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Prophylactic properties of biofloc- or Nile tilapia-conditioned water against *Vibrio parahaemolyticus* infection of whiteleg shrimp (*Penaeus vannamei*)

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Running title: AHPND, biofloc and tilapia-conditioned water

Key words: Acute hepatopancreatic necrosis disease (AHPND), bath challenge, disease management, greenwater, Imhoff cone, *Oreochromis niloticus*, shrimp disease.

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Research highlights:

1. Whiteleg shrimp *Penaeus vannamei* were bath challenged with *Vibrio parahaemolyticus* and shrimp had significantly greater survival in biofloc than clear seawater during 96 h post-challenge.

2. Shrimp stocking density (1 to 5 shrimp per 400 mL) did not influence survival of shrimp bath challenged with *V. parahaemolyticus* in either biofloc or clear seawater conditions.

3. Survival of shrimp bath challenged with *V. parahaemolyticus* was significantly greater in Nile tilapia-conditioned water prepared at 5 ppt and 10 ppt compared to at 15 ppt and in clear seawater at 5, 10 and 15 ppt.

4. Biofloc and Nile tilapia-conditioned water may protect against acute hepatopancreas necrosis disease (AHPND), and these are inexpensive potential disease management control strategies that could be adopted by the shrimp industry.

Statement of relevance:

Managed biofloc and Nile tilapia-conditioned water culture conditions can reduce whiteleg shrimp losses due to *Vibrio parahaemolyticus* (134 characters with spaces)

Abstract

Isolates of *Vibrio parahaemolyticus* (Vp_{AHPND}) that carry a plasmid encoding two *Pir*-like toxins cause acute hepatopancreatic necrosis disease (AHPND), a disease that has caused devastating economic losses to the shrimp industry, particularly in Asia. However, lower prevalence of AHPND infection has been associated with farms that operate with biofloc or lower salinity culture water. Therefore, the aim of this present study was to investigate the effects of biofloc, different culture water salinity and Nile tilapia (*Oreochromis niloticus*)-conditioned water on survival of whiteleg shrimp (*Penaeus vannamei*) bath challenged experimentally with Vp_{AHPND}. First, groups of shrimp were bath challenged with Vp_{AHPND} in clear 15 ppt seawater (CW) or in the presence of a pre-cultured biofloc at 25%, 50% and 100% (v/v). Survival during 96 h post-challenge was significantly greater in groups cultured in 50% and 100% biofloc ($p<0.05$). In a second trial, the effect of shrimp stocking density on biofloc protection against bath challenge with Vp_{AHPND} was determined and shrimp challenged in 100% biofloc again had significantly greater survival ($p<0.05$) compared to the CW group, whilst under our experimental conditions stocking density had no significant influence on survival post-challenge. In a third trial, shrimp were challenged with Vp_{AHPND} in three different salinities of CW or Nile tilapia-conditioned (NTC) water (5 ppt, 10 ppt and 15 ppt). Survival in this final trial was 33% at 96 h in 5 ppt CW compared to just 7% in the 10 ppt and the 15 ppt CW groups, though these differences were not statistically significant. Moreover, shrimp survival in the 5 ppt and 10 ppt NTC water groups was significantly greater than in the 15 ppt NTC water group ($p<0.05$), while significantly greater survival was observed in 10 ppt NTC water compared to 10 ppt CW ($p<0.05$). The results indicate that biofloc and NTC water may provide some protection against AHPND, whilst low salinity culture water may also offer a degree of protection against this bacterium. These findings may allow for the implementation of inexpensive strategies in the shrimp industry to assist in minimising the impact of Vp_{AHPND} as part of pond management practices.

1. Introduction

Infection of tiger shrimp (*Penaeus monodon*) and whiteleg shrimp (*Penaeus vannamei*) by pathogenic isolates of *Vibrio parahaemolyticus* that carry a plasmid encoding two *Pir*-like toxins can cause progressive degeneration of the hepatopancreas resulting in high mortalities of juvenile shrimp and often entire loss of stocks within 30 days (Lightner *et al*., 2012; Network of Aquaculture Centres Asia-Pacific [NACA], 2012; Zorriehzahra & Banaederakhshan, 2015). Since 2009 this infection, known as acute hepatopancreatic necrosis disease (AHPND), has resulted in collective losses exceeding an estimated US$ 43 bn across Asia (China, Malaysia, Thailand, Vietnam) and in Mexico (Flegel, 2012; Tran *et al*., 2013; Chonsin *et al*., 2016; Pakingking *et al*., 2016; Office International des Epizooties [OIE], 2017; Shinn *et al*., in press b). AHPND infections, however, are also known in India (Ananda Raja *et al.*, 2017), the Philippines (Dabu *et al.*, 2015; de la Peña *et al.*, 2015), Costa Rica and Honduras (Jun *et al.*, 2016), while mortalities of *P. monodon* attributed to an AHPND-like condition have been reported from Cambodian ponds (Lang and Sothea, 2016). In Thailand, AHPND caused whiteleg shrimp production to reduce from *ca.* 600,000 tons in 2011 to *ca.* 200,000 tons by 2015. In turn, this meant that Thailand has been surpassed by Vietnam, China and India as the largest exporters of shrimp (Pakingking *et al*., 2016; Portley, 2016), and these differentials correlate positively with levels intensification in these countries, *i.e.* Vietnam, China and India retains a greater mix of less and more intensive culture systems with the former type being less impacted by AHPND.

