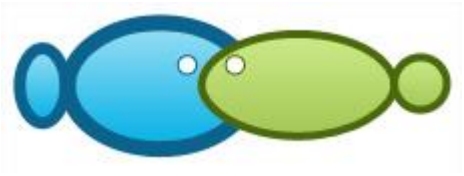

Histopathological analysis of pacific white shrimp (*Litopenaeus vannamei*) experimentally infected with *Vibrio harveyi* in a laboratory scale polyculture with seaweed (*Gracilaria verrucosa*)

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Abstract. The pacific white shrimp (*Litopenaeus vannamei*) is one of the main aquaculture commodities in the world that faces the threat of diseases. The recently discovered disease, AHPND, was related to the pathogen causing the vibriosis diseases. Furthermore, diseases caused by *Vibrio harveyi* bacteria cause losses to farmers. The aim of this study was to observe the histopathology of *L. vannamei* polyculture with seaweed *Gracilaria verrucosa* in a post-challenge test with *V. harveyi*. The research method comprised the following steps: seaweed and shrimp preparation, cultivation of bacteria, post-challenge test and histopathological examination. The treatment (T8) consisted of seaweed 800 g 100 L^{-1} brackish water in polyculture with shrimp, meanwhile the treatment (T4) consisted of seaweed 400 g 100 L^{-1} brackish water in polyculture with shrimp. Control shrimps were cultured without using seaweed. The first control (C1) was designed without challenge test with *V. harveyi* and the second control (C2) with challenge test. The result showed that polyculture seaweed and shrimp affect the bacterial growth. The bacterial population in the treatments was noticeably lower than in the control. Microphotographs of the muscles showed that C1 and T8 scored 0, meaning a normal histological structure. C2 scored 2, corresponding to a moderate damage, cell nucleus apoptosis, and moderately necrotic muscle fibers. T4 had score 3, corresponding to a high damage, nucleus apoptosis and fully necrotic muscle fibers. Furthermore, microphotographs of the hepatopancreas showed score 0 for C1 and T8, meaning normal. C2 and T4 had a score of 2, meaning a moderate damage, inclusion bodies, proliferating cells, wide organ spacing, dilated vacuoles, cell hyperplasia and non-dilated spacing. In conclusion, the results indicate that the polyculture with seaweed and shrimp affects the shrimp health and determines a bacterial population reduction.

Key Words: bacteria, challange test, diseases, microphotographs, vibriosis.

Introduction. Pacific white shrimp (*Litopenaeus vannamei*) is one of the main aquaculture commodities in the world. The main countries producing the pacific white shrimp include Japan, Taiwan, China, Philippines, Malaysia, Indonesia (FAO 2020). The global pacific white shrimp market demand is estimated to reach five million tons each year, while the industry is lacking the supply (Halim & Juanri 2016). Currently, one of the most important challenges in shrimp farming is a disease (Anderson et al 2019). Intensifying the shrimp farming leads to disease outbreaks incurring big losses to farmers. The presence of diseases decrease the production and becomes a limiting factor (Lavilla-Pitogo et al 2000). In the last four decades of shrimp production has been uncertain and were characterized by major disease events (Shinn et al 2018). The most common etiological agents that damaged the shrimp cultures are viruses and bacteria (Lightner 2011). In the Asian shrimp industry it has been estimated that 60% of losses in the shrimp production have been caused by viral diseases and 20% by bacterial pathogens, mostly by vibriosis (Flegel 2012).

Vibriosis or luminous disease is one of the important diseases in shrimp, that is mainly caused by *Vibrio harveyi* (Chiu et al 2007). Vibriosis as a bacterial disease is still a production problem in shrimp farming. The disease is associated with *L. vannamei* in larvae, post-larvae, broodstock and hatchery probionts (Vandenberghe et al 1999). Luminous *V. harveyi* is mostly capable of causing mortality of penaeid shrimp in

hatcheries and culture ponds (Souza & Wan 2021). In Mexico, a disease causing big losses to farmers is the Bright-Red Syndrome (BRS) caused by *V. harveyi* (Soto-Rodriguez et al 2012). Recent researches showed that Acute Hepatopancreatic Necrosis Disease (AHPND) is not only caused by *V. parahaemolyticus*, but also by *V. harveyi.* Identification using molecular methods showed that the related bacteria in AHPND was also closely *V. harveyi*. The yellow colony has been found more virulent than the green colony (Muthukrishnan et al 2019).

