Application of *Halomonas* sp. HIB-F to *Litopenaeus vannamei* aquaculture system

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Abstract. An increase in stocking density under intensification cultivation systems of whiteleg shrimp, *Litopenaeus vannamei*, accompanied by high feeding can lead to the formation of toxic inorganic nitrogen compounds in water, such as ammonia/ammonium (NH_3/NH_4^+) and nitrate (NO_3^-). The application of nitrifying bacteria is expected to be an innovative strategy to reduce the ammonia/ammonium and nitrate concentration in shrimp farming environments. This study aimed to examine the environmental performance and shrimps' survival treated with *Halomonas* sp. HIB-F as an endemic nitrificant from Indonesian seawater. The shrimps used in this study had an average weight of 0.4±0.1 shrimp^{-1} and an average length of 3.8±0.5 cm shrimp^{-1} and stocked by 50 shrimps m^{-2}. The shrimps' rearing system was treated by two treatments, i.e. with the application of *Halomonas* sp. HIB-F (N) and without the application of *Halomonas* sp. HIB-F (K). Results showed a direct and strong relationship due to the addition of *Halomonas* sp. HIB-F against ammonium and nitrate formed in the maintenance media. The survival of *Litopenaeus vannamei* was significantly different between the two treatments. Application of *Halomonas* sp. HIB-F led to a higher survival rate of culture shrimps accounting for 94±1.8%, while their survival rate without bacterial application reached 54±6.1%. *Halomonas* sp. HIB-F is able to be applied to improve the water quality over the shrimps' cultivation period.
Key Words: growth performance, mariculture, nitrifying bacteria, nitrogen cycling, whiteleg shrimp.

Introduction. Whiteleg shrimp (*Litopenaeus vannamei*) is one of the important fisheries commodities that is continuously cultivated in Asia, including Indonesia. According to shrimp export data issued by the Indonesian Central Bureau of Statistics (BPSN 2017), Indonesian shrimp was exported to varying destination countries accounting for 113,937 tons in 2010 and reached 145,077.90 tons in 2015. It means that there has been an increase in demand by 6,228.18 tons year^{-1} or an increase rate of 5.46% year^{-1}. The high demand in European, American, and Asian markets, such as Japan and China, is caused by the nutritional content of whiteleg shrimp. Based on the proximate analysis, the shrimp carcass (whole body) reared in an outdoor environment for 8 weeks and fed with different diets contains a crude protein content of 17.86-19.82%, a crude lipid content of 1.41-1.67%, an ash content of 2.50-2.88% and a moisture of 73.89-76.65% (Tacon et al 2002). In addition, whiteleg shrimp has a high amino acid and omega3 content, and low fat, which is beneficial to the human body (Pires et al 2018).

L. vannamei is one of the adaptive crustaceans which can survive against environmental changes and resist disease, and it isrelatively easy to handle (Darwin et al 2017). The shrimps are capable of residing in the water column, so it can be cultivated by applying intensive systems. An increase in the stocking density of aquatic biota often causes various problems, including an increase in aquaculture wastes. The waste is resulted from organic matter decomposition of the uneaten feed, feces, and urine from cultivated aquatic organisms (Coldebella et al 2018).

Accumulation of organic matter in aquaculture environment is able to trigger the formation of toxic compounds in inorganic ammonium nitrogen (NH_3/NH_4^+-N) and nitrite (NO_2^--N) form. These compound accumulation in the environment and fish bodies has been reported to damage the function and structure of fish organs (Han et al 2017). Ammonium and nitrite cause a decrease in the ability of blood to bind oxygen and in the body's resistance to disease (Chang et al 2015). In certain concentrations, these compounds can cause aquatic animals' death (Hastuti et al 2017).