Lower prevalence of infection has been associated with lower salinity culture conditions,and use of biofloc systems and lined ponds (Gabaudan, 2012; NACA, 2012; De Schryver *et al.*, 2014; Boonyawiwat *et al*., 2017). Juvenile shrimp, *i.e.* post-larvae (PL) stage 1 to PL stage 30, are reared typically in clear water with the addition of phytoplankton, zooplankton (*Artemia* sp.), commercial feeds and other supplementary feeds such as microalgae, *e.g. Chaetoceros* spp. (Suita, 2016). As detritivores, shrimp can also feed on biofloc, a flocculent, organic, protein-rich suspension consisting of prokaryotic and eukaryotic microbes. Moreover, the basic principle of a biofloc system is to recycle the ammonia and nitrite resulting from uneaten food and faeces into microbial biomass that can be used either *in situ* by the cultured animals as a source of protein or subsequently harvested and processed into a feed (Avnimelech, 1999; Hari *et al*., 2004; De

Schryver *et al*., 2008; Kuhn *et al.*, 2009; Crab *et al*., 2012; Avnimelech, 2014; Ekasari *et al*., 2014).

The use of biofloc can increase feed utilization, growth, survival and the reproductive performance of cultured animals (Xu *et al*., 2012; Ekasari *et al*., 2014; Suita, 2016; Ballester *et al*., 2017). Moreover, some studies have investigated the beneficial immunological effects of the organisms found in biofloc, and their cellular components and metabolites can act as immunostimulants to enhance the shrimp innate immune system and provide improved protection against pathogens (Vazquez *et al*., 2009; Crab *et al*., 2010; Ekasari *et al*., 2014; de Jesus Becerra-Dorame *et al*., 2014; Xu *et al*., 2014; Kim *et al*., 2014; Shinn *et al*., in press a). Critically, biofloc may also have a direct 'probiotic' effect in the pond or gut microbiome, *i.e.* as benign commensal heterotrophic bacteria with potential to displace pathogenic *Vibrio* spp. (facultative anaerobes) under intensively aerated production conditions (Arias-Moscoso *et al*., 2018)

Production of biofloc is optimised through managed addition of organic carbon under highly aerated and minimal water-exchange culture conditions, and biofloc may be managed *in* or *ex situ*, *i.e.* directly with the target culture species or separately (Avnimelech, 1999). Biofloc systems are further differentiated as brown or green water systems contingent on lighting levels and thus the relative mix of 'brown' heterotrophic bacteria and 'green' phytoplankton (Taw, 2012). Hereafter, we differentiate between this interpretation and a more generalised use of 'greenwater' (conjoined) to describe any phytoplankton dominated culture system, with or (more typically in the case of tilapia culture) without aeration and lacking any directed carbon:nitrogen management.

Biofloc and greenwater approaches to culture shrimp precede the recent emergence of AHPND-causing strains of *V. parahaemolyticus* (*Vp*$_{\text{AHPND}}$) in many places (Hargreaves, 2013) but, in the Philippines and Vietnam, greenwater technology has been adopted alongside improved biosecurity practices at grow-out pond sites to prevent AHPND (Usero & Apostol-Albaladejo, 2015; Cadiz *et al*., 2016; Pakingking, 2016). Since 1996, tilapia-conditioned water with a high *Chlorella* content has been used in shrimp farming to prevent *Vibrio* spp. infections (Dash *et al*., 2017). Meanwhile, bacteria isolated from tilapia skin and mucus from the gut and skin have demonstrated potent anti-*Vibrio* spp. properties (Lio-Po *et al.*, 2005). Aside from reducing the burden of certain bacteria in the water, co-

culture of tilapia in shrimp ponds is recommended for improving soil and water quality (Tendencia *et al.*, 2015). Tendencia *et al.* (2004, 2015) reported that rearing tilapia at >300 g m^{-3} inhibited the growth of *Vibrio* spp. in shrimp biomass stocked at 80 g m^{-3} and improved shrimp survival.

This present study aimed to investigate the effects of biofloc and Nile tilapia (*Oreochromis niloticus*)-conditioned (NTC) water prepared at different salinities to protect whiteleg shrimp against experimental bath challenge with a pathogenic *Vp*$_{AHPND}$ isolate.

2. Materials and methods

2.1 Bacteria, shrimp and tilapia

The *Vp*$_{AHPND}$ isolate FVG0001 was used for all challenge trials. During May to June 2017, batches of juvenile whiteleg shrimp were acquired from a commercial shrimp hatchery located in Chachoengsao Province, Thailand, and transferred to the quarantine unit at the Fish Vet Group Asia Limited (FVGAL) Research Aquarium in Chonburi, Thailand. On receipt of each shipment, the shrimp were surface-disinfected with 0.1 mg L^{-1} povidone iodine and a sub-sample (n = 20 individuals; mean of 0.4 g) were confirmed to be negative for seven major shrimp diseases (*Vp*$_{AHPND}$; the microsporidian *Enterocytozoon hepatopenaei* [EHP]; infectious hypodermal and haemotopoietic necrosis virus [IHHNV]; infectious myonecrosis virus [IMNV]; Taura syndrome virus [TSV]; white-spot syndrome virus [WSSV]; and, yellow head virus [YHV]) by iiPCR test kits (GeneReach Biotechnology Corporation, Taichung, Taiwan) and OIE approved methodologies (OIE, 2017). Furthermore, 24 mixed sex Nile tilapia were sourced from a commercial farm (119.8 ± 33.4 g) and transferred to the FVGAL Diagnostic Laboratory (*ca.* 2 km from the FVGAL Research Aquarium).