Bacteria from the *Vibrio* genus are ubiquitous in the aquatic environments and aquaculture production systems. *V. harveyi* has been recognized as a serious pathogen for important aquatic organisms, such as the pacific white shrimp. Extensive research has been carried out to find alternatives in anticipating *V. harveyi* infection in shrimp, starting from the utilization of natural ingredients (Kurniaji et al 2020; Rudi et al 2019), probiotics (Sukenda 2003; Balcázar & Rojas-Luna 2007; Harpeni et al 2018) symbionts (Oktaviana et al 2014; Munaeni et al 2014) and vaccination (Pope et al 2011; Vinay et al 2019). Various research results have the potential to prevent the *V. harveyi* infection and to stimulate the shrimp growth. The main obstacle faced is the problem of practicality and the application cost for both traditional and extensive farmers. Around 80% of the Indonesian shrimp aquaculture entities still practice traditional or extensive farming (Halim & Juanri 2016). Limitations to adopt the modern technology make it difficult for shrimp farmers to solve the disease problems.

Polyculture of *L. vannamei* and seaweed (*Gracilaria verrucosa*) is a technology with many advantages and can be easily used by extensive farmers. Integration of algae with shrimp in the aquaculture system has been suggested as an effective technology to improve water quality and to control diseases (Rongbin et al 2013). Some research on polyculture seaweed and shrimp showed a significant effect on the growth performance and survival rate of shrimp (Samidjan et al 2019; Ihsan et al 2019; Susilowati et al 2014). Seaweed has an important role as a biofilter to absorb waste nutrients, to control eutrophication and to improve the health of water (Chopin et al 2001). In natural conditions, seaweed is highly efficient to remove nutrients, such as ammonia nitrogen, nitrate and phosphate. Seaweed *G. verrucosa* also has been studied as a biocontrol agent and can prevent the *V. harveyi* infections on pacific white shrimp (Anton et al 2020). Phytochemical tests showed that *G. verrucosa* contains alkaloids, flavonoids and steroids, which have an antioxidant and antibacterial activity (Febrianto et al 2019). One of the potential functions of the seaweed in the polyculture systems is to maintain the shrimp health. This study aimed to observe the histopathology of the *L. vannamei* polyculture in *G. verrucosa* seaweed, post-challenged with *V. harveyi.*

Material and Method

Research design. This study used a completely randomized design with 2 treatments and 1 control. Each treatment had 3 replications. The first treatment (T8) was seaweed 800 g 100 L^{-1} brackish water in polyculture with shrimp. The second treatment (T4) was seaweed 400 g 100 L^{-1} brackish water in polyculture with shrimp. Control shrimp was cultured without using seaweed. The first control (C1) was designed without a challenge test with *V. harveyi* and the second control (C2) with a challenge test. Research stages were aquarium preparation, seaweed and shrimp rearing, challenge test and observation. The aquarium, with the dimensions of 70×50×50 cm, has been disinfected to prevent contamination. As much as 100 L of brackish water was used in all experimental aquariums. Seaweed was added to the aquarium according to the treatment design, for 5 days. The bacterial population was counted 6 days after the seaweed was cultured. Each aquarium contains 10 shrimp were added to the aquarium and kept with seaweed starting on 6 days post-seaweed cultured. The *V. harveyi* bacterial challenge test was carried out after 10 days of rearing. After 10 days of challenge test, the samples were taken for histological observations. The stages of the research can be seen in Figure 1.

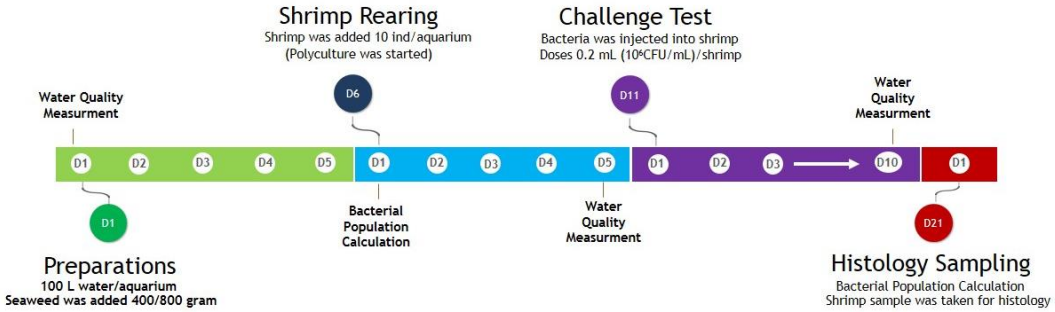

Figure 1. Stages of research.