Ammonium is a ionized nitrogen compound that can be used by nitrifying bacteria as a source of nitrogen (Caceres et al 2017). The presence of ammonium in water is actually not dangerous, yet it will be toxic when pH and temperature increase. According to Ogbonna & Chinomso (2010), ammonium concentration is influenced by pH and temperature. The higher the pH and temperature, the more ammonium transformed into ammonia so the toxicity in the water rises (Hibban et al 2016). Resistance to ammonium and nitrate varies in aquatic organisms. The optimal concentration of ammonia and total ammonia for aquatic biota are <0.1 mg L^{-1} and <1.0 mg L^{-1}, respectively (Carbajal-Hernandez et al 2013). Ammonia exposure are able to reduce quality and productivity in aquaculture activities. Efforts to minimize ammonium/ammonia concentration could be conducted by using microorganisms, such as bacteria. Muthukrishnan et al (2015) reported that some indigenous bacteria are capable of reducing ammonia concentration in water. According to Sangnoi et al (2017), *Halomonas* sp. possesses the ability to reduce ammonium by 23-71% and a high tolerance level for salinity. *Halomonas* sp. is a gram-negative bacterium belonging to halophilic heterotrophic group. *Halomonas campisalis* removes inorganic nitrogen compound (ammonium, nitrite, and nitrate) simultaneously, and grow well in medium containing up to 20% ($^{w}/_{v}$) of NaCl (Guo et al 2013).

Water quality management is essential in shrimp farming activities. Various technologies are used for maintaining proper water conditions for shrimp growth and survival. Application of a closed recirculation system with 500 post larva shrimp individuals m^{-3} stocking density produces shrimps with a productivity of 5.20 kg m^{-3} during 84 days rearing period (Suantika et al 2018). Shrimp feeding leading to a significant proportion of dissolved primary amines (DPA 23%) in feces and 26% of urea impacts the microbial community in pond water (Burford & Williams 2001). In a shrimp recirculation aquaculture system, it has been revealed that *Nitrospira marina* 16S rRNA gene ranged from 5 to 1×10^6 gene copies μL^{-1} of template DNA (Brown et al 2013). The presence and density of nitrifying bacteria in the recirculation system need to be maintained. Application of nitrifying bacteria *Nitrosomonas eutropha* and *Nitrobacter winogradskyi* in the recirculation system leads to water quality with ammonia content <1.0 mg L^{-1} (Kuhn et al 2010). An innovative strategy which could be performed to improve the water quality through nitrogen wastewater reduction is applying *Halomonas* sp. The application of this nitrifying bacteria is expected to decline ammonium concentration in whiteleg shrimps' rearing media, and subsequently, it could be applied in a massive industry. In the current work, we determine the environmental performance and the survival of whiteleg shrimp *L. vannamei* reared in a culture environment applied with *Halomonas* sp. HIB-F as an endemic nitrificant from Indonesian seawater.

Material and Method

Experimental design. This research was conducted by comparing treatments under a recirculation system without replication. The replication is only performed in shrimps' rearing containers. A recirculation package has two water treatment tubes (filter tubes and empty/adapted tubes) and three shrimps' rearing containers. This study consisted of two treatments, i.e. 1) with the application of nitrifying bacteria *Halomonas* sp. HIB-F in one of the filter tubes (N) as shown in Figure 1, and 2) without the application of nitrifying bacteria *Halomonas* sp. HIB-F as control (K).

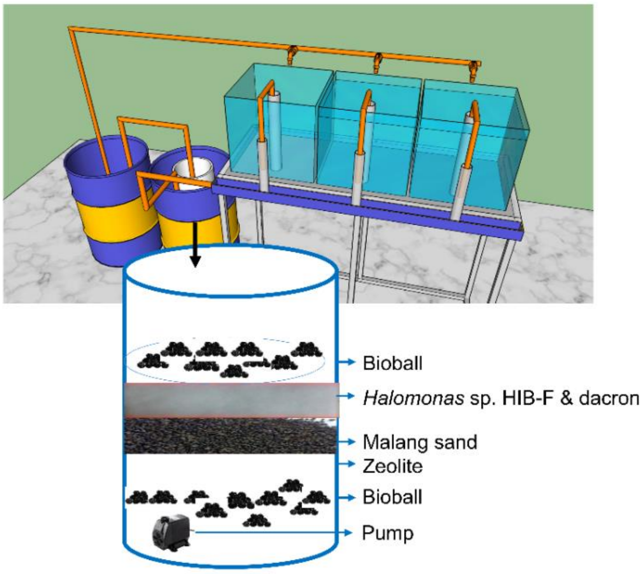

Figure 1. The design of the application system of *Halomonas* sp. HIB-F in a recirculation aquaculture system of *Litopenaeus vannamei* (Hastuti et al 2017; Hastuti et al 2019 modified).

