1 **Prophylactic properties of biofloc- or Nile tilapia-conditioned water against *Vibrio***

2 ***parahaemolyticus* infection of whiteleg shrimp (*Penaeus vannamei*)**

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12 Running title: AHPND, biofloc and tilapia-conditioned water

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14 *Key words:* Acute hepatopancreatic necrosis disease (AHPND), bath challenge, disease

15 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)

50 **Abstract**

51

52 Isolates of *Vibrio parahaemolyticus* (Vp_{AHPND}) that carry a plasmid encoding two *Pir*-like

53 toxins cause acute hepatopancreatic necrosis disease (AHPND), a disease that has caused

54 devastating economic losses to the shrimp industry, particularly in Asia. However, lower

55 prevalence of AHPND infection has been associated with farms that operate with biofloc

56 or lower salinity culture water. Therefore, the aim of this present study was to investigate

57 the effects of biofloc, different culture water salinity and Nile tilapia (*Oreochromis

58 niloticus*)-conditioned water on survival of whiteleg shrimp (*Penaeus vannamei*) bath

59 challenged experimentally with Vp_{AHPND}. First, groups of shrimp were bath challenged

60 with Vp_{AHPND} in clear 15 ppt seawater (CW) or in the presence of a pre-cultured biofloc at

61 25%, 50% and 100% (v/v). Survival during 96 h post-challenge was significantly greater in

62 groups cultured in 50% and 100% biofloc ($p<0.05$). In a second trial, the effect of shrimp

63 stocking density on biofloc protection against bath challenge with Vp_{AHPND} was determined

64 and shrimp challenged in 100% biofloc again had significantly greater survival ($p<0.05$)

65 compared to the CW group, whilst under our experimental conditions stocking density had

66 no significant influence on survival post-challenge. In a third trial, shrimp were challenged

67 with Vp_{AHPND} in three different salinities of CW or Nile tilapia-conditioned (NTC) water (5

68 ppt, 10 ppt and 15 ppt). Survival in this final trial was 33% at 96 h in 5 ppt CW compared

69 to just 7% in the 10 ppt and the 15 ppt CW groups, though these differences were not

70 statistically significant. Moreover, shrimp survival in the 5 ppt and 10 ppt NTC water

71 groups was significantly greater than in the 15 ppt NTC water group ($p<0.05$), while

72 significantly greater survival was observed in 10 ppt NTC water compared to 10 ppt CW

73 ($p<0.05$). The results indicate that biofloc and NTC water may provide some protection

74 against AHPND, whilst low salinity culture water may also offer a degree of protection

75 against this bacterium. These findings may allow for the implementation of inexpensive

76 strategies in the shrimp industry to assist in minimising the impact of Vp_{AHPND} as part of

77 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

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

114

115 The use of biofloc can increase feed utilization, growth, survival and the reproductive
116 performance of cultured animals (Xu *et al*., 2012; Ekasari *et al*., 2014; Suita, 2016;
117 Ballester *et al*., 2017). Moreover, some studies have investigated the beneficial
118 immunological effects of the organisms found in biofloc, and their cellular components
119 and metabolites can act as immunostimulants to enhance the shrimp innate immune system
120 and provide improved protection against pathogens (Vazquez *et al.*, 2009; Crab *et al*.,
121 2010; Ekasari *et al*., 2014; de Jesus Becerra-Dorame *et al*., 2014; Xu *et al*., 2014; Kim *et*
122 *al*., 2014; Shinn *et al*., in press a). Critically, biofloc may also have a direct 'probiotic'
123 effect in the pond or gut microbiome, *i.e.* as benign commensal heterotrophic bacteria with
124 potential to displace pathogenic *Vibrio* spp. (facultative anaerobes) under intensively
125 aerated production conditions (Arias-Moscoso *et al*., 2018)

126

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

136

137 Biofloc and greenwater approaches to culture shrimp precede the recent emergence of
138 AHPND-causing strains of *V. parahaemolyticus* (*Vp*AHPND) in many places (Hargreaves,
139 2013) but, in the Philippines and Vietnam, greenwater technology has been adopted
140 alongside improved biosecurity practices at grow-out pond sites to prevent AHPND (Usero
141 & Apostol-Albaladejo, 2015; Cadiz *et al*., 2016; Pakingking, 2016). Since 1996, tilapia-
142 conditioned water with a high *Chlorella* content has been used in shrimp farming to
143 prevent *Vibrio* spp. infections (Dash *et al*., 2017). Meanwhile, bacteria isolated from tilapia
144 skin and mucus from the gut and skin have demonstrated potent anti-*Vibrio* spp. properties
145 (Lio-Po *et al.*, 2005). Aside from reducing the burden of certain bacteria in the water, co-

146 culture of tilapia in shrimp ponds is recommended for improving soil and water quality

147 (Tendencia *et al*., 2015). Tendencia *et al*. (2004, 2015) reported that rearing tilapia at >300

148 g m^{-3} inhibited the growth of *Vibrio* spp. in shrimp biomass stocked at 80 g m^{-3} and

149 improved shrimp survival.

150

151 This present study aimed to investigate the effects of biofloc and Nile tilapia (*Oreochromis*

152 *niloticus*)-conditioned (NTC) water prepared at different salinities to protect whiteleg

153 shrimp against experimental bath challenge with a pathogenic Vp_{AHPND} isolate.

154

155 **2. Materials and methods**

156

157 *2.1 Bacteria, shrimp and tilapia*

158

159 The Vp_{AHPND} isolate FVG0001 was used for all challenge trials. During May to June 2017,

160 batches of juvenile whiteleg shrimp were acquired from a commercial shrimp hatchery

161 located in Chachoengsao Province, Thailand, and transferred to the quarantine unit at the

162 Fish Vet Group Asia Limited (FVGAL) Research Aquarium in Chonburi, Thailand. On

163 receipt of each shipment, the shrimp were surface-disinfected with 0.1 mg L^{-1} povidone

164 iodine and a sub-sample (n = 20 individuals; mean of 0.4 g) were confirmed to be negative

165 for seven major shrimp diseases (Vp_{AHPND}; the microsporidian *Enterocytozoon*

166 *hepatopenaei* [EHP]; infectious hypodermal and haemotopoietic necrosis virus [IHHNV];

167 infectious myonecrosis virus [IMNV]; Taura syndrome virus [TSV]; white-spot syndrome