Shrimp and seaweed preparation. The experimental organisms were *L. vannamei* specimens from the ponds of Marine and Fisheries Polytechnic, with the bone weight of 8.87±0.55 g and the length of 11.15±0.31 cm, obtained from shrimp seeds (from CP. Central Proteina Prima) which have been reared for 2 months. Shrimp has been checked for health and was found in normal conditions. *G. verrucosa* was obtained from farmers in Waetuo Village, Bone Regency, South Sulawesi. Seaweed has been reared for 1 month in traditional ponds and it was cleaned of mud and various organisms before using.

Bacterial V. harveyi cultivation and challenge test. The bacterial isolate of *V. harveyi* was obtained from the stock of the Fish Health and Environmental Laboratory, Brackish Water Cultivation Center, Takalar, South Sulawesi. Bacteria was confirmed as *V. harveyi* and were rejuvenated twice using TCBS (Thiosulfate Citrate Bile Salt Sucrose) media. Bacterial preparation was carried out by dilution using a physiological solution of 0.9% NaCl. The concentration of bacteria used for challenge test was 10^6 CFU mL^{-1}. A total of 0.2 mL of *V. harveyi* bacterial solution was injected intramuscularly using a syringe into the third segment of the shrimp's abdomen. During the challenge test, shrimp were given commercial feed of 32% protein.

Calculation of bacterial population. The calculation of bacterial population was carried out using the hepatopancreas of shrimps. The bacterial population sampling procedure refers to the method of Romano et al (2015). For each treatment, 3 shrimps were sampled at the end of the challenge test. The head of shrimp was opened using sterile scissors. The hepatopancreas of each shrimp was weighed and homogenized with a sterile physiological solution, at a 10-fold dilution. A total of 100 µL of hepatopancreatic solution supernatant was spread on TCBS media and incubated for 24 hours at 30°C. Bacteria were counted based on the colony formation on plates having 30-300 CFU according to Marwiyah et al (2019).

Histopathological analysis. The histopathological analysis was carried out at Maros Veterinary Center, South Sulawesi. The examination following the standard laboratory procedures refers to the method Luna (1971) with modifications according to Manan et al (2015) and Anjaini et al (2018). Organ samples were obtained from the hepatopancreas and muscle of shrimp. The specimens for histopathological examination were put in a fixation solution (including 10% Neutral Formalin Buffer) and stored for about 2 days before cutting and proceeding to histopathological testing. Cell morphology changes in tissues were observed after Hematoxylin and Eosin staining of the infected tissue preparations. Tissue samples were fixed with Buffered Neutral Formalin (BNF), in a volume at least 10 times the tissue's volume. In general, the time required for complete fixation is 48 hours. The next stages were the tissue blocking and cutting process, depression process, coloring process, dehydration, purification and finishing. Histological preparations have been analyzed by professionals (veterinarians) from MPC Clinic. Specimens were classified according to a histopathological scoring: score 0=no change/normal, score 1=a minimal damage, score 2=a moderate damage, score 3=a high damage.

Water quality assessment. The measured water quality parameters were: temperature, pH, salinity and Dissolved Oxygen (DO). Temperature was measured daily during the rearing of seaweed and shrimp, while DO, pH and salinity were measured at the beginning, middle and end of the study.

Data analysis. The bacterial population data were analyzed using the ANOVA (analysis of variance) analysis method at 95% confidence interval, through the Statistical Program Software System (SPSS Version 16.0). The determination of the significant differences between the treatments was continued with the Duncan's test. The histopathological parameters and water quality were analyzed descriptively.