2.2 Shrimp holding conditions and preparation of biofloc

Disease-free shrimp were stocked into 400-L tanks (positioned out of direct sunlight) containing mature biofloc. The biofloc in each tank had been established in 300 L of 15 ppt seawater. This water had been pre-treated with 50 mg L^{-1} chlorine and then treated with a further 10 mg L^{-1} chlorine for at least 1 h by addition of calcium hypochlorite, with any

residual chlorine driven off with vigorous aeration. Absence of residual chlorine was confirmed using an orthotolidine-based chlorine test kit (Monitor®; Pet Wonderland Group, Thailand). The biofloc was initiated by adding 5 g rice bran, 1.5 g ground shrimp feed and 3 g white sugar (as sources of carbon) to each tank and incubating for 2 days at 28–29°C with intensive aeration (this provided greenwater biofloc given the ambient lighting typical of sub-tropical shrimp pond production conditions). Thereafter, 1 g ground shrimp feed and 3 g white sugar were added on a daily basis. At day 3, physicochemical water parameters were measured *in situ* and adjusted by changing rates of carbon substrate addition to adhere within the following limits: <0.03 mg L^{-1} ammonia and <1 mg L^{-1} nitrite (measured with a Tetra^{TM} test kit; Tetra GmbH, Melle, Germany), pH 7.5–8.0 (maintained through the addition of calcium carbonate as necessary), alkalinity 80-150 mg L^{-1} $CaCO_3$, 15 ppt salinity and 28–29°C (measured using a hand-held automatic temperature compensation refractometer; Bellingham & Stanley Ltd, United Kingdom), and >5 mg L^{-1} dissolved oxygen (DO) (measured with a hand-held DO meter; YSI 550A; Xylem Inc., United States). A system of inverted air pipes provided continuous aeration to maintain DO at >5 mg L^{-1} and salinity, DO and temperature readings were taken daily thereafter. The shrimp were maintained on commercial feed (Starbird 5093 S shrimp feed; Charoen Pokphand Co., Bangkok, Thailand) at 10% body wt d^{-1}, given daily in three equal rations at 08:00, 14:00 and 18:00. Additionally, white sugar was added at a ratio of white sugar:shrimp feed (2.3:1). The condition of the shrimp and biofloc were monitored microscopically every day to ensure that the shrimp were in good condition (*i.e.*, no evidence of necrosis, biofouling or infection of the shrimp). The biofloc was considered to be ready for application when Imhoff cone readings were >10 ml L^{-1} after a 30-min settlement period, and 10–15 mL L^{-1} is considered ideal for shrimp culture (Hargreaves, 2013). Total suspended solids readings were confirmed by filtering 1 L of biofloc suspension through pre-weighed filter paper (Whatman No. 93; GE Healthcare UK Limited, Buckinghamshire, UK) and then drying for 24 h at 50°C before massing the dried matter. Biofloc was collected at >14 d and used for the experimental challenge trials. Generally, 10–15% of the water volume was exchanged daily with pre-treated and dechlorinated 15 ppt seawater (except for the day prior to the start of a challenge trial to preserve the condition of the biofloc); however, volume exchanges deviated occasionally to ensure Imhoff cone readings were maintained at 10–15 mL L^{-1}.

2.3 Tilapia holding conditions and preparation of NTC water

The tilapia were stocked into a single 600-L aerated (70 L min^{-1}) tank containing dechlorinated freshwater (partially shaded from direct sunlight) and allowed to acclimate for 7 days. Water temperature (32.1 ± 2.6 °C) and surface light (mean intensity of 60,346 lux d^{-1} [maximum = 297,602 lux d^{-1}] and mean duration of 12.91 ± 0.18 h sunlight d^{-1} [range: 12.5–13 h d^{-1}]) was recorded every 15 min with data loggers (Onset HOBO UA-001-64; Bourne, MA, USA). After acclimation, the fish were split such that 8 fish were assigned at random to each of three 200-L tanks (biomass of *ca.* 960 g $tank^{-1}$). Aeration was then split between the three tanks, while DO, pH, ammonia and nitrate were measured daily and adjusted where necessary to maintain >5 mg L^{-1} DO, 7.5–8.0 pH, <0.03 mg L^{-1} ammonia and <1 mg L^{-1} nitrite. Salinity and temperature were also measured daily. Salinity in each tank was adjusted at a rate of 2 ppt each day until salinities of 5 ppt, 10 ppt and 15 ppt were achieved. The fish were maintained on a 2% body wt d^{-1} feeding regime using a commercial pelleted feed (CP 9921; Charoen Pokphand Co., Bangkok, Thailand) for >14 d before the NTC water was collected and used for the experimental challenge trials. At collection, the chlorophyll *a* content of each tank was determined from 1-L samples collected in acid-washed polyethylene bottles and analysed by the Institute of Marine Science at Burapha University (Chonburi, Thailand) following the procedure described by Strickland & Parsons (1972). The chlorophyll *a* concentration of the NTC water was determined to be 1,150 mg m^{-3} (5 ppt), 1,917 mg m^3 (10 ppt) and 1,292 mg m^3 (15 ppt). Meanwhile, total suspended solids in 1 L from each tank was determined by Imhoff cone (readings were between 11–15 mL L^{-1}) and filtering as described above, and the dry weight of organic material from each of the three tanks was 4.7 mg L^{-1} (5 ppt), 6.8 mg L^{-1} (10 ppt) and 4.9 mg L^{-1} (15 ppt).

