weighing.

Analysis of Biofloc

Samples of biofloc used for each experimental trial were taken from the shrimp culture tanks and evaluated. For the first trial, a 1.5 L sample was taken and subsequently passed through a 0.45 µm cellulose membrane, after which 8 mm discs were cut and placed onto trypticase soy agar (TSA) plates supplemented with 2 % (w/v) NaCl and inoculated with 100 µL of the VP_{AHPND} (concentration = 1.28×10^8 cfu.mL^{-1}). Three biofloc filter discs were placed on two TSA plates which were subsequently incubated at 28 °C for 14–16 h, after which the plates were visually assessed for clearance zones around each disc.

Statistical Analysis

Pairwise Kaplan-Meier survival analyses with subsequent Mantel-Cox log-rank tests conducted in Excel 2016 were applied to the mortality data to calculate the survival probabilities and to compare the survival distributions of the shrimp in each experimental group. Statistical significance was set at $P < 0.05$.

Ethics Statement

These experimental procedures were reviewed by and conducted under the approval of Fish Vet Group's internal ethical review board. Scientists conducting the aquatic pathogen trials hold licences for the use of "Animals for Scientific Purposes" issued by the Institute for Animals for Scientific Purpose Development (IAD), National Research Council of Thailand (NRCT). Fish Vet Group's laboratories and challenge facilities are registered with the relevant Thai authorities and are inspected as required under current Thai legislation.

Results

Trial One

Following the experimental challenge with VP_{AHPND}, water samples were taken from a random selection of the test vessels and used to verify the dose of bacteria added (Table 1). The shrimp were subsequently assessed every three hours and any mortalities were noted; the cumulative mortality curves are presented in Figure 1. The trial was terminated 96 h post-infection after the pattern of mortalities had stabilized.

Two doses of VP_{AHPND} were used to challenge the shrimp (i.e. 2.11–2.63 × 10^5 cfu.mL^{-1} and 2.84–4.54 × 10^5 cfu.mL^{-1}; n = 60 shrimp per dose) against a control group (n = 60 shrimp; Table 1). The highest mortalities were seen in the shrimp groups that had been most recently transferred from biofloc, i.e. those that were immediately transferred from biofloc into clear water and challenged (Bio-CW Vp; mortalities of 80 % and 60 %) and those that had been reared in biofloc but maintained for 5 days in clear water and then challenged (5D Vp; mortalities of 60 % and 73.3 %; Fig. 1).

The rates of mortality were lower (i.e. 33.3 % and 20 %) in the shrimp that were transferred from biofloc and then held in clear water for 10 days (10D Vp) before they were challenged in clear water, but the lowest mortality of 0 % and 6.7 % were in the two groups of shrimp that were reared and challenged in biofloc (Biofloc Vp). All shrimp were handled identically, in that they were transferred from their culture environment into their relevant vessels approximately 4 hr prior to experimental challenge.

Significant differences between the culture conditions are presented in Table 2. The lower rates of mortality in the shrimp reared and challenged in biofloc were statistically lower ($P < 0.05$) than those determined in the other experimentally challenged groups.