Results

Population of V. harveyi bacteria. The observation of *V. harveyi* population in shrimp's hepatopancreas was carried out before the challenge test and 10 days after challenge test. The results showed that 10 days after the challenge test there was an increase in the population, for all treatments and control. The population in the hepatopancreas, after the challenge test of the shrimp reared with seaweed, was significantly lower than in the shrimp reared without seaweed (control; $P<0.05$). The bacterial populations in the control and in the treatments were not significantly different from $1.53\text{-}2.31\times10^4$ CFU m L^{-1}. 10 days after the challenge test, the bacterial population in the control was significantly higher than in the T4 and T8 treatments. The *V. harveyi* bacterial population densities in the shrimp's hepatopancreas can be seen in Table 1.

Table 1

Population of bacteria *Vibrio harveyi* in hepatopancreas of shrimp

Treatments	*Population of Vibrio harveyi (10^4 CFU mL^{-1})*	
	Pre-challenge test	*10 Days post-challenge test*
C2	1.80^a	22.66^a
T4	2.31^a	12.56^b
T8	1.53^a	8.40^b

C2-control; T4-shrimp reared with 400 g 100 L^{-1} brackish water; T8-shrimp reared with 800 g 100 L^{-1} brackish water. Different superscripts in the same column show that there are significant differences ($P<0.05$).

The polyculture of seaweed and shrimp has an effect on the bacterial growth. The bacterial populations between the T4 and T8 treatments were not significantly different ($8.40–12.56\times10^4$ CFU mL^{-1}). The antibacterial potential of the seaweed was analyzed for some of its compounds, such as: polysaccharides, fatty acids, phlorotannins, pigments, lectins, alkaloids, terpenoids and halogenated compounds (Pérez et al 2016). The *G. verrucosa* active antibacterial fractions were determined by column chromatography. Antibacterial compounds contained: alkaloids, flavonoids, tannins and phenols, with an antibacterial activity against the *Pseudomonas aeruginosa, P. putida, V. harveyi* and *V. algynoloticus* bacteria (Maftuch et al 2016). The mechanism of the antibacterial inhibition of the compounds from seaweed on the *V. harveyi* in water was not yet found. Most of the research concerns their bacterial inhibition activity only as seaweed extract, while an in-depth research on the isolated compounds would be necessary.

The polyculture between seaweed and shrimp in the same aquaculture system showed the seaweed potential to maintain the health of shrimp by preventing the bacterial infections (Figure 2). *V. harveyi* bacteria is known to be a virulent pathogen for shrimp in all life stages (Vandenberghe et al 1999). The result of seaweed research has found different mechanisms for the inhibition of *Vibrio. Vibrio* bacteria inhibition is caused by chemical compounds produced by bacteria associated with seaweed. *Vibrio* bacteria have a quorum sensing mechanism with an increased cell-to-cell communication which increases the virulence level. The AHL lactones from *B. licheniformis* DAHB1 inhibit *Vibrio* biofilm formation in the shrimp's intestine. DAHB1 quorum-quencing AiiA might be

developed for inhibiting *Vibrio* colonization and mortality of shrimps in aquaculture (Vinoj et al 2014). *Bacillus* are bacteria associated to seaweed (Singh 2014). *Bacillus* are epiphytic and endophytic bacteria associated with red macroalgae *Gracillaria* (Singh et al 2015). These bacteria can grow as epiphytic bacteria associated to the seaweed without being affected by the antibacterial activity of the seaweed. *Gracilaria* inhibited the growth of *Staphylococcus eureus*, *P. aeruginosa, Streptococcus faecalis*, except for *B. cereus* (Kolanjinathan & Govindarajan 2009). The virulence factors and their roles in the bacterial diseases were documented. Both of these factors are being regulated by quorum sensing, including for the *V. harveyi* bacteria. Quorum sensing depends on population density of bacteria by auto-inducer production. Quorum quenching is a disruption of the quorum sensing, considered as an alternative to prevent the bacterial infection in shrimp (Subramani & Jayaprakashvel 2019).