2.4 Preparation for shrimp bath challenge with Vp_{AHPND}

The inoculum for the challenge trial was prepared by inoculating the Vp_{AHPND} isolate FVG0001 into tryptone soya broth (TSB) supplemented with 2% NaCl and culturing for 12 h at 28°C with shaking (*ca.* 250 rpm). Bacterial cells were collected by centrifugation at 900 ×g for 10 min at 10°C and then the bacterial pellet was re-suspended in sterile 15 ppt seawater. The number of colony-forming units (CFU) mL^{-1} in the suspension was estimated by measuring the optical density at 600 nm (OD_{600}), as an OD_{600} of 1.0 AU equated to *ca.* 3.0×10^8 CFU mL^{-1}. The suspension was adjusted to the desired OD_{600} (=1.0

AU) with sterile 15 ppt seawater, and then CFU mL^{-1} verified by diluting and plating suspensions across tryptone soya agar and incubating at 28°C until CFU could be enumerated. Each challenge trial was performed in 1-L vessels and the quantity of bacteria required for each challenge was determined from virulence pre-tests performed typically <48 h earlier. Each virulence pre-test was conducted on shrimp from the same population intended for use in the trial and under the same conditions as the actual challenge. The pre-tests used a minimum of three bacterial concentrations and three individually-housed shrimp per dose to determine the CFU mL^{-1} required to give *ca.* 66% mortality at 48 h post-infection. For all challenge trials, a semi-randomised block design was used to allocate the test vessels on the benching within the challenge room; however, the negative (non-challenged) control shrimp vessels were isolated on a separate bench to minimise potential cross-contamination.

2.5 Trial 1: Effect of biofloc on survival of shrimp bath challenged with Vp_{AHPND}

Shrimp (0.36 ± 0.12 g) were maintained in clear 15 ppt seawater (CW) for ≥7 days prior to challenge in 200-L tanks. The day before the challenge, the shrimp were transferred to static 1-L glass vessels in a temperature-controlled challenge room maintained at 27.2 ± 0.2°C and monitored every 15 min with data loggers (Onset HOBO UA-001-64) placed inside two additional glass vessels in the challenge room. Then 3 shrimp were placed into each 1-L glass vessel containing 400 mL of 25%, 50% or 100% (v/v) biofloc, where 100% biofloc was from a 14-day old culture, with an Imhoff cone reading of 11 mL L^{-1} (0.54 g dry matter [DM] L^{-1}). Each vessel was aerated at *ca.* 5 L min^{-1}. From the virulence pre-test, 3.2 mL of *Vp* inoculum was added to each challenge group vessel. Then the shrimp were monitored for survival every 3 h up to 96 h and mortalities were recorded and carcasses removed. At 24 h, a further 400 mL of the appropriate culture medium was added to each vessel (*i.e.*, 15 ppt CW or a biofloc suspension as appropriate) and shrimp were fed *ad libitum* with commercial feed (Starbird 5093 S shrimp feed). At 48 h and 72 h, 400 mL of tank water was removed and replaced with 400 mL of appropriate culture medium. In total, 15 replicates per treatment were prepared in addition to 15 negative (non-challenged) control vessels.

2.6 Trial 2: Effect of shrimp stocking density on biofloc-conferred survival of shrimp bath challenged with Vp_{AHPND}

Earlier studies have reported correlation between shrimp stocking density and increased risk of AHPND (Boonyawiwat *et al*., 2017; OIE, 2018). As before, shrimp (0.36 ± 0.12 g) were maintained in 15 ppt CW for ≥7 days prior to challenge. Then the shrimp were transferred into 1-L glass vessels containing 400 mL of 50% or 100% biofloc at 1, 3 or 5 shrimp per vessel (temperature and aeration conditions as described in Section 2.5). In addition, a positive control group was prepared such that these vessels contained a single shrimp in 15 ppt CW and were challenged with Vp_{AHPND}. From the virulence pre-test (single shrimp held in 50% biofloc in this case), 6.2 mL *Vp* inoculum was added to each challenge group vessel. Shrimp survival was determined as described in Section 2.5, while culture medium exchange was also performed as before. In total, 10 replicates per treatment were prepared in addition to 10 negative (non-challenged) control vessels.