Preparation of maintenance system. The maintenance system used in this study was a recirculation aquaculture system (RAS) with filters (Hastuti et al 2017; Hastuti et al 2019 modified). The water that has been used was treated in the first filter tube with a capacity of 220 L and a volume of 180 L, adapted in the second filter tube, then used in maintenance containers with a water discharge of 125 mL s^{-1}. The maintenance container was a plastic box with a size of 0.6 x 0.4 x 0.4 m (L x W x H). We used three boxes for each treatment as replication. Maintenance containers were filled with 36 L seawater, then installed with two aeration sites, and two additional shelters made of raffia rope (Fonna et al 2018). The filter tubes were constructed using plastic tubes containing various filter materials, including bioball, Malang sand, and dacron. The container and the filter tube was connected with a 5 cm-diameter pipe.

The application of nitrifying bacteria Halomonas sp. HIB-F. The bacterium used in this study was *Halomonas* sp. HIB-F isolated from Ancol seawater, Jakarta, Indonesia (Hastuti et al 2019). Bacterial suspension with a cell abundance of 10^9 CFU mL^{-1} within a total volume of 200 mL was introduced into a filtered tube system.

Preparation and cultivation of shrimps. This study used *L. vannamei* obtained from PT Suri Tani Pemuka (STP), Indramayu, West Java, Indonesia. Prior to stocking, *L. vannamei* were acclimatized for 24 hours to reduce stress due to environmental differences. The stocking density of *L. vannamei* for each maintenance container was 50 shrimps m^{-2}. These shrimps have an initial average weight of 0.4±0.1 g $shrimp^{-1}$ and an initial average length of 3.8±0.5 cm $shrimp^{-1}$. *L. vannamei* were reared within 12 weeks and fed with Fengli pellets. Culture shrimps were fed with 3% of shrimp biomass, 5 times day^{-1}, namely at 08:00, 12:00, 16:00, 20:00, and 23:00 (Tacon et al 2013).

Water quality assessment. Seawater as culture media was taken from Ancol, Jakarta, which was adjusted to a salinity of 25 ppt by the addition of freshwater (Hastuti et al 2015). The water quality parameters measured in this study were dissolved oxygen (DO), salinity, ammonium, nitrate, total organic matter (TOM), and biological oxygen demand (BOD). DO and salinity were measured using DO meter and salinometer, respectively. Ammonium and nitrate were determined following the phenate and brucin method, respectively (APHA 2005). TOM was calculated based on the titration method

(Indriyastuti et al 2014). BOD in the shrimp rearing environment was measured using the Winkler method according to APHA (2005).

The abundance of nitrifying bacteria. Bacterial abundance was calculated using the Total Plate Count (TPC) method on the specific nitrification medium and expressed in units of CFU mL^{-1} (colony forming units mL^{-1}) (Hastuti et al 2017).

Blood glucose level of L. vannamei. *L. vannamei* blood glucose level was measured using the Wedemeyer & Yasutake (1977) methods.

The survival rate of L. vannamei. Survival rate was calculated by the ratio of surviving *L. vannamei* number at initial to final rearing day. Observation of dead *L. vannamei* was carried out every day, and weight and length of the shrimps were recorded. Calculation of *L. vannamei*' survival rate was carried out using a formula described by Goddard (1996).

Data analysis. Water quality parameters and bacterial abundance were analyzed using quantitative descriptive linear regression correlation tests. Blood glucose level and survival rates were analyzed using the two independent sample t-test at a significant level of 0.05. Statistical analysis was performed using SPSS 24.0 software.

Results and Discussion

Water quality characteristics. During the *L. vannamei* rearing period, DO in water environment applied with *Halomonas* sp. HIB-F ranged from 5.1 to 7.9 mg L^{-1}, which was similar to the control (5.0-7.9 mg L^{-1}) (Table 1). According to the Indonesian National Standard (SNI) 8008:2014 (SNI 2014), the optimum DO for *L. vannamei* cultivation is >4 mg L^{-1}. The result indicates that DO in both treatments were still safe for *L. vannamei* growth and survival. Salinity is related to the organism's osmoregulation process and the body's resistance of crustaceans (Li et al 2008). We maintained the salinity in the two treatments at 25 g L^{-1}, supporting a stable water condition. Consequently, culture *L. vannamei* did not spend much energy on the osmoregulation process during their rearing period.