168 virus [WSSV]; and, yellow head virus [YHV]) by iiPCR test kits (GeneReach

169 Biotechnology Corporation, Taichung, Taiwan) and OIE approved methodologies (OIE,

170 2017). Furthermore, 24 mixed sex Nile tilapia were sourced from a commercial farm

171 (119.8 ± 33.4 g) and transferred to the FVGAL Diagnostic Laboratory (*ca.* 2 km from the

172 FVGAL Research Aquarium).

173

174 *2.2 Shrimp holding conditions and preparation of biofloc*

175

176 Disease-free shrimp were stocked into 400-L tanks (positioned out of direct sunlight)

177 containing mature biofloc. The biofloc in each tank had been established in 300 L of 15 ppt

178 seawater. This water had been pre-treated with 50 mg L^{-1} chlorine and then treated with a

179 further 10 mg L^{-1} chlorine for at least 1 h by addition of calcium hypochlorite, with any

180 residual chlorine driven off with vigorous aeration. Absence of residual chlorine was
181 confirmed using an orthotolidine-based chlorine test kit (Monitor®; Pet Wonderland
182 Group, Thailand). The biofloc was initiated by adding 5 g rice bran, 1.5 g ground shrimp
183 feed and 3 g white sugar (as sources of carbon) to each tank and incubating for 2 days at
184 28–29°C with intensive aeration (this provided greenwater biofloc given the ambient
185 lighting typical of sub-tropical shrimp pond production conditions). Thereafter, 1 g ground
186 shrimp feed and 3 g white sugar were added on a daily basis. At day 3, physicochemical
187 water parameters were measured *in situ* and adjusted by changing rates of carbon substrate
188 addition to adhere within the following limits: <0.03 mg L^{-1} ammonia and <1 mg L^{-1} nitrite
189 (measured with a Tetra^{TM} test kit; Tetra GmbH, Melle, Germany), pH 7.5–8.0 (maintained
190 through the addition of calcium carbonate as necessary), alkalinity 80-150 mg L^{-1} CaCO$_3$,
191 15 ppt salinity and 28–29°C (measured using a hand-held automatic temperature
192 compensation refractometer; Bellingham & Stanley Ltd, United Kingdom), and >5 mg L^{-1}
193 dissolved oxygen (DO) (measured with a hand-held DO meter; YSI 550A; Xylem Inc.,
194 United States). A system of inverted air pipes provided continuous aeration to maintain
195 DO at >5 mg L^{-1} and salinity, DO and temperature readings were taken daily thereafter.
196 The shrimp were maintained on commercial feed (Starbird 5093 S shrimp feed; Charoen
197 Pokphand Co., Bangkok, Thailand) at 10% body wt d^{-1}, given daily in three equal rations
198 at 08:00, 14:00 and 18:00. Additionally, white sugar was added at a ratio of white
199 sugar:shrimp feed (2.3:1). The condition of the shrimp and biofloc were monitored
200 microscopically every day to ensure that the shrimp were in good condition (*i.e.*, no
201 evidence of necrosis, biofouling or infection of the shrimp). The biofloc was considered to
202 be ready for application when Imhoff cone readings were >10 ml L^{-1} after a 30-min
203 settlement period, and 10–15 mL L^{-1} is considered ideal for shrimp culture (Hargreaves,
204 2013). Total suspended solids readings were confirmed by filtering 1 L of biofloc
205 suspension through pre-weighed filter paper (Whatman No. 93; GE Healthcare UK
206 Limited, Buckinghamshire, UK) and then drying for 24 h at 50°C before massing the dried
207 matter. Biofloc was collected at >14 d and used for the experimental challenge trials.
208 Generally, 10–15% of the water volume was exchanged daily with pre-treated and
209 dechlorinated 15 ppt seawater (except for the day prior to the start of a challenge trial to
210 preserve the condition of the biofloc); however, volume exchanges deviated occasionally
211 to ensure Imhoff cone readings were maintained at 10–15 mL L^{-1}.
212
213 *2.3 Tilapia holding conditions and preparation of NTC water*

214

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

248   AU) with sterile 15 ppt seawater, and then CFU mL^{-1} verified by diluting and plating

249   suspensions across tryptone soya agar and incubating at 28°C until CFU could be

250   enumerated. Each challenge trial was performed in 1-L vessels and the quantity of bacteria

251   required for each challenge was determined from virulence pre-tests performed typically

252   <48 h earlier. Each virulence pre-test was conducted on shrimp from the same population

253   intended for use in the trial and under the same conditions as the actual challenge. The pre-

254   tests used a minimum of three bacterial concentrations and three individually-housed

255   shrimp per dose to determine the CFU mL^{-1} required to give *ca.* 66% mortality at 48 h

256   post-infection. For all challenge trials, a semi-randomised block design was used to

257   allocate the test vessels on the benching within the challenge room; however, the negative

258   (non-challenged) control shrimp vessels were isolated on a separate bench to minimise

259   potential cross-contamination.

260

261   *2.5 Trial 1: Effect of biofloc on survival of shrimp bath challenged with* Vp$_{AHPND}$

262

263   Shrimp (0.36 ± 0.12 g) were maintained in clear 15 ppt seawater (CW) for ≥7 days prior to

264   challenge in 200-L tanks. The day before the challenge, the shrimp were transferred to

265   static 1-L glass vessels in a temperature-controlled challenge room maintained at 27.2 ±

266   0.2°C and monitored every 15 min with data loggers (Onset HOBO UA-001-64) placed

267   inside two additional glass vessels in the challenge room. Then 3 shrimp were placed into

268   each 1-L glass vessel containing 400 mL of 25%, 50% or 100% (v/v) biofloc, where 100%

269   biofloc was from a 14-day old culture, with an Imhoff cone reading of 11 mL L^{-1} (0.54 g

270   dry matter [DM] L^{-1}). Each vessel was aerated at *ca.* 5 L min^{-1}. From the virulence pre-test,

271   3.2 mL of *Vp* inoculum was added to each challenge group vessel. Then the shrimp were

272   monitored for survival every 3 h up to 96 h and mortalities were recorded and carcasses

273   removed. At 24 h, a further 400 mL of the appropriate culture medium was added to each

274   vessel (*i.e.*, 15 ppt CW or a biofloc suspension as appropriate) and shrimp were fed *ad*

275   *libitum* with commercial feed (Starbird 5093 S shrimp feed). At 48 h and 72 h, 400 mL of

276   tank water was removed and replaced with 400 mL of appropriate culture medium. In total,

277   15 replicates per treatment were prepared in addition to 15 negative (non-challenged)

278   control vessels.