2.7 Trial 3: Effect of Nile tilapia-conditioned water at different salinities on survival of shrimp bath challenged with Vp_{AHPND}

Shrimp (0.36 ± 0.12 g) were maintained in three salinities of CW (5 ppt, 10 ppt and 15 ppt) for 14 days prior to challenge, and then transferred into 1-L glass vessels (1 shrimp per vessel) containing 400 mL of 5 ppt, 10 ppt or 15 ppt CW or 5 ppt, 10 ppt or 15 ppt NTC water (to avoid salinity shock the shrimp were transferred to identical salinity conditions). Temperature and aeration conditions of the vessels were as described in Section 2.5. From the virulence pre-test, 3.2 mL *Vp* inoculum was added to each challenge group vessel, and again culture medium exchange was performed as described in Section 2.5. In total, 10 replicates per treatment were prepared in addition to an equivalent number of negative (non-challenged) control vessels.

2.8 Disposal of experimental materials

On completion of each trial, all remaining shrimp were euthanized in icy water and incinerated. All glass vessels and tank water were sterilised with 70 mg L^{-1} calcium hypochlorite for ≥24 h. Thereafter, the water was dechlorinated, airlines and airstones were discarded, while glass vessels were scrubbed, rinsed and allowed to dry.

2.9 Ethics statement

These experiments were reviewed by and conducted under the approval of the University of Stirling Animal Welfare and Ethical Review Body and the FVGAL internal ethical review board. Scientists conducting aquatic pathogen trials at FVGAL Research Aquarium hold licences for the use of "Animals for Scientific Purposes" issued by the Institute for Animals for Scientific Purpose Development, National Research Council of Thailand. The FVGAL laboratories and challenge facilities are registered with the relevant authorities and are inspected as required under current Thai legislation.

2.10 Statistical analysis

Survival in each shrimp group was plotted for each trial and Mantel-Cox log-rank tests (two-way) were performed to determine whether significant differences existed in survival between groups. A statistically significant difference was accepted at $p<0.05$ and Holm's correction was applied to account for multiple comparisons (Holm, 1979).

3. Results

3.1 Trial 1: Effect of biofloc on survival of shrimp bath challenged with Vp$_{AHPND}$

In the trial to determine whether different concentrations of biofloc would protect against a bath challenge with *Vp*$_{AHPND}$, few mortalities were observed in the non-challenged control groups (Figure 1). Indeed, no significant differences existed in shrimp survival between the 15 ppt CW control and each control group maintained in 25%, 50% and 100% biofloc ($p>0.05$; Figure 1), thus indicating neither 15 ppt CW nor biofloc affected shrimp survival *per se*.

For shrimp challenged with *Vp*$_{AHPND}$, greatest mortality was observed for those maintained in 15 ppt CW (60% mortality at 96 h post-challenge; Figure 1), which confirmed that the *Vp*$_{AHPND}$ challenge had been successful. Importantly, there was significantly greater survival during 96 h post-challenge for shrimp maintained in 50% and 100% biofloc compared to those maintained in 15 ppt CW ($p<0.05$; Figure 1).

3.2 *Trial 2: Effect of shrimp stocking density on biofloc-conferred survival of shrimp bath challenged with* Vp$_{AHPND}$

In the trial to determine whether shrimp stocking density affected biofloc protection against a bath challenge with *Vp*$_{AHPND}$, few mortalities were observed in the non-challenged control groups and no significant differences existed in percentage shrimp survival between the 15 ppt CW control (1 shrimp per vessel) and each control group maintained in 25%, 50% and 100% biofloc and containing 1, 3 or 5 shrimp per vessel (p>0.05; Figure 2), which again indicated that neither 15 ppt CW nor biofloc affected shrimp survival *per se* at any of the stocking densities.

For shrimp challenged with *Vp*$_{AHPND}$, the greatest percentage mortality was observed for shrimp maintained in 15 ppt CW (100% mortality at 33 h post-challenge; Figure 2), which confirmed that the *Vp*$_{AHPND}$ challenge had been successful. There was significantly greater survival during 96 h post-challenge for shrimp maintained in 100% biofloc (1 shrimp per vessel) compared to the 15 ppt CW (1 shrimp per vessel) group (p<0.05); however, there was no difference in survival between the 50% biofloc (1 shrimp per vessel) and 15 ppt CW (1 shrimp per vessel) groups (p>0.05; Figure 2). The absence of a protective effect by the 50% biofloc compared to the first trial where a significant enhancement in survival was observed compared to shrimp challenged in CW may be due to the greater dose of bacteria used in this second trial (means of 2.65 and 4.47 $\times 10^6$ CFU mL^{-1} in trials 1 and 2, respectively). Furthermore, shrimp density in the vessels did not influence shrimp survival during 96 h post-challenge, as there were no significant differences between the 50% biofloc group containing 1 shrimp per vessel and the 50% biofloc groups containing either 3 or 5 shrimp per vessel, or between the 100% biofloc group containing 1 shrimp per vessel and the 100% biofloc groups containing either 3 or 5 shrimp per vessel (p>0.05; Figure 2).