Table 1

Daily water quality of *Litopenaeus vannamei* cultivation with (N) and without the bacterial application of *Halomonas* sp. HIB-F (K)

Water quality parameters	*Treatments*		*Optimal range (SNI 8008:2014)*
	K	*N*	
DO (mg L^{-1})	5.0-7.9	5.1-7.9	>4.0
Salinity (g L^{-1})	25	25	10-32

K-without the bacterial application of *Halomonas* sp. HIB-F; N-with the bacterial application of *Halomonas* sp. HIB-F.

Ammonium (NH_4^+) is a nitrogen compound derived from organic matter degradation of uneaten feed, feces, and metabolic processing of protein by aquatic organisms (Coldebella et al 2018). Ammonium concentrations increased from week 1 to 4 in the two treatments, then declined until week 6. Application of *Halomonas* sp. HIB-F (N) led to ammonia concentration ranging from 0.008 to 0.107 mg L^{-1}, whereas in control (K) ranged between 0.010 and 0.126 mg L^{-1} (Figure 2). This result indicated that ammonium in the application of Halomonas sp. HIB-F (N) was relatively lower than in the control. It might be caused by the nitrifying bacterial activity, which oxidizes ammonium to nitrite and subsequently to nitrate. Wasielesky et al (2017) showed that the optimum ammonium concentration for shrimps' growth and survival is <0.91 mg L^{-1}. In this study, ammonium in the two treatments were still below the maximum limit. The decrease in ammonium concentration is presumably due to the activity of nitrifying bacteria (Caceres et al 2017). The analysis showed that there was a direct relationship between the application of *Halomonas* sp. HIB-F with a decrease in ammonium concentration compared to the control.

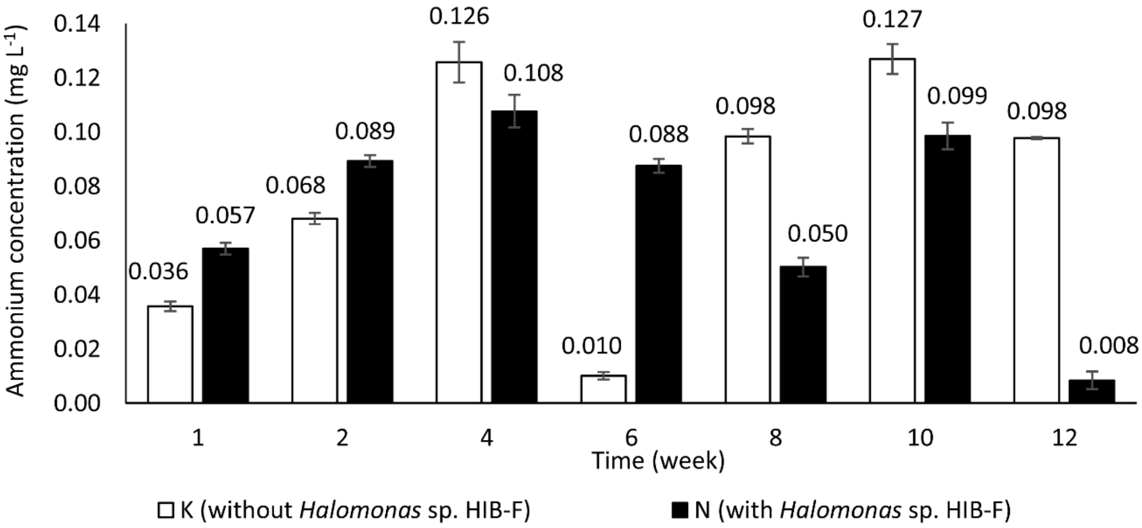

Figure 2. Ammonium concentration in the rearing media of *Litopenaeus vannamei* with (N) and without the application of *Halomonas* sp. HIB-F (K).