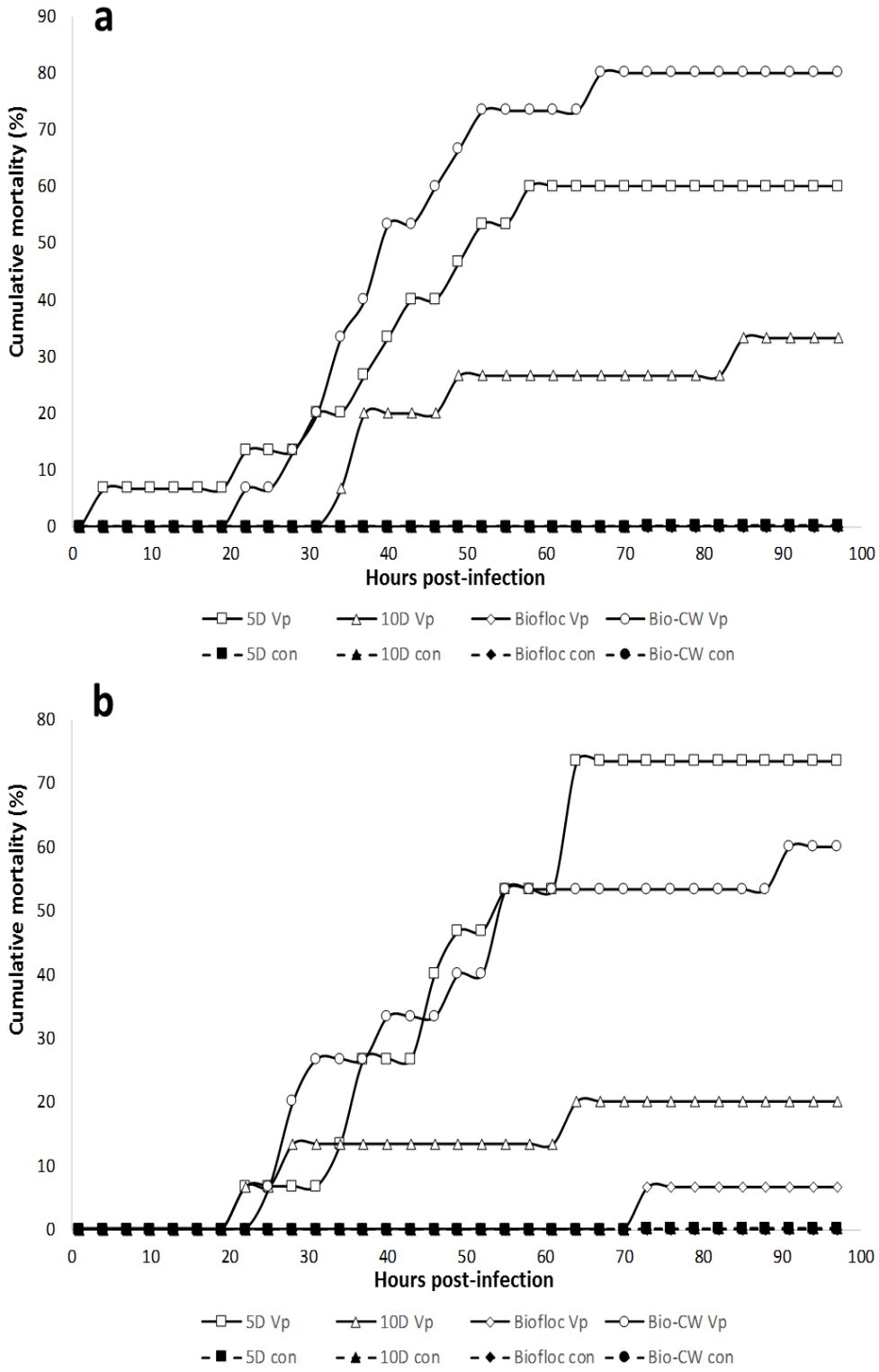

Fig. 1. Cumulative mortality curves for *Penaeus vannamei* reared in biofloc and then subjected to a water treatment before being challenged with two different doses of *Vibrio parahaemolyticus* (Vp isolate FVG0001) and compared against corresponding controls. Figure 1a = 1.2 mL VP_{AHPND} culture.vessel^{-1} (dose 1), while Figure 1b = 1.8 ml VP_{AHPND} culture.vessel^{-1} (dose 2), where the initial concentration of the inoculum was 1.28 ×10^{8} cfu.mL^{-1}. Shrimp were either held for five (5D Vp) or ten days (10D Vp) in non-biofloc, clear, pretreated 15 ppt seawater before challenge or were transferred immediately from biofloc to clear non-biofloc pretreated water (Bio-CW Vp). A fourth group of shrimp was reared and challenged in biofloc (Biofloc Vp). A further four groups of shrimp, 5D con, 10D con, Biofloc con and Bio-CW con are the corresponding control groups to each VP_{AHPND} -challenged group.