279

280   *2.6 Trial 2: Effect of shrimp stocking density on biofloc-conferred survival of shrimp bath*

281   *challenged with* Vp$_{AHPND}$

282

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

294

295 *2.7 Trial 3: Effect of Nile tilapia-conditioned water at different salinities on survival of*
296 *shrimp bath challenged with* Vp_{AHPND}

297

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

307

308 *2.8 Disposal of experimental materials*

309

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

314

315 *2.9 Ethics statement*

316

317   These experiments were reviewed by and conducted under the approval of the University

318   of Stirling Animal Welfare and Ethical Review Body and the FVGAL internal ethical

319   review board. Scientists conducting aquatic pathogen trials at FVGAL Research Aquarium

320   hold licences for the use of "Animals for Scientific Purposes" issued by the Institute for

321   Animals for Scientific Purpose Development, National Research Council of Thailand. The

322   FVGAL laboratories and challenge facilities are registered with the relevant authorities and

323   are inspected as required under current Thai legislation.

324

325   *2.10 Statistical analysis*

326

327   Survival in each shrimp group was plotted for each trial and Mantel-Cox log-rank tests

328   (two-way) were performed to determine whether significant differences existed in survival

329   between groups. A statistically significant difference was accepted at $p<0.05$ and Holm's

330   correction was applied to account for multiple comparisons (Holm, 1979).

331

332   **3. Results**

333

334   *3.1 Trial 1: Effect of biofloc on survival of shrimp bath challenged with Vp_{AHPND}*

335

336   In the trial to determine whether different concentrations of biofloc would protect against a

337   bath challenge with Vp_{AHPND}, few mortalities were observed in the non-challenged control

338   groups (Figure 1). Indeed, no significant differences existed in shrimp survival between the

339   15 ppt CW control and each control group maintained in 25%, 50% and 100% biofloc

340   ($p>0.05$; Figure 1), thus indicating neither 15 ppt CW nor biofloc affected shrimp survival

341   *per se*.

342

343   For shrimp challenged with Vp_{AHPND}, greatest mortality was observed for those maintained

344   in 15 ppt CW (60% mortality at 96 h post-challenge; Figure 1), which confirmed that the

345   Vp_{AHPND} challenge had been successful. Importantly, there was significantly greater

346   survival during 96 h post-challenge for shrimp maintained in 50% and 100% biofloc

347   compared to those maintained in 15 ppt CW ($p<0.05$; Figure 1).

348

349   3.2 *Trial 2: Effect of shrimp stocking density on biofloc-conferred survival of shrimp bath*

350   *challenged with* Vp$_{AHPND}$

351   In the trial to determine whether shrimp stocking density affected biofloc protection

352   against a bath challenge with *Vp*$_{AHPND}$, few mortalities were observed in the non-

353   challenged control groups and no significant differences existed in percentage shrimp

354   survival between the 15 ppt CW control (1 shrimp per vessel) and each control group

355   maintained in 25%, 50% and 100% biofloc and containing 1, 3 or 5 shrimp per vessel

356   (*p*>0.05; Figure 2), which again indicated that neither 15 ppt CW nor biofloc affected

357   shrimp survival *per se* at any of the stocking densities.

358

359   For shrimp challenged with *Vp*$_{AHPND}$, the greatest percentage mortality was observed for

360   shrimp maintained in 15 ppt CW (100% mortality at 33 h post-challenge; Figure 2), which

361   confirmed that the *Vp*$_{AHPND}$ challenge had been successful. There was significantly greater

362   survival during 96 h post-challenge for shrimp maintained in 100% biofloc (1 shrimp per

363   vessel) compared to the 15 ppt CW (1 shrimp per vessel) group (*p*<0.05); however, there

364   was no difference in survival between the 50% biofloc (1 shrimp per vessel) and 15 ppt

365   CW (1 shrimp per vessel) groups (*p*>0.05; Figure 2). The absence of a protective effect by

366   the 50% biofloc compared to the first trial where a significant enhancement in survival was

367   observed compared to shrimp challenged in CW may be due to the greater dose of bacteria

368   used in this second trial (means of 2.65 and 4.47 $\times 10^6$ CFU mL^{-1} in trials 1 and 2,

369   respectively). Furthermore, shrimp density in the vessels did not influence shrimp survival

370   during 96 h post-challenge, as there were no significant differences between the 50%

371   biofloc group containing 1 shrimp per vessel and the 50% biofloc groups containing either

372   3 or 5 shrimp per vessel, or between the 100% biofloc group containing 1 shrimp per

373   vessel and the 100% biofloc groups containing either 3 or 5 shrimp per vessel (*p*>0.05;

374   Figure 2).

375

376   3.3 *Trial 3: Effect of Nile tilapia-conditioned water at different salinities on survival of*

377   *shrimp bath challenged with* Vp$_{AHPND}$

378

379   In the trial to determine whether NTC water at different salinities could protect against a

380   bath challenge with *Vp*$_{AHPND}$, again few mortalities were recorded in non-challenged

381   control groups (Figure 3). There were no significant differences in shrimp survival

382   between the 5 ppt, 10 ppt and 15 ppt CW control groups (*p*>0.05), or between the 5 ppt, 10

383   ppt and 15 ppt NTC water control groups ($p>0.05$), or when comparing each 5 ppt, 10 ppt

384   and 15 ppt CW control group with each respective salinity NTC water control group

385   ($p>0.05$). These observations for the non-challenged control groups indicate that neither

386   salinity nor the NTC water affected shrimp survival *per se*.

387

388   For shrimp challenged with Vp_{AHPND}, there was no significant differences in shrimp

389   survival between 5 ppt, 10 ppt and 15 ppt CW groups ($p>0.05$), though this may be due to

390   the stringency of our statistical analyses because survival in the 5 ppt CW group was much

391   greater at 96 h (33%) compared to the 10 ppt and the 15 ppt CW groups (both 7%).

392   Moreover, shrimp survival in the 5 ppt and 10 ppt NTC water groups was significantly

393   greater than in the 15 ppt NTC water group ($p<0.05$; Figure 3). Indeed, it is interesting that

394   there were no significant differences ($p>0.05$) in shrimp survival between the 5 ppt CW

395   and 5 ppt NTC water groups (relatively high survival of 33% and 80%, respectively) or

396   between the 15 ppt CW and 15 ppt NTC water groups (relatively low survival of 7% and

397   13%, respectively). Taken together, these observations indicate that low salinity may in

398   itself provide a degree of protection against a bath challenge with Vp_{AHPND}. Furthermore,

399   and confirming the trend of a protective effect of NTC water against a bath challenge with

400   Vp_{AHPND}, significantly greater shrimp survival was confirmed in the 10 ppt NTC water

401   group compared to the 10 ppt CW group ($p<0.05$; Figure 3).