Nitrifying bacteria plays a major role in oxidizing ammonium to nitrite and nitrate. Nitrate, as a final product of the nitrification process, is an inorganic nitrogen compound that is safe for aquatic organisms and relatively stable compared to other inorganic nitrogen compounds (Auffret et al 2013). Our results showed that nitrates formed in the application of *Halomonas* sp. HIB-F (N) accounted for 1.501-2.201 mg L^{-1}, which was relatively higher than in the control (K), ranging from 1.274-1.871 mg L^{-1} (Figure 3). Yao & Peng (2017) states that a high nitrate concentration is caused by optimal nitrite oxidation by bacteria, especially nitrifying bacteria. However, nitrate content in the culture environment is also influenced by the activity of denitrifying bacteria, which reduces nitrate to nitrite (Pajares & Bohannan 2016).

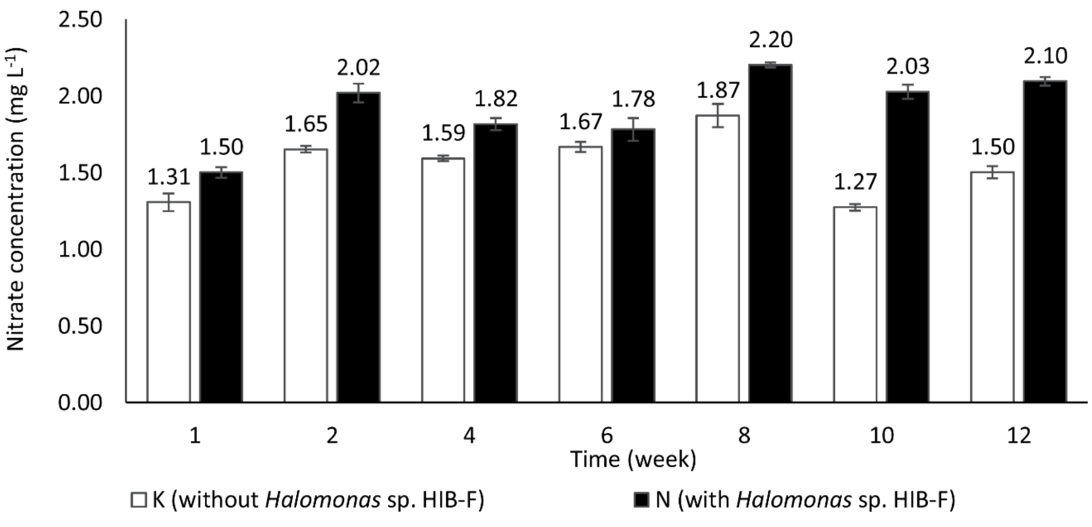

Figure 3. Nitrate concentration in the *Litopenaeus vannamei* rearing media added with *Halomonas* sp. HIB-F (N) and the control (K).

Another critical environmental parameter in the culture environment is organic matter. High organic matter content is an indicator of the high level of input waste, such as from feed and metabolic waste. Organic matter content of the 2 treatments increased from week 1 to 6, then declined in week 8 until the end of the rearing period. The highest organic matter content in both treatments occurred at week 6, accounting for 15.51 mg L^{-1} and 13.36 mg L^{-1} in the application of *Halomonas* sp. HIB-F and the control, respectively. Organic matter in the rearing medium added with *Halomonas* sp. HIB-F (N) tends to be higher in comparison with control (K) (Figure 4). However, the organic matter content was still under the normal range. A previous study reported that organic matter in 41-day old shrimps' pond reaches 68.4 mg L^{-1} (Wulandari et al 2015). Apart from uneaten feed and the secretion products of aquatic organisms, according to Hastuti et al (2011), organic matters in sediment can also come from the molting activity and died biota.