Table 1. The average bacterial dose (cfu) per experimental vessel and per water condition. Following the challenge of the experimental *Penaeus vannamei* with a pathogenic isolate of *Vibrio parahaemolyticus* (isolate FVG0001), the bacterial load was determined from 1 mL water samples taken from a random selection of test vessels in each water treatment (5-days = 5 days in clear water before challenge in clear water; 10-days = 10 days in clear water before challenge in clear water; Biofloc = reared in biofloc and challenged in biofloc; Bio-CW = reared in biofloc and then transferred to clear water immediately before challenge).

Condition	Water treatment	Number of bacteria ($cfu.mL^{-1}$) on TCBS	
		Green colonies	Yellow colonies
Vp Dose 1	5-days	2.62×10^5	
	10-days	2.63×10^5	
	Biofloc	3.90×10^5	
	Bio-CW	2.11×10^5	
Vp Dose 2	5-days	2.82×10^5	
	10-days	3.26×10^5	
	Biofloc	5.96×10^5	
	Bio-CW	4.54×10^5	
Control	5-days	1.60×10^2	4.00×10
	10-days	4.00×10	2.00×10
	Biofloc	2.60×10^3	9.20×10^3
	Bio-CW	2.00×10	2.00×10

Table 2. Pairwise Mantel-Cox log-rank tests applied to the Kaplan-Meier survival probabilities for each test group of shrimp (n = 15 replicates). Survival of *Penaeus vannamei* challenged with different doses of *Vibrio parahaemolyticus* was assessed against a control group. Shrimp reared to ca. 1 g in biofloc were either maintained in non-biofloc, pretreated 15 ppt seawater for ten days (10D), five days (5D) or were immediately transferred (approximately 4 h) before being challenged (Bio-CW). A fourth group of shrimp reared in biofloc was also challenged in biofloc (Biofloc). Figures in a bold font highlight significant differences ($P < 0.05$) in shrimp survival between the different test conditions.

		Dose 1				Dose 2			
		5D	10D	Biofloc	Bio-CW	5D	10D	Biofloc	Bio-CW
Dose 1	5D								
	10D	0.136							
	Biofloc	**< 0.001**	**0.017**						
	Bio-CW	0.272	**0.009**	**< 0.001**					
Dose 2	5D	0.852	**0.047**	**< 0.001**	0.364				
	10D	**0.030**	0.482	0.073	**0.002**	**0.009**			
	Biofloc	**0.001**	0.068	0.317	**< 0.001**	**< 0.001**	0.267		
	Bio-CW	0.897	0.147	**< 0.001**	0.217	0.634	**0.038**	**0.002**	
Control	5D	**0.001**	**0.004**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
	10D	0.068	0.175	**0.017**	**0.017**	0.267	0.562	0.073	0.073
	Biofloc	0.317	0.150	1.000	1.000	1.000	**< 0.001**	0.317	0.317
	Bio-CW	**<0.001**	**<0.001**	**< 0.001**	**< 0.001**	**0.011**	**0.023**	**0.004**	**0.004**

Trial Two

The second trial, using VP_{AHPND} doses of 1.33–1.44 × 10^6 cfu.mL^{-1} (Table 3), yielded similar results to that of the first (Figure 2). The trial, however, included filtered biofloc as a test condition. Again, the highest level of mortality (73.3 %) was seen in the shrimp transferred from biofloc to clear water prior to challenge, which differed significantly ($P < 0.001$) from those reared and challenged in biofloc which had the lowest level of mortality (13.3 %). A high level of mortality (53.3 %) was also seen in the shrimp that were reared in clear water for 7 days prior to challenge. Lower levels of mortality were seen in the other groups challenged in biofloc, i.e. 26.7 % for those reared in clear water for 7 days but then challenged in biofloc, and only 20 % mortality among the shrimp reared in biofloc but challenged in < 2µm filtered biofloc. Significant differences in the survival rates of the shrimp in the different experimental groups are given in Table 4.