402

403   **4. Discussion**

404

405   This present study aimed to investigate the effects of biofloc and NTC water prepared at

406   different salinities to protect whiteleg shrimp against an experimental bath challenge with a

407   pathogenic Vp_{AHPND} isolate. Shrimp challenged with Vp_{AHPND} in biofloc and NTC water

408   prepared at 10 ppt had significantly increased survival during 96 h post-challenge.

409

410   In the first trial, a direct relationship was found to exist between biofloc concentration and

411   shrimp survival post-challenge with Vp_{AHPND}, and 50% and 100% biofloc (14-day old

412   culture; dry weight of 0.54 g L^{-1}) provided significant protection, which is in agreement

413   with an earlier study performed under similar conditions (Shinn *et al.*, in press a).

414   Moreover, the observations are in line with other reports that demonstrate biofloc to be

415   beneficial against shrimp pathogens, possibly through probiotic or immunostimulatory

416   effects as distinct from simply reducing the probability of exposure to pathogens through

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

430

431 A further aim of this present study was to investigate whether NTC water was effective to
432 protect whiteleg shrimp against a Vp_{AHPND} challenge. Farming shrimp at lower salinity has
433 been associated with reduced risk of an AHPND outbreak (Gabaudan, 2012; NACA,
434 2012). However, little information is available regarding whether different salinities of
435 tilapia-conditioned water exert differential effects on bacterial pathogens such as Vp_{APHND}.
436 In this present study, NTC water prepared at 10 ppt increased shrimp survival significantly
437 during 96 h post-challenge with Vp_{AHPND} compared to the 10 ppt CW control, thus
438 confirming a protective effect of the NTC water against Vp_{APHND}. The protection afforded
439 by the NTC water at 10 ppt could be due to the microbial community in this milieu, as
440 microorganisms with anti-*Vibrio* effects have been isolated from tilapia skin and gut
441 mucus and from NTC water shrimp ponds (Lio-Po *et al.*, 2005; Dash *et al.*, 2017).
442 Nevertheless, DM and chlorophyll *a* concentrations in the 10 ppt NTC water were greater
443 than in the 5 ppt and 15 ppt NTC water, and this additional material may explain the
444 greater survival of the shrimp in this group. Interestingly, the shrimp groups challenged in
445 5 ppt and 10 ppt NTC water had significantly greater survival than in the 15 ppt group, and
446 it could be that the different NTC waters were composed of distinct microbial communities
447 with differential effects on Vp_{APHND} and shrimp. Meanwhile, the 5 ppt CW group had
448 greater survival than the 10 ppt and the 15 ppt CW groups, though no significant difference
449 was detected but, taken in conjunction with the NTC water observations, the findings
450 suggest that low water salinity may provide some protection against Vp_{AHPND}, which is

451 worthy of further investigation. The results suggest that tilapia-conditioned water at low

452 salinity could be employed as a strategy to reduce incidence of AHPND.

453

454 One shortcoming of the present study is that the shrimp challenged in the biofloc

455 conditions continued to feed throughout the trial, whereas those challenged in CW did not,

456 meaning that the shrimp in biofloc would have had a better nutritional status and thus

457 likely to be less susceptible to infection by Vp_{APHND}. Furthermore, the data from this

458 present study do not allow for determining whether the biofloc and NTC water led to

459 increased survival of challenged shrimp through stimulating the immune response or direct

460 inactivation of the pathogen. As the Vp_{APHND} bacterium was introduced concurrently with

461 the biofloc or NTC water, antibacterial effects perhaps explain the protective effects on

462 shrimp survival because immunostimulation would be expected to take longer to take

463 effect, though this requires experimental confirmation. Therefore, follow up studies should

464 examine the direct effects of biofloc, NTC water and water salinity on the Vp_{AHPND}

465 bacterium, such as reductions in cell division rates and viability, because these data may

466 reveal the mechanisms underlying the increased shrimp survival. Importantly, the

467 experimental Vp_{APHND} challenge used may not well mimic what happens in the culture

468 ponds where there is a slow build-up of bacteria, and therefore the development of

469 improved challenge models that more closely reflect field conditions is warranted. Finally,

470 the Nile tilapia and whiteleg shrimp used in the NTC water trial were gradually adjusted to

471 the desired salinities at a rate of 2 ppt each day and the stress of this procedure may have

472 impacted subsequent shrimp survival and this requires further investigation. Indeed, the

473 population of whiteleg shrimp used in this present study was reared at 15 ppt from PL

474 stage 14, and it would be interesting to rear shrimp at 5 ppt, 10 ppt and 15 ppt and repeat

475 the trial to see if further survival improvements could be achieved.

476

477 In conclusion, NTC water prepared at 10 ppt and biofloc protected whiteleg shrimp against

478 experimental bath challenge with a pathogenic Vp_{AHPND} isolate. This suggests that

479 inexpensive strategies could be developed by the shrimp industry that would reduce the

480 impact of Vp_{AHPND}.