3.3 Trial 3: Effect of Nile tilapia-conditioned water at different salinities on survival of shrimp bath challenged with Vp$_{AHPND}$

In the trial to determine whether NTC water at different salinities could protect against a bath challenge with *Vp*$_{AHPND}$, again few mortalities were recorded in non-challenged control groups (Figure 3). There were no significant differences in shrimp survival between the 5 ppt, 10 ppt and 15 ppt CW control groups (p>0.05), or between the 5 ppt, 10

ppt and 15 ppt NTC water control groups (p>0.05), or when comparing each 5 ppt, 10 ppt and 15 ppt CW control group with each respective salinity NTC water control group (p>0.05). These observations for the non-challenged control groups indicate that neither salinity nor the NTC water affected shrimp survival *per se*.

For shrimp challenged with *Vp*$_{AHPND}$, there was no significant differences in shrimp survival between 5 ppt, 10 ppt and 15 ppt CW groups (p>0.05), though this may be due to the stringency of our statistical analyses because survival in the 5 ppt CW group was much greater at 96 h (33%) compared to the 10 ppt and the 15 ppt CW groups (both 7%). Moreover, shrimp survival in the 5 ppt and 10 ppt NTC water groups was significantly greater than in the 15 ppt NTC water group (p<0.05; Figure 3). Indeed, it is interesting that there were no significant differences (p>0.05) in shrimp survival between the 5 ppt CW and 5 ppt NTC water groups (relatively high survival of 33% and 80%, respectively) or between the 15 ppt CW and 15 ppt NTC water groups (relatively low survival of 7% and 13%, respectively). Taken together, these observations indicate that low salinity may in itself provide a degree of protection against a bath challenge with *Vp*$_{AHPND}$. Furthermore, and confirming the trend of a protective effect of NTC water against a bath challenge with *Vp*$_{AHPND}$, significantly greater shrimp survival was confirmed in the 10 ppt NTC water group compared to the 10 ppt CW group (p<0.05; Figure 3).

4. Discussion

This present study aimed to investigate the effects of biofloc and NTC water prepared at different salinities to protect whiteleg shrimp against an experimental bath challenge with a pathogenic *Vp*$_{AHPND}$ isolate. Shrimp challenged with *Vp*$_{AHPND}$ in biofloc and NTC water prepared at 10 ppt had significantly increased survival during 96 h post-challenge.

In the first trial, a direct relationship was found to exist between biofloc concentration and shrimp survival post-challenge with *Vp*$_{AHPND}$, and 50% and 100% biofloc (14-day old culture; dry weight of 0.54 g L^{-1}) provided significant protection, which is in agreement with an earlier study performed under similar conditions (Shinn *et al.*, in press a). Moreover, the observations are in line with other reports that demonstrate biofloc to be beneficial against shrimp pathogens, possibly through probiotic or immunostimulatory effects as distinct from simply reducing the probability of exposure to pathogens through

operating a very low-water exchange system (Crab *et al*., 2010; Haslun *et al*., 2012; Moss *et al*., 2012; Zhao *et al*., 2012, Dash *et al*., 2017). Excessive biofloc concentrations might exert detrimental effects on shrimp health, *e.g.* by causing a reduction in gill function, biofouling of the carapace and induction of a stressful state; however, our findings demonstrate potential for highly effective protection against *Vp*$_{AHPND}$ at relatively low biofloc concentrations (e.g., < 0.6 g DM L^{-1}). Furthermore, prophylactic biofloc treatment limited to the most AHPND-sensitive first 30 days of shrimp culture may also preclude biofloc build-up and instability problems associated with extended culture and elevated feed inputs under closed production conditions (Little *et al*., 2008). In addition, this prophylactic approach would also support policy objectives to reduce antimicrobial usage in intensive shrimp culture, linked to food safety and antimicrobial resistance concerns. Notably, a number of studies have reported on antimicrobial-resistant strains of *V. parahaemolyticus* (Han *et al*., 2007; Lai *et al*., 2015; Saifedden *et al*., 2016)

A further aim of this present study was to investigate whether NTC water was effective to protect whiteleg shrimp against a *Vp*$_{AHPND}$ challenge. Farming shrimp at lower salinity has been associated with reduced risk of an AHPND outbreak (Gabaudan, 2012; NACA, 2012). However, little information is available regarding whether different salinities of tilapia-conditioned water exert differential effects on bacterial pathogens such as *Vp*$_{APHND}$. In this present study, NTC water prepared at 10 ppt increased shrimp survival significantly during 96 h post-challenge with *Vp*$_{AHPND}$ compared to the 10 ppt CW control, thus confirming a protective effect of the NTC water against *Vp*$_{APHND}$. The protection afforded by the NTC water at 10 ppt could be due to the microbial community in this milieu, as microorganisms with anti-*Vibrio* effects have been isolated from tilapia skin and gut mucus and from NTC water shrimp ponds (Lio-Po *et al*., 2005; Dash *et al*., 2017). Nevertheless, DM and chlorophyll *a* concentrations in the 10 ppt NTC water were greater than in the 5 ppt and 15 ppt NTC water, and this additional material may explain the greater survival of the shrimp in this group. Interestingly, the shrimp groups challenged in 5 ppt and 10 ppt NTC water had significantly greater survival than in the 15 ppt group, and it could be that the different NTC waters were composed of distinct microbial communities with differential effects on *Vp*$_{APHND}$ and shrimp. Meanwhile, the 5 ppt CW group had greater survival than the 10 ppt and the 15 ppt CW groups, though no significant difference was detected but, taken in conjunction with the NTC water observations, the findings suggest that low water salinity may provide some protection against *Vp*$_{AHPND}$, which is

worthy of further investigation. The results suggest that tilapia-conditioned water at low salinity could be employed as a strategy to reduce incidence of AHPND.