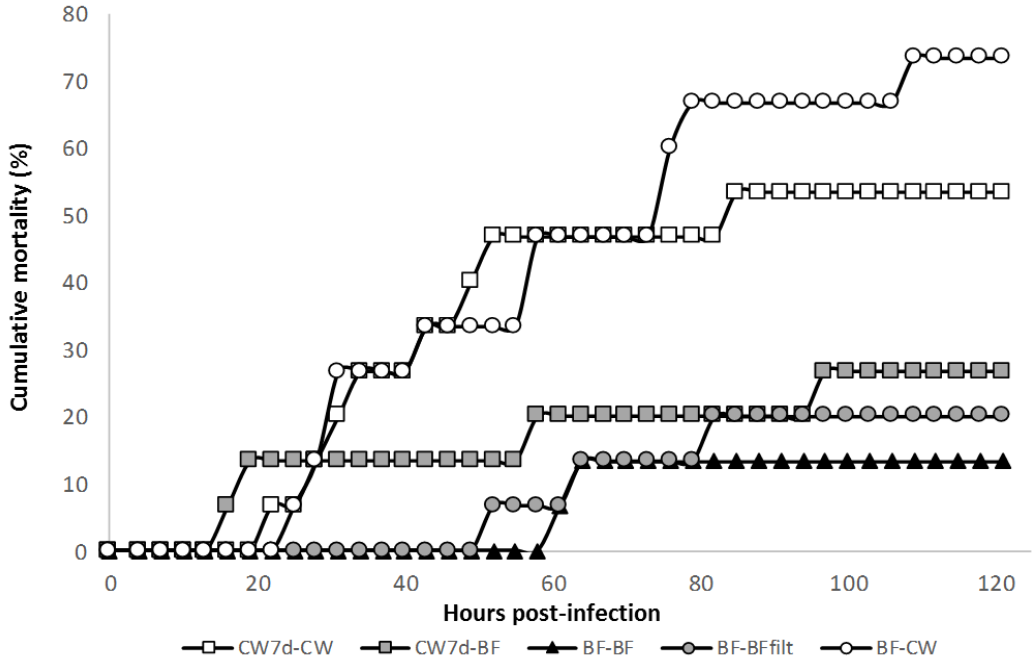

Fig. 2. Cumulative mortality curves of *Penaeus vannamei* challenged with *Vibrio parahaemolyticus* in the second biofloc-based experiment CW7d-CW = shrimp held for seven days in clear water before challenge in clear water; CW7d-BF = shrimp held for seven days in clear water and then challenged in biofloc; BF-BF = shrimp reared and challenged in biofloc; BF-BFfilt = shrimp reared in biofloc but challenged in filtered biofloc; and BF-CW = shrimp reared in biofloc but challenged in clear water. Each group consists of 15 shrimp; corresponding controls were used for each challenge condition. The control curves are not shown but there was the loss of two shrimp in the CW7d-CW control at 49 h post-inoculation (p.i.) and three shrimp in the CW7d-BF control at 49 h (1 shrimp) and 97 h p.i. (two shrimp). There was no other loss of control shrimp.

Table 3. Average bacterial dose (cfu.mL^{-1}) per experimental vessel and per water condition in the second biofloc-based *Vibrio parahaemolyticus* challenge trial. Following bacterial challenge, the bacterial load was determined in a random selection of test vessels within each water treatment (CW7d-CW = 7 days in clear water before challenge in clear water; CW7d-BF = 7 days in clear water before challenge in biofloc; BF-BF = reared in biofloc and challenged in biofloc; BF-BFfilt = reared in biofloc and then challenged in filtered biofloc water; BF-CW = reared in biofloc and then challenged in clear water).