481

482 **Acknowledgements**

483

494
495 **References**
496

497 Ananda Raja, R., Sridhar, R., Balachandran, C., Palanisammi, A., Ramesh, S., &
498 Nagarajan, K. (2017). Pathogenicity profile of *Vibrio parahaemolyticus* in farmed Pacific
499 white shrimp, *Penaeus vannamei*. *Fish and Shellfish Immunology*, 67, 368-381. doi:
500 10.1016/j.fsi.2017.06.020.
501
502 Arias-Moscoso, J.L., Espinoza-Barrón, L.G., Miranda-Baeza, A., Rivas-Vega, M.E., &
503 Nieves-Soto, M. (2018). Effect of commercial probiotics addition in a biofloc shrimp farm
504 during the nursery phase in zero water exchange. *Aquaculture Reports*, 11, 47-52.
505 doi.org/10.1016/j.aqrep.2018.06.001.
506
507 Avnimelech, Y. (1999). Carbon/nitrogen ratio as a control element in aquaculture systems.
508 *Aquaculture*, 176, 227-235. doi.org/10.1016/S0044-8486(99)00085-X.
509
510 Avnimelech, Y. (2014). *Biofloc Technology. A Practical Guide Book*. 3rd ed. Baton Rouge,
511 United States: The World Aquaculture Society. ISBN: 978-188880-7226.
512
513 Ballester, E.L.C., Marzarotto, S.A., de Castro, C.S., Frozza, A., Postore, I., & Abreu, P.C.
514 (2017). Productive performance of juvenile freshwater prawns *Macrobrachium rosenbergii*
515 in biofloc system. *Aquaculture Research*, 48, 4748-4755. doi: 10.1111/are.13296.
516
517 Boonyawiwat, B.V., Patanasatienkul, T., Kasornchandra, J., Poolkhet, C. Yaemkasem, S.,
518 Hammel, L., & Davidson, J. (2017). Impact of farm management on expression of early
519 mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) on penaeid
520 shrimp farms in Thailand. *Journal of Fish Diseases*, 40, 649-659. doi: 10.1111/jfd.12545.
521
522 Cadiz, R.E., Traifalgar, R.F.M., Sanares, R.C., Andrino-Felarca, K.G.S., & Corre, J.V.L.
523 (2016). Comparative efficacies of tilapia green water and biofloc technology (BFT) in
524 suppressing population growth of green *Vibrios* and *Vibrio parahaemolyticus* in the
525 intensive tank culture of *Penaeus vannamei. AACL Bioflux*, 9, 195-203.
526
527 Chonsin, K., Matsuda, S., Theethakaew, C., Kodama, T., Junjhon, J., Suzuki, Y.,
528 Suthienkul, O., & Iida, T. (2016). Genetic diversity of *Vibrio parahaemolyticus* strains

529 isolated from farmed Pacific white shrimp and ambient pond water affected by acute
530 hepatopancreatic necrosis disease outbreak in Thailand. *FEMS Microbiology Letters*, 363,
531 222. doi: 10.1093/femsle/fnv222.

533 Crab, R., Defoirdt, T., Bossier, P., & Verstraete, W. (2012). Biofloc technology in
534 aquaculture: beneficial effects and future challenges. *Aquaculture*, 356-357, 351-356.
535 doi.org/10.1016/j.aquaculture.2012.04.046.

537 Crab, R., Lambert, A., Defoirdt, T., Bossier, P., & Verstraete, W. (2010). The application
538 of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic
539 *Vibrio harveyi. Journal of Applied Microbiology*, 109, 1643-1649. doi: 10.1111/j.1365-
540 2672.2010.04791.x.

542 Dabu, I.M., Lim, J.J., Arabit, P.M.T., Orense, S.J.A.B., Tabardillo Jr., J.A., Corre Jr., V.E.,
543 & Maningas, M.B.B. (2015) The first record of acute hepatopancreatic necrosis disease in
544 the Philippines. *Aquaculture Research*, 48, 792-799. doi:10.1111/are.12923.

546 Dash, P., Avunje, S., Tandel, R.S., Sandeep, K.P., & Panigrahi, A. (2017). Biocontrol of
547 luminous vibriosis in shrimp aquaculture: a review of current approaches and future
548 perspectives. *Reviews in Fisheries Science & Aquaculture*, 25, 245-255.
549 doi.org/10.1080/23308249.2016.1277973.

551 de Jesus Becerra-Dorame, M.J., Martinez-Cordova, L.R., Martínez-Porchas, M.,
552 Hernandez-Lopez, J., Lopez-Elías, J.A., & Mendoza-Cano, F. (2014). Effect of using
553 autotrophic and heterotrophic microbial-based systems for the pre-grown of *Litopenaeus*
554 *vannamei,* on the production performance and selected haemolymph parameters.
555 *Aquaculture Research*, 45, 944-948. doi: 10.1111/are.12033.

557 de la Peña, L.D., Cabillon, N.A.R., Catedral, D.D., Amar, E.C., Usero, R.C., Monotilla,
558 W.D., Calpe, A.T., Fernandez, D.D.G., & Saloma, C.P. (2015). Acute hepatopancreatic
559 necrosis disease (AHPND) outbreak in the *Penaeus vannamei* and *P. monodon* cultured in
560 the Philippines. *Diseases of Aquatic Organisms*, 116, 251-254. doi: 10.3354/dao02919.

562 De Schryver, P., Crab, R., Defoirdt, T., Boon, N., & Verstraete, W. (2008). The basics of
563 bio-flocs technology: The added value for aquaculture. *Aquaculture*, 277, 125-137.
564 doi.org/10.1016/j.aquaculture.2008.02.019.

566 De Schryver, P., Defoirdt, T., & Sorgeloos, P. (2014). Early mortality syndrome outbreaks:
567 A microbial management issue in shrimp farming? *PLoS Pathogens*, 10, e1003919.
568 https://doi.org/10.1371/journal.ppat.1003919.

570 Ekasari, J., Hanif Azhar, M., Surawidjaja, E.H., Nuryati, S., De Schryver, P., & Bossier, P.
571 (2014). Immune response and disease resistance of shrimp fed biofloc grown on different
572 carbon sources. *Fish and Shellfish Immunology*, 41, 332-339. doi:
573 10.1016/j.fsi.2014.09.004.

575 Flegel, T.W. (2012). Historic emergence, impact and current status of shrimp pathogens in
576 Asia. *Journal of Invertebrate Pathology*, 110, 166-173. doi: 10.1016/j.jip.2012.03.004.
577