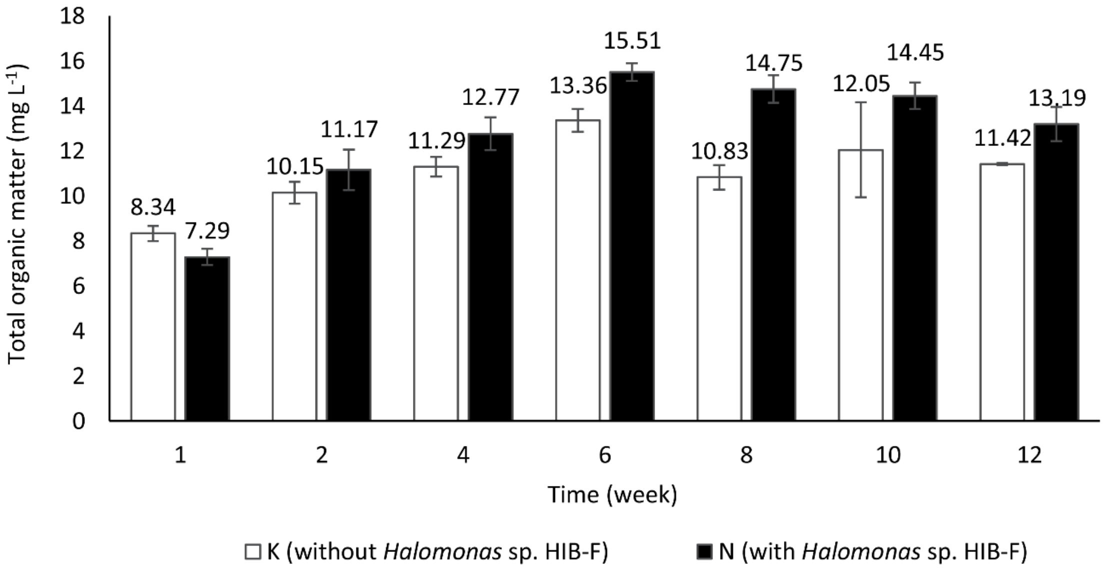

Figure 4. Total organic matter (TOM) in *Litopenaeus vannamei* rearing media applied with *Halomonas* sp. HIB-F (N) and the control (K).

Decomposition of organic matter is carried out by microorganisms in the environment. The amount of oxygen consumed by microbes to decompose organic matter under aerobic condition is represented as BOD. We found that the rearing medium added with *Halomonas* sp. HIB-F tends to have a relatively higher BOD in every week compared to control, which was 5.1 mg L^{-1} at week 8 (Figure 5). BOD in the rearing medium without the addition of *Halomonas* sp. HIB-F (K) increased until week 6 and started to decrease in week 8. It is considered to be in line with the organic matter content in the culture environment applied with *Halomonas* sp. HIB-F (Figure 4). There was a direct relationship between the amount of organic matter and BOD in the waters. The addition of nitrifying bacteria can affect BOD in the culture environment. Meanwhile, high organic matter will not be separated from high waste entering the cultivation system. It will depend on the number of living biota and their growth. The higher the organic matter content in the water, the higher the oxygen needed by microorganisms to degrade organic matter (Simanjuntak 2009), which is in accordance with the number of bacteria. BOD values inferior to 4 mg L^{-1} indicate that organic matter concentration compatible with the rate of decomposition by microorganisms, whereas BOD>4 mg L^{-1} indicates high concentrations of organic matter which cannot be optimally degraded by bacteria (Coldebella et al 2018).