Condition	Water treatment	Number of bacteria (cfu.mL^{-1}) on TCBS	
		Green colonies	Yellow colonies
Vp	CW7d-CW	1.33×10^6	3.33×10^4
	CW7d-BF	1.44×10^6	6.67×10^3
	BF-BF	1.92×10^6	
	BF-BFfilt	1.27×10^6	
	BF-CW	1.46×10^6	
Control	CW7d-CW	2.00×10	6.00×10
	CW7d-BF	5.00×10^2	6.20×10^2
	BF-BF	2.60×10^2	4.60×10^2
	BF-BFfilt	3.80×10^2	2.40×10^2
	BF-CW		

Table 4. Pairwise Mantel-Cox log-rank tests applied to the Kaplan-Meier survival probabilities from *Penaeus vannamei* challenged with *Vibrio parahaemolyticus* under different water conditions (CW7d-CW = 7 days in clear water before challenge in clear water; CW7d-BF = 7 days in clear water before challenge in biofloc; BF-BF = reared in biofloc and challenged in biofloc; BF-BFfilt = reared in biofloc and then challenged in filtered biofloc water; BF-CW = reared in biofloc and then challenged in clear water). Figures shown in a bold font highlight significant differences ($P < 0.05$) in the survival of shrimp between the different water conditions.

		Vp challenged				
		CW7d-CW	CW7d-BF	BF-BF	BF-BFfilt	BF-CW
Vp chal	CW7d-CW					
	CW7d-BF	0.148				
	BF-BF	**0.014**	0.340			
	BF-BFfilt	**0.038**	0.634	0.644		
	BF-CW	0.452	**0.021**	**0.001**	**0.003**	

		Control				
		CW7d-CW	CW7d-BF	BF-BF	BF-BFfilt	BF-CW
Vp chal	CW7d-CW	**0.018**	**0.034**	**0.001**	**0.001**	**0.001**
	CW7d-BF	0.365	0.603	**0.035**	**0.035**	**0.035**
	BF-BF	0.957	0.668	0.150	0.150	0.150
	BF-BFfilt	0.690	0.949	0.073	0.073	0.073
	BF-CW	**0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**

Control	CW7d-CW					
	CW7d-BF	0.679				
	BF-BF	0.157	0.076			
	BF-BFfilt	0.157	0.076	NS		
	BF-CW	0.157	0.076	NS	NS	

Analysis of Biofloc

No demonstrable antimicrobial activity (i.e. no clear zones around the discs through which the biofloc was filtered) was detected in the samples of biofloc taken from the shrimp culture tank.

Discussion

Biofloc has also been shown to have a positive impact on the survival, growth and the digestive enzyme activities of *P. vannamei* (Xu et al. 2013). The studies conducted by the latter authors found that the levels of digestive enzyme activity were significantly higher in the stomachs of biofloc-reared shrimp than in those of shrimp raised in non-biofloc water.

These findings were also in agreement with an earlier study conducted by Moss et al. (2001), who had found that the activity of digestive enzymes in the hepatopancreas was higher in *P. vannamei* reared in ponds than in those raised in well water. Suita et al. (2015) stated that biofloc positively affects intestinal peristalsis and the synthesis of digestive enzymes by the hepatopancreas, with an increased thickness to the hepatopancreatic tubules and a rise in the number of enzyme-producing B cells. Changes to the hepatopancreas may be associated with essential amino acids, vitamins and minerals present in biofloc (Decamp et al. 2002).