578 Gabaudan, J. (2012). 17th Aquaculture Conference Asia Pacific. *AQUA Culture Asia*
579 *Pacific Magazine*, 1, 17-22.
580
581 Han, F., Walker, R.D., Janes, M.E., Prinyawiwatkul, W., & Ge, B. (2007). Antimicrobial
582 susceptibilities of *Vibrio parahaemolyticus* and *Vibrio vulnificus* isolates from Louisiana
583 Gulf and retail raw oysters. *Applied and Environmental Microbiology*, 73, 7096-7098. doi:
584 10.1128/AEM.01116-07.
585
586 Hari, B., Madhusoodana, K., Varghese, J.T., Schrama, J.W., & Verdegem, M.C.J. (2004).
587 Effects of carbohydrate addition on production in extensive shrimp culture systems.
588 *Aquaculture*, 241, 179-194. doi.org/10.1016/j.aquaculture.2004.07.002.
589
590 Hargreaves, J.A. (2013). Biofloc Production Systems for Aquaculture. Southern Regional
591 Aquaculture Center. United States: Department of Agriculture, National Institute of Food
592 and Agriculture.
593
594 Haslun, J., Correia, E., Strychar, K., Morris, T., & Samocha, T. (2012). Characterization of
595 bioflocs in a no water exchange super-intensive system for the production of food size
596 Pacific white shrimp *Litopenaeus vannamei. International Journal of Aquaculture*, 2, 29-
597 39. doi: 10.5376/ija.2012.02.0006.
598
599 Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian*
600 *Journal of Statistics*, 6, 65-70.
601
602 Jun, J.W., Han, J.E., Tang, K.F.J., Lightner, D.V., Kim, J., Seo, S.W., & Park, S.C. (2016).
603 Potential application of bacteriophage pVp-1: agent combating *Vibrio parahaemolyticus*
604 strains associated with acute hepatopancreatic necrosis disease (AHPND) in shrimp.
605 *Aquaculture*, 457, 100-103. doi.org/10.1016/j.aquaculture.2016.02.018.
606
607 Kim, S.-K., Pang, Z., Seo, H.-C., Cho, Y.-R., Samocha, T. & Jang, I.K. (2014). Effect of
608 bioflocs on growth and immune activity of Pacific white shrimp, *Litopenaeus vannamei*
609 postlarvae. *Aquaculture Research*, 45, 362-371. doi: 10.1111/are.12319.
610
611 Kuhn, D.D., Boardman, G.D., Lawrence, A.L., Marsh, L., & Flick, G.J. (2009). Microbial
612 floc meals as a replacement ingredient for fish meal and soybean protein in shrimp feed.
613 *Aquaculture*, 296, 51-57. doi: 10.1016/j.aquaculture.2009.07.025.
614
615 Lai, H.C., Ng, T.H., Ando, M., Lee, C.T., Chen, I.T., Chuang, J.C., Mavichak, R., Chang,
616 S.H., Yeh, M.D., Chiang, Y.A., Takeyama, H., Hamaguchi, H., Lo, C.F., Aoki, T., &
617 Wang, H.C. (2015). Pathogenesis of acute hepatopancreatic necrosis disease (AHPND) in
618 shrimp. *Fish and Shellfish Immunology*, 47, 1006-1014.
619
620 Lang, O., & Sothea, M. (2016). Current status of shrimp farming and diseases in
621 Cambodia. In R.V. Pakingking Jr., E.G.T de Jesus-Ayson, & B.O. Acosta, eds. *Addressing*
622 *acute hepatopancreatic necrosis disease (AHPND) and other transboundary diseases for*
623 *improved aquatic animal health in Southeast Asia*. Proceedings of the ASEAN Regional
624 Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved
625 Aquatic Animal Health in Southeast Asia, pp. 33-36. Iloilo, Philippines, Southeast Asian
626 Fisheries Development Center/Aquaculture Department.
627

628 Lightner, D.V., Redman, R.M., Pantoja, C.R., Noble, B.L., & Tran, L. (2012). Early
629 mortality syndrome affects shrimp in Asia. *Global Aquaculture Advocate*, 15, 40.

631 Lio-Po, G.D., Leaño, E.M., Peñaranda, M.M.D., Villa-Franco, A.U., Sombito, C.D., &
632 Guanzon Jr., N.G. (2005). Anti-luminous *Vibrio* factors associated with the 'green water'
633 grow-out culture of the tiger shrimp *Penaeus monodon*. *Aquaculture*, 250, 1-7.
634 doi.org/10.1016/j.aquaculture.2005.01.029.

636 Little, D.C., Murray, F.J., Azim, E., Leschen, W., Boyd, K, Watterson, A., & Young, J.A.
637 (2008). Options for producing a warm-water fish in the UK: Limits to "Green Growth"?
638 *Trends in Food Science and Technology*, 19, 255-264. doi.org/10.1016/j.tifs.2007.12.003.

640 Moss, S.M., Moss, D.R., Arce, S.M., Lightner, D.V., & Lotz, J.M. (2012). The role of
641 selective breeding and biosecurity in the prevention of disease in penaeid shrimp
642 aquaculture. *Journal of Invertebrate Pathology,* 110, 247-250.
643 doi.org/10.1016/j.jip.2012.01.013.

645 NACA (2012). *Report of the Asia Pacific Emergency Regional Consultation on the
646 Emerging Shrimp Disease: Early Mortality Syndrome (EMS)/Acute Hepatopancreatic
647 Necrosis Syndrome (AHPNS), 9-10 Aug 2012*. Bangkok, Thailand: Network of
648 Aquaculture Centres in Asia-Pacific. 131 pp.

650 OIE (2017). *OIE-Listed diseases, infections and infestations in force in 2017*. Retrieved on
651 1 July 2017 from http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2017/.

653 OIE (2018). *Manual of diagnostic tests for aquatic animals*. Acute hepatopancreatic
654 necrosis disease. Retrieved on 16 August 2018 from
655 http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/chapitre_ahpnd.pd
656 f.

658 Pakingking, R.V., Jr., de Jesus-Ayson, E.G.T., & Acosta, B.O. (Eds.) (2016). *Addressing
659 acute hepatopancreatic necrosis disease (AHPND) and other transboundary diseases for
660 improved aquatic animal health in Southeast Asia.* Proceedings of the ASEAN Regional
661 Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved
662 Aquatic Animal Health in Southeast Asia, 22-24 February 2016, Makati City, Philippines.
663 Tigbauan, Iloilo, Philippines: Aquaculture Dept., Southeast Asian Fisheries Development
664 Center. 109 pp. ISBN: 9789719931065.

666 Portley, N. (2016). *Report on the Shrimp Sector. Asian Shrimp Trade and Sustainability.*
667 Retrieved on 1 July 2017 from http://cmsdevelopment.sustainablefish.org.s3.amazonaws.
668 com/2016/04/07/Asian%20shrimp_long%20form-05098e04.pdf.

670 Saifedden, G., Farinazleen, G., Nor-Khaizura, A., Kayali, A.Y., Nakaguchi, Y.,
671 Nishibuchi, M., & Son, R. (2016) Antibiotic susceptibility profile of *Vibrio
672 parahaemolyticus* isolated from shrimp in Selangor, Malaysia. *International Food
673 Research Journal*, 23, 2732-2736.