One shortcoming of the present study is that the shrimp challenged in the biofloc conditions continued to feed throughout the trial, whereas those challenged in CW did not, meaning that the shrimp in biofloc would have had a better nutritional status and thus likely to be less susceptible to infection by *Vp*$_{\text{APHND}}$. Furthermore, the data from this present study do not allow for determining whether the biofloc and NTC water led to increased survival of challenged shrimp through stimulating the immune response or direct inactivation of the pathogen. As the *Vp*$_{\text{APHND}}$ bacterium was introduced concurrently with the biofloc or NTC water, antibacterial effects perhaps explain the protective effects on shrimp survival because immunostimulation would be expected to take longer to take effect, though this requires experimental confirmation. Therefore, follow up studies should examine the direct effects of biofloc, NTC water and water salinity on the *Vp*$_{\text{AHPND}}$ bacterium, such as reductions in cell division rates and viability, because these data may reveal the mechanisms underlying the increased shrimp survival. Importantly, the experimental *Vp*$_{\text{APHND}}$ challenge used may not well mimic what happens in the culture ponds where there is a slow build-up of bacteria, and therefore the development of improved challenge models that more closely reflect field conditions is warranted. Finally, the Nile tilapia and whiteleg shrimp used in the NTC water trial were gradually adjusted to the desired salinities at a rate of 2 ppt each day and the stress of this procedure may have impacted subsequent shrimp survival and this requires further investigation. Indeed, the population of whiteleg shrimp used in this present study was reared at 15 ppt from PL stage 14, and it would be interesting to rear shrimp at 5 ppt, 10 ppt and 15 ppt and repeat the trial to see if further survival improvements could be achieved.

In conclusion, NTC water prepared at 10 ppt and biofloc protected whiteleg shrimp against experimental bath challenge with a pathogenic *Vp*$_{\text{AHPND}}$ isolate. This suggests that inexpensive strategies could be developed by the shrimp industry that would reduce the impact of *Vp*$_{\text{AHPND}}$.

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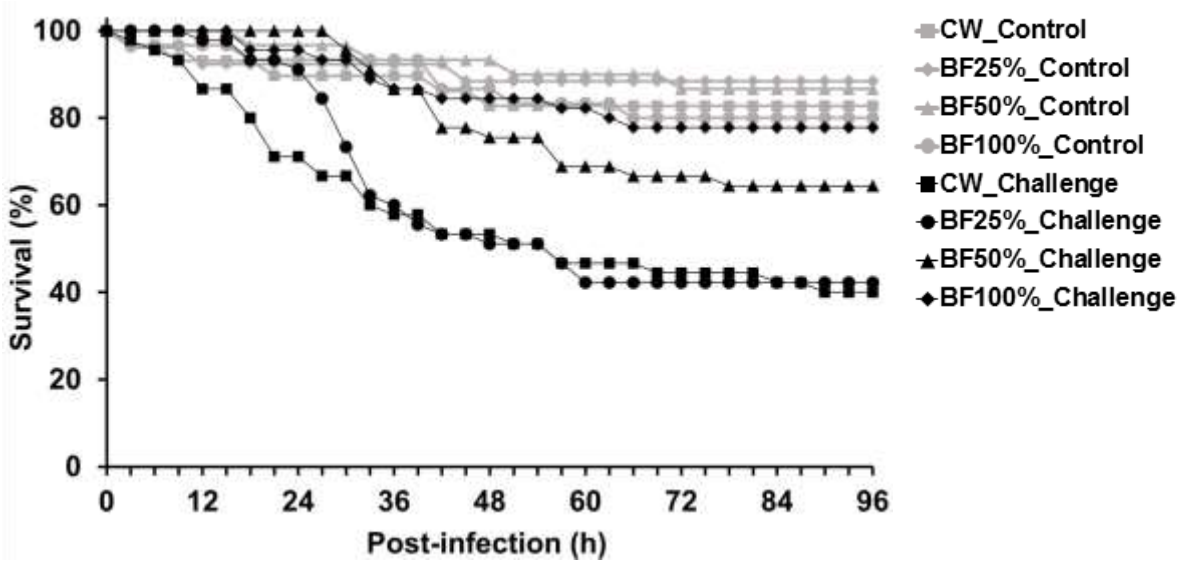

Figure 1. Survival of *Penaeus vannamei* bath challenged with the pathogenic AHPND-causing *Vibrio parahaemolyticus* isolate FVG0001 in clear 15 ppt seawater (CW) or biofloc (BF) at 25%, 50% and 100% (v/v) at 28–29°C in 1-L vessels containing 3 shrimp per vessel. n=15 vessels per group.