The current study set out to compare the survival rates of whiteleg shrimp when reared in two main water types (i.e. clear water or in biofloc) for different periods of time and then challenged with $VP_{AHPND.}$ The mortality of shrimp within each water condition can be ranked as follows:

Trial 1a: Biofloc (0 %) > 10D (33.3 %) > 5D (60 %) > Bio-CW (80 %)

Trial 1b: Biofloc (6.7 %) > 10D (20 %) > Bio-CW (60 %) > 5D (73.3 %)

Trial 2: Biofloc (13.3 %) > Filtered biofloc (20 %) > CW7d-Biofloc (26.7 %) > CW7d - CW (53.3 %) > BF-CW (73.3 %)

These findings show that the shrimp, when challenged in biofloc regardless of their prior culture conditions, have lower levels of mortality than those challenged in clear water. Within the biofloc-challenged group, those that were reared and infected in biofloc had the lowest mortality (i.e. 0 %, 6.7 % and 13.3 %). There appears to be little difference in the mortality of the shrimp that were reared in biofloc and then challenged in < 2 µm filtered biofloc (20 %) and those reared for 7 days in clear water but then transferred to biofloc ca. 4 h prior to challenge (26.7 %) – a difference in mortality of only a single shrimp. For the shrimp challenged in clear water, there is a suggested correlation in mortality and the duration spent in clear water prior to challenge, i.e. 10D (ave. 26.65 %) > 5D (ave. 66.7 %) > 4 h (ave. 71.1 %). All shrimp were initially reared in biofloc until they were transferred into clear water.

The results suggest that the movement into clear water places a stress on the shrimp that has an immediate impact on their feeding activity and intake, with potential consequential impacts on enzymatic activity within the hepatopancreas and the immune status of the shrimp. The change in culture environment will also effect a change in the intestinal bacterial community of the gut, changing the shrimp's resistance to pathogenic bacteria by, for example, affecting virulence by quorum quenching (Pande et al. 2013; Luis-Villaseñor et al. 2015; Zheng et al. 2016). The lower rates of mortality correlated to the time spent in clear water suggest that accommodation to the new environment is required in: 1) re-establishing a stable intestinal microflora; and, 2) switching from the option to feed continuously on biofloc supplemented with a commercial pelleted diet to a regime where diet is not continuously available. On termination of the trial, the shrimp maintained in biofloc had dark guts indicating that they had continued to feed throughout the challenge, whereas the shrimp maintained in clear water had very little within their guts despite being fed throughout.

From the current trial, shrimp that were reared in biofloc but then were transferred to clear water for the VP_{AHPND} challenge had the highest rates of mortality (Figs. 1 and 2). This suggests that the preculture in biofloc, and the shrimp's intestinal bacterial community, offers no protection when transferred and then challenged in clear water under the experimental conditions used here. In a marked contrast to this, the benefits of biofloc appear to be immediate. There was only a 26.7 % mortality in the shrimp that were reared in clear water for 7 days but then were immediately transferred to biofloc for the VP_{AHPND} challenge, whereas there was a 73.3 % mortality in the shrimp reared in biofloc but then transferred to clear water for the VP_{AHPND} challenge (Fig. 2). Under the conditions used here, it would appear that biofloc has an impact on the virulence of VP_{AHPND}, theoretically by disrupting quorum sensing. This appears to be unaffected by filtration. In conclusion, *P. vannamei* challenged with VP_{AHPND} in biofloc had the highest rates of survival (ave. 86.7 % survival for those challenged in biofloc versus ave. 43.4 % for shrimp challenged in clear water). The benefits appear to be immediate; however, the protection is immediately lost once shrimp are transferred into clear water and challenged. The findings suggest that careful management of the microbial community within aquaculture ponds can assist in controlling bacterial infections.

Acknowledgements

The views expressed in this article are those of the authors and do not necessarily represent the views of, and should not be attributed to Benchmark Holdings PLC and its subsidiaries including the Fish Vet Group, Benchmark Animal Health, and INVE Aquaculture, nor to Chiang Mai University. While no funding was received to support this study, in-kind contributions and use of wet lab and challenge test facilities was provided by Fish Vet Group Asia Ltd. This was an invited paper and work on this was conducted in free personal time.

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