675 Shinn, A.P., Jiravanichpaisal, J., Griffiths, D., Pokharatsiri, A., Burana, P., Sumon, T.,
676 Tongmee, C., Decamp, O., & Galli, L. (in press a). Effect of biofloc on the survival of
677 whiteleg shrimp, *Penaeus vannamei*, when challenged with a pathogenic strain of *Vibrio*

678 *parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease
679 (AHPND). *Asian Fisheries Science*.

681 Shinn, A.P., Pratoomyot, J., Griffiths, D., Trong, T.Q., Vu, N.T., Jiravanichpaisal, J., &
682 Briggs, M. (in press b). Asian shrimp production and the economic costs of disease. *Asian*
683 *Fisheries Science*.

685 Strickland, J.D.H. & Parsons, T.R. (1972) *A practical handbook of seawater analysis*. 2nd
686 edition. *Fisheries Research Board of Canada Bulletin*, 167, 310 pp.

688 Suita, S.M. (2016). Biofloc consumption by Pacific white shrimp postlarvae. *Global*
689 *Aquaculture Advocate*. Retrieved on 1 July 2017 from
690 https://www.aquaculturealliance.org/advocate/biofloc-consumption-by-pacific-white-
691 shrimp-postlarvae/.

693 Taw, N. (2012). Recent developments in biofloc technology. *Global Aquaculture*
694 *Advocate*, 15. Retrieved on 21 August 2018 from http://www.aqtinfo.com/2016/07/recent-
695 developments-in-biofloc.html.

697 Tendencia, E.A., Bosma, R.H., Verdegem, M.C.J., & Verreth, J.A.J. (2015). The potential
698 effect of greenwater technology on water quality in the pond culture of *Penaeus monodon*
699 Fabricius. *Aquaculture Research*, 46, 1-13. doi: 10.1111/are.12152.

701 Tendencia, E.A., dela Peña, M.R., Fermin, A.C., Lio-Po, G., Choresca Jr., C.H., & Inui, Y.
702 (2004). Antibacterial activity of tilapia *Tilapia hornorum* against *Vibrio harveyi*.
703 *Aquaculture*, 232, 145-154. doi.org/10.1016/S0044-8486(03)00531-3.

705 Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., &
706 Lightner, D.V. (2013). Determination of the infectious nature of the agent of acute
707 hepatopancreatic necrosis syndrome affecting penaeid shrimp. *Diseases of Aquatic*
708 *Organisms*, 105, 45-55. doi: 10.3354/dao02621.

710 Usero, R., & Apostol-Albaladejo, M.A.G. (2015). *Philippine Shrimp Grow-Out Farm*
711 *Management Practices Against Acute Hepatopancreatic Necrosis Disease (AHPND) with*
712 *Empahasis On Green Water Technology*. Bureau of Fisheries and Aquatic Resources
713 (BFAR) and Negros Prawn Producers Cooperative (NPPC).

715 Vazquez, L., Alpuche, J., Maldonado, G., Agundis, C., Pereyra-Morales, A., & Zenteno, E.
716 (2009). Immunity mechanisms in crustaceans. *Innate Immunity*, 15, 179-188. doi:
717 10.1177/1753425909102876.

719 Wyban, J., & Sweeney, J.N. (1991) *Intensive Shrimp Production Technology: The Oceanic*
720 *Institute Shrimp Manual*. Honolulu, Hawaii: The Oceanic Institute. ISBN: 0961701633.

722 Xu, W.J., Pan, L.Q., Sun, X.H., & Huang, J. (2012). Effects of bioflocs on water quality,
723 and survival, growth and digestive enzyme activities of *Litopenaeus vannamei* (Boone) in
724 zerowater exchange culture tanks. *Aquaculture Research,* 44, 1093-1102.
725 doi.org/10.1111/j.1365-2109.2012.03115.x.

727 Xu, W.J., & Pan, L.Q. (2014). Evaluation of dietary protein level on selected parameters of
728 immune and antioxidant systems, and growth performance of juvenile *Litopenaeus*
729 *vannamei* reared in zero-water exchange biofloc-based culture tanks. *Aquaculture*, 426-
730 427, 181-188. doi.org/10.1016/j.aquaculture.2014.02.003.

731

732 Zhao, H., Cao, J., Wang, A., Du, Z., Ye, C., Huang, Y., Lan, H., Zhou, T., & Li, G.L.
733 (2012). Effect of long-term administration of dietary β-1,3-glucan on growth,
734 physiological, and immune responses in *Litopenaeus vannamei* (Boone, 1931).
735 *Aquaculture International*, 20, 145-158. doi: 10.1007/s10499-011-9448-6.