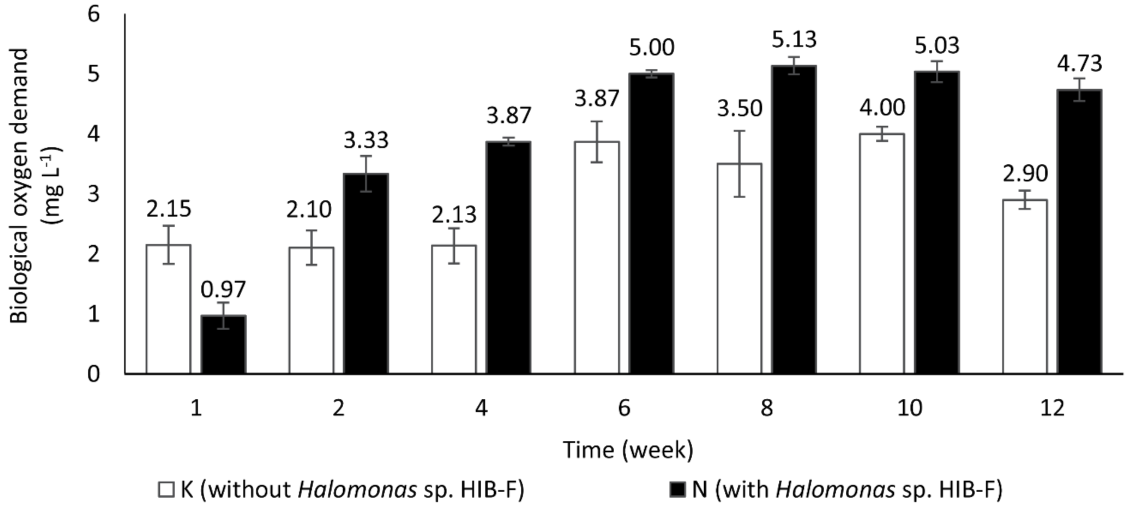

Figure 5. Biological oxygen demand (BOD) in *Litopenaeus vannamei* rearing media with (N) and without *Halomonas* sp. HIB-F (K).

The abundance of nitrifying bacteria. Nitrifying bacteria play a pivotal role in ammonium oxidation. The higher the abundance of nitrifying bacteria in water, the higher the ammonia decomposition process (Kuhn et al 2010). Nitrifying bacterial abundance in the rearing media applied with *Halomonas* sp. HIB-F in week 1 reached 14.0×10^5 CFU mL^{-1} and declined until week 12 accounting for 1.5×10^5 CFU mL^{-1}. According to the measurement of nitrifying bacterial abundance, the number of nitrifying bacteria in the rearing water applied with *Halomonas* sp. HIB-F was relatively higher than the control throughout the 90 days of the rearing period (Table 2). Bacterial abundance is influenced by the bacterial addition to the treatment and by the resident bacterial growth. In addition, bacterial abundance is altered by the amount of oxygen, number and type of bacteria, nutrients availability, temperature, pH, and salinity (Subagiyo et al 2015). When environmental and nutrients conditions are optimal, bacteria can grow. Carbon sources play an important role in bacterial growth under nitrification and denitrification process (Hastuti 2011). However, if the nutrients are not available, the bacteria will die, causing a decrease in the bacterial abundance of in the final week of the shrimp-rearing period.

Table 2

The abundance of nitrifying bacteria (CFU mL^{-1}) in the *Litopenaeus vannamei* rearing media with (N) and without the application of *Halomonas* sp. HIB-F (K)

Time (Week)	*Treatments*	
	K	*N*
1	13.3×10^5	14.0×10^5
6	4.7×10^5	10.6×10^5
12	1.5×10^5	3.8×10^5

K-without the bacterial application of *Halomonas* sp. HIB-F; N-with the bacterial application of *Halomonas* sp. HIB-F.

Blood glucose level. The application of nitrifying bacteria is expected to improve water quality, especially inorganic nitrogen parameters. Changes in the water profile cause stress to aquatic biota (Lorenzon et al 2007), which can affect their growth, reproduction, eating habits, and behavior (Schreck & Tort 2016). Stress responses in aquatic biota triggered by unsupportive water conditions are capable of increasing pathogenic infections. The stress level in crustaceans can be proven by an increase in haemolymph glucose levels (hyperglycemia) (Mercier et al 2009; Hastuti et al 2017). In this study, *L. vannamei* blood glucose level at the end of the rearing period with *Halomonas* sp. HIB-F application reached 23.62±7.64 mg dL^{-1}, which was slightly lower than the control (26.31±6.39 mg dL^{-1}) (Figure 6). Our result suggests that the shrimps reared without the application of *Halomonas* sp. HIB-F appears to be more stressed.