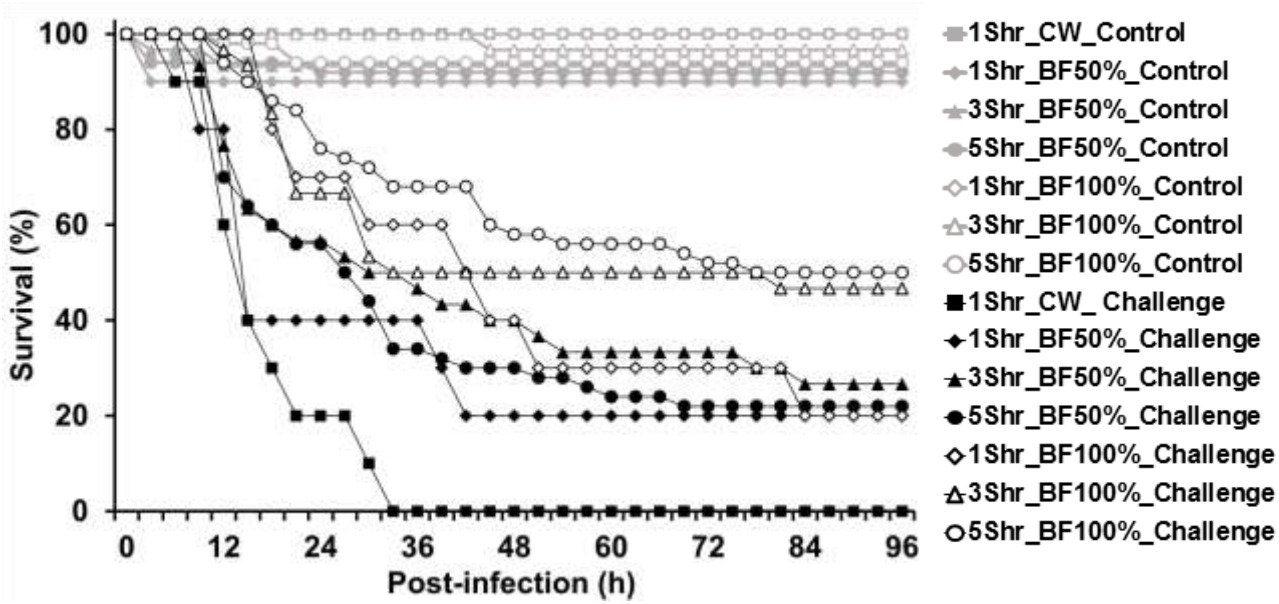

Figure 2. Survival of *Penaeus vannamei* bath challenged with the pathogenic AHPND-causing *Vibrio parahaemolyticus* isolate FVG0001 in clear 15 ppt seawater (CW) or biofloc (BF) at 50% and 100% (v/v) at 28–29°C in 1-L vessels containing 1, 3 and 5 shrimp per vessel. n=10 vessels per group.

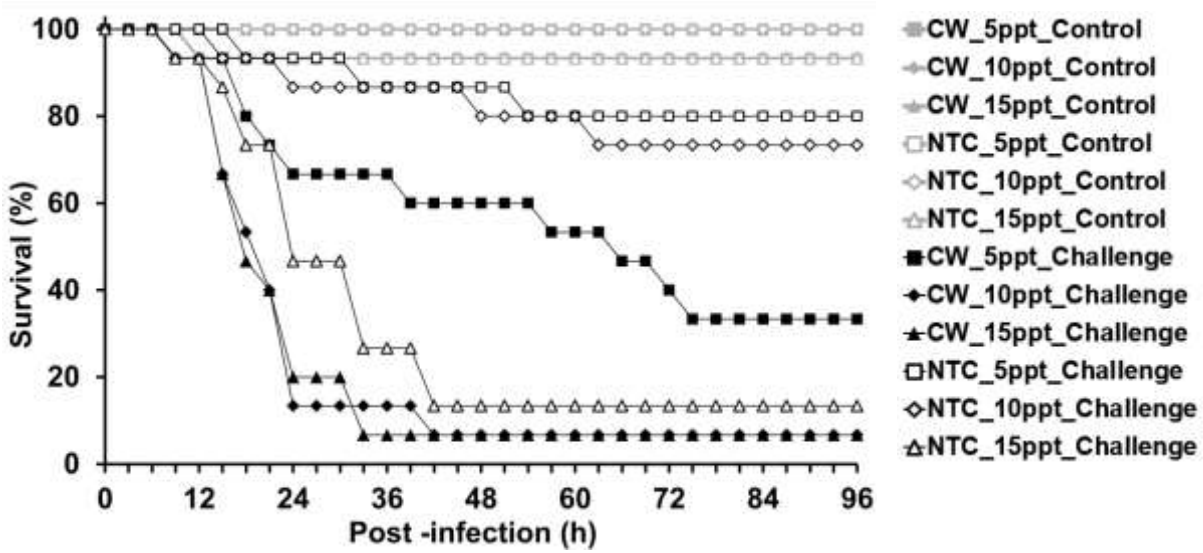

Figure 3. Survival of *Penaeus vannamei* bath challenged with the pathogenic AHPND-causing *Vibrio parahaemolyticus* isolate FVG0001 in clear seawater (CW) at 5 ppt, 10 ppt and 15 ppt or Nile tilapia-conditioned (NTC) water at 5 ppt, 10 ppt and 15 ppt at 28–29°C in 1-L vessels containing 1 shrimp per vessel. n=10 vessels per group.