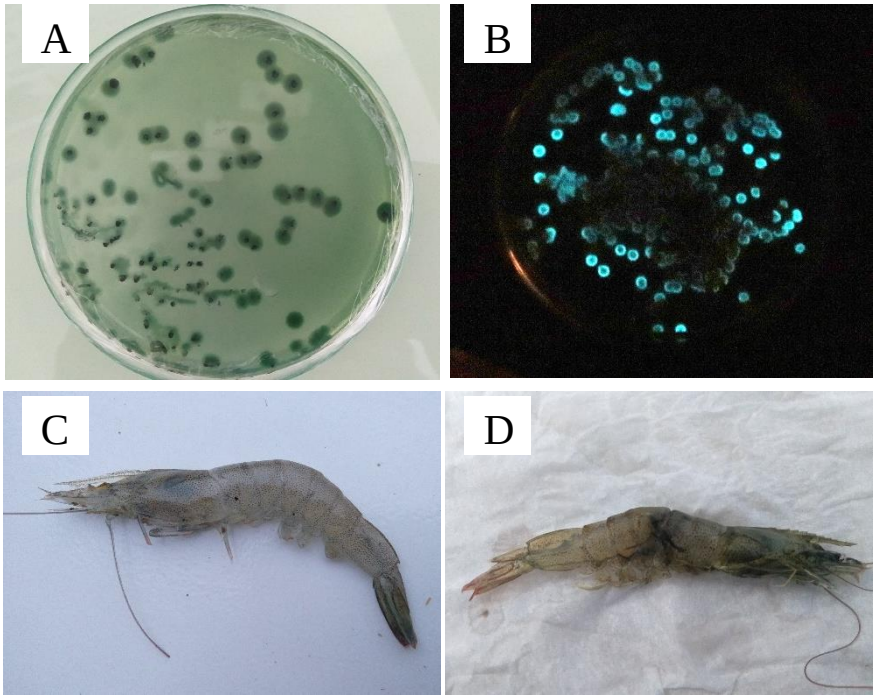

Figure 2. Research documentation: *Vibrio harveyi* colony on TCBS (A), *Vibrio harveyi* colony on TCBS in blackout (B), normal shrimp (C), abnormal shrimp (D).

Histopathological analysis in muscle. A histopathological analysis was performed on the muscle, in all treatments and controls. The result showed that there are differences between shrimp reared with and without seaweed. The observation results for the muscle can be seen in Figure 3.

One of target organs for the *V. harveyi* infection is the muscle. Four microphotographs of muscle after 10 days post challenge test showed different conditions. Control 1 (C1) consisted of the shrimp reared without seaweed and without challenge test with *V. harveyi*. C1 was given the score 0 that means no change (normal) in the cell nucleus and muscle fibers (shrimp without infection). Uninfected shrimp was showing a normal histology in muscle and hepatopancreas (Yatip et al 2017). Histological intraperitoneally observations in shrimps infected by *V. harveyi* showed distrupted mucus and basophilic nuclei in the skin, increased vacuolation (hepatocyte disruption) and hepatic necrosis in liver (Xie et al 2020). Control 2 (C2) consisted of the shrimp reared without seaweed and experimentally challenged with *V. harveyi.* C2 was given the score 2 that means moderate damage, apoptosis of the cell nucleus and moderately necrotic muscle fibers. General necrosis of the muscle bands was related to *V. harveyi* infection. Microphotographs of the skeletal muscle of *L. vannamei* were showing haemocytic infiltration, inflammatory response and melanization after experimental infection with *V. harveyi*, according to the observations of Soto-Rodriguez et al (2012). The same condition was found in the treatment T4. Shrimps reared with seaweed 400 g 100 L^{-1}

brackish water and experimentally challenged with *V. harveyi* were given score 3, according to the microphotographs. This score means high damage, nucleus under apoptosis and completely necrotic muscle fibers. Muscle necrosis in shrimp after *V. harveyi* infection is similar to the viral infection in the muscle, such as the *Penaeus vannamei nodavirus* (PvNV) (Tang et al 2007) and the Infectious Myonecrosis Virus (IMNV) (Poulos et al 2006). The similarity between bacteria and virus infection in muscle must be confirmed by using histology/molecular examinations (Soto-Rodriguez et al 2012). The treatment T8 consisted of rearing shrimps with seaweed 800 g 100 L^{-1} brackish water and experimentally challenging them with *V. harveyi.* T8 was given the score 0, that means normal cells (no change), cell nucleus and muscle fibers. Normality of muscle tissue (according to the microphotographs) in shrimp reared with seaweed 800 g 100 L^{-1} indicates the positive effect of seaweed on shrimp health.