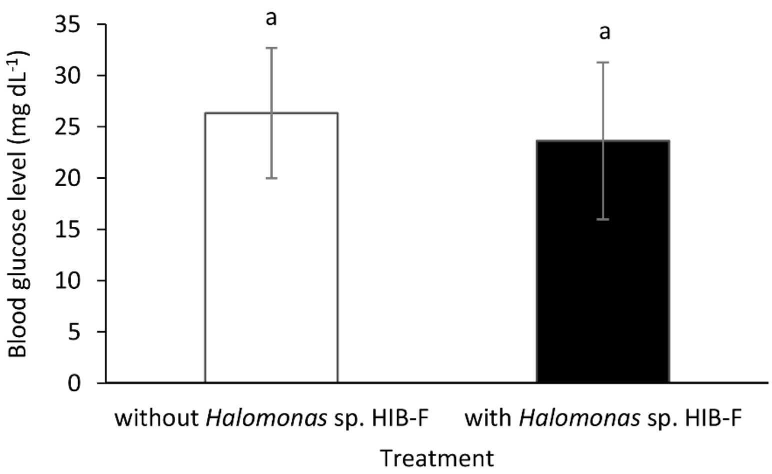

Figure 6. Blood glucose level of *Litopenaeus vannamei* reared in the culture media added with (N) and without *Halomonas* sp. HIB-F (K). Same letters indicate no statistical difference according to the independent sample t-test at a significant level of 0.05.

Survival rate. Relative stable water quality in aquaculture environments encourage the physiological responses and survival of *L. vannamei*. We detected that the survival rate of culture shrimps had a significant difference among treatments ($P<0.05$). *L. vannamei* survival rate in the application of *Halomonas* sp. HIB-F was 94±1.8%, while the control reached 52±6.1% (Figure 7).

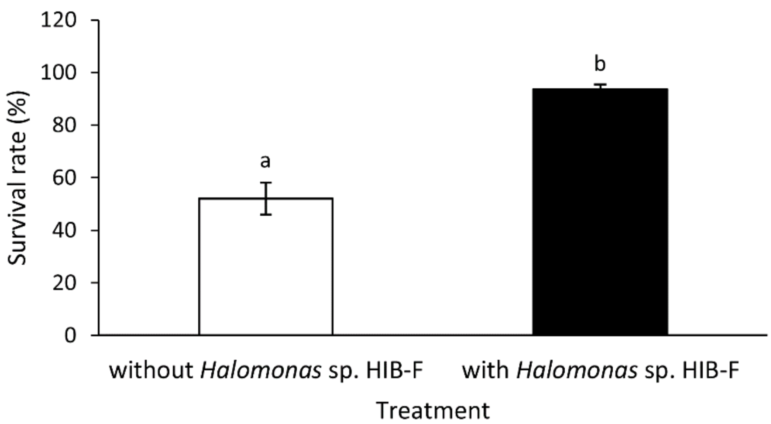

Figure 7. The survival rate of *Litopenaeus vannamei* reared in the culture media added with *Halomonas* sp. HIB-F (N) and the control (K). Same letters indicate no statistical difference according to the independent sample t-test at a significant level of 0.05.

At the end of the rearing period (90 days rearing), the application of the nitrifying bacteria *Halomonas* sp. HIB-F with the cell density of 10^9 CFU mL^{-1} in a total volume of 200 mL for 200 L water was able to decrease ammonium concentration in the water environment by 90.81%, at a value largely inferior (0.0083 mg L^{-1}) to the concentration in the control (0.0977 mg L^{-1}). *Halomonas* sp. HIB-F was also able to improve other environmental parameters in the rearing media, such as organic matter, nitrates, and

BOD, and indirectly the survival rate of shrimps. Wasielesky et al (2017) reported that ammonia concentration is one of the water quality parameters, which is difficult to be tolerated by shrimp's body and therefore it needs to be reduced.

Conclusions. Application of *Halomonas* sp. HIB-F in the culture environment is able to improve environmental quality, including ammonium, nitrate, and organic matter concentrations. These tend to be more stable and to reach values stimulating an increased survival rate of *L. vannamei*, reaching 94%. Further research needs to be conducted regarding the optimal abundance of nitrifying bacteria to reduce ammonium and nitrite, timely and gradually applied, in an appropriate dosage, in order to support the growth of *L. vannamei*.

Acknowledgements. The authors would like to thank Ir. H. Hardi Pitoyo, the Chairman of the Banyuwangi Shrimp Club, East Java, Indonesia, for supporting this research.

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Received: 30 April 2020. Accepted: 04 August 2020. Published online: 12 August 2020.
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How to cite this article:
Hastuti Y. P., Syarifuddin N. I., Tridesianti S., Fatma Y. S., Supriyono E., 2020 Application of *Halomonas* sp. HIB-F to *Litopenaeus vannamei* aquaculture system. AACL Bioflux 13(4):2116-2126.