736

737 Zorriehzahra, M.J., & Banaederakhshan, R. (2015). Early mortality syndrome (EMS) as
738 new emerging threat in shrimp industry. *Advances in Animal and Veterinary Sciences*, 3,
739 64-72. doi.org/10.14737/journal.aavs/2015/3.2s.64.72.
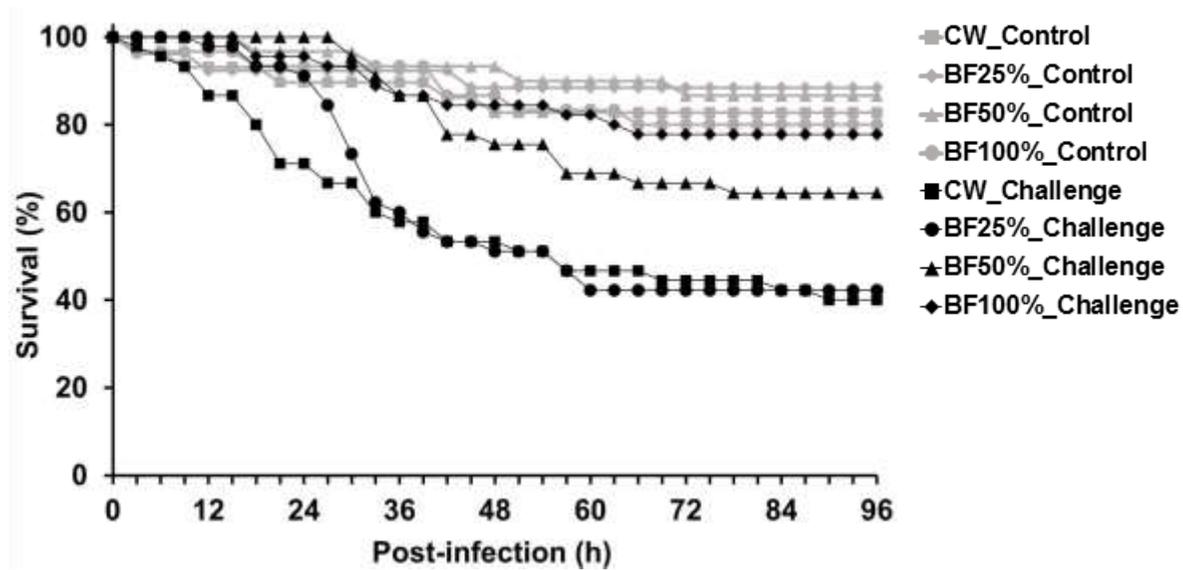
Figure 1. Survival of *Penaeus vannamei* bath challenged with the pathogenic AHPND-causing *Vibrio parahaemolyticus* isolate FVG0001 in clear 15 ppt seawater (CW) or biofloc (BF) at 25%, 50% and 100% (v/v) at 28–29°C in 1-L vessels containing 3 shrimp per vessel. n=15 vessels per group.
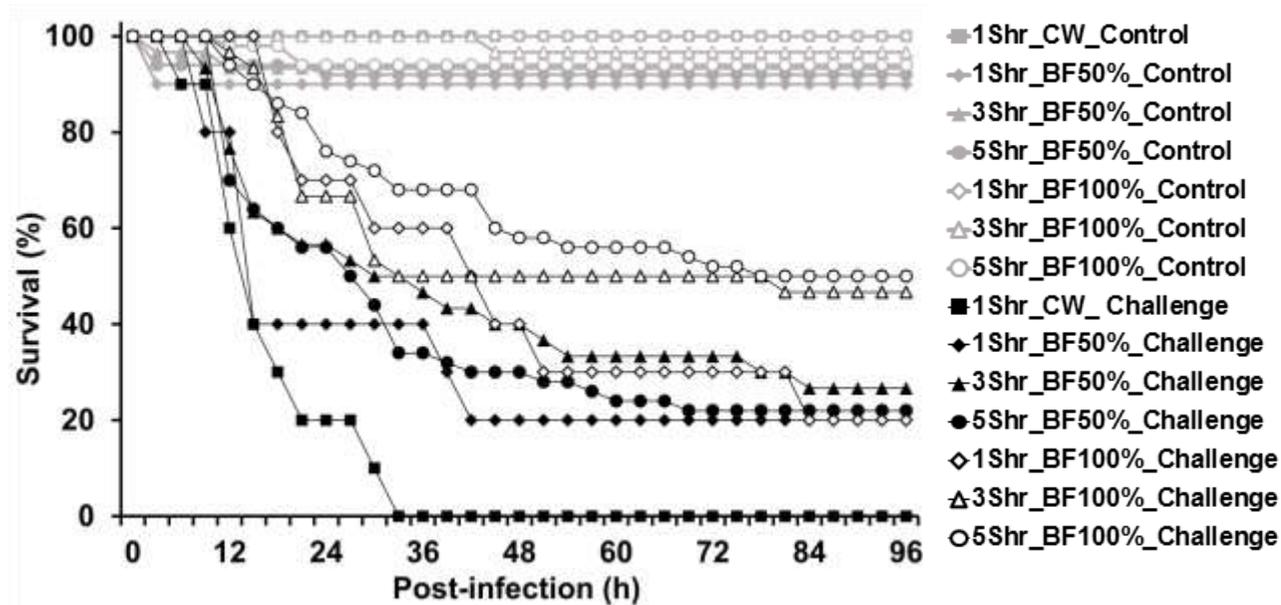
Figure 2. Survival of *Penaeus vannamei* bath challenged with the pathogenic AHPND-causing *Vibrio parahaemolyticus* isolate FVG0001 in clear 15 ppt seawater (CW) or biofloc (BF) at 50% and 100% (v/v) at 28–29°C in 1-L vessels containing 1, 3 and 5 shrimp per vessel. n=10 vessels per group.
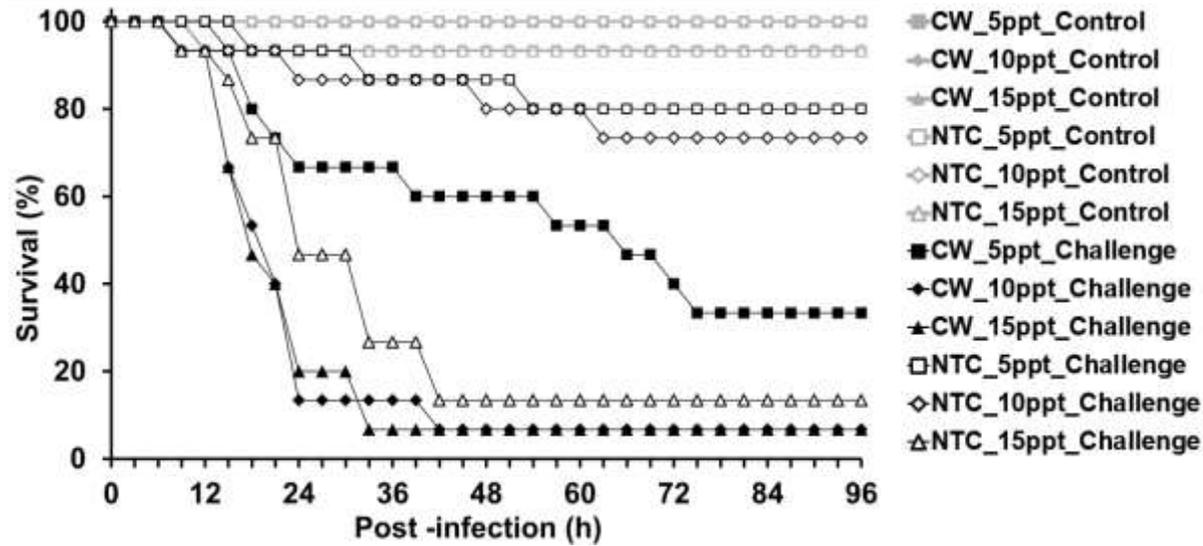
Figure 3. Survival of *Penaeus vannamei* bath challenged with the pathogenic AHPND-causing *Vibrio parahaemolyticus* isolate FVG0001 in clear seawater (CW) at 5 ppt, 10 ppt and 15 ppt or Nile tilapia-conditioned (NTC) water at 5 ppt, 10 ppt and 15 ppt at 28–29°C in 1-L vessels containing 1 shrimp per vessel. n=10 vessels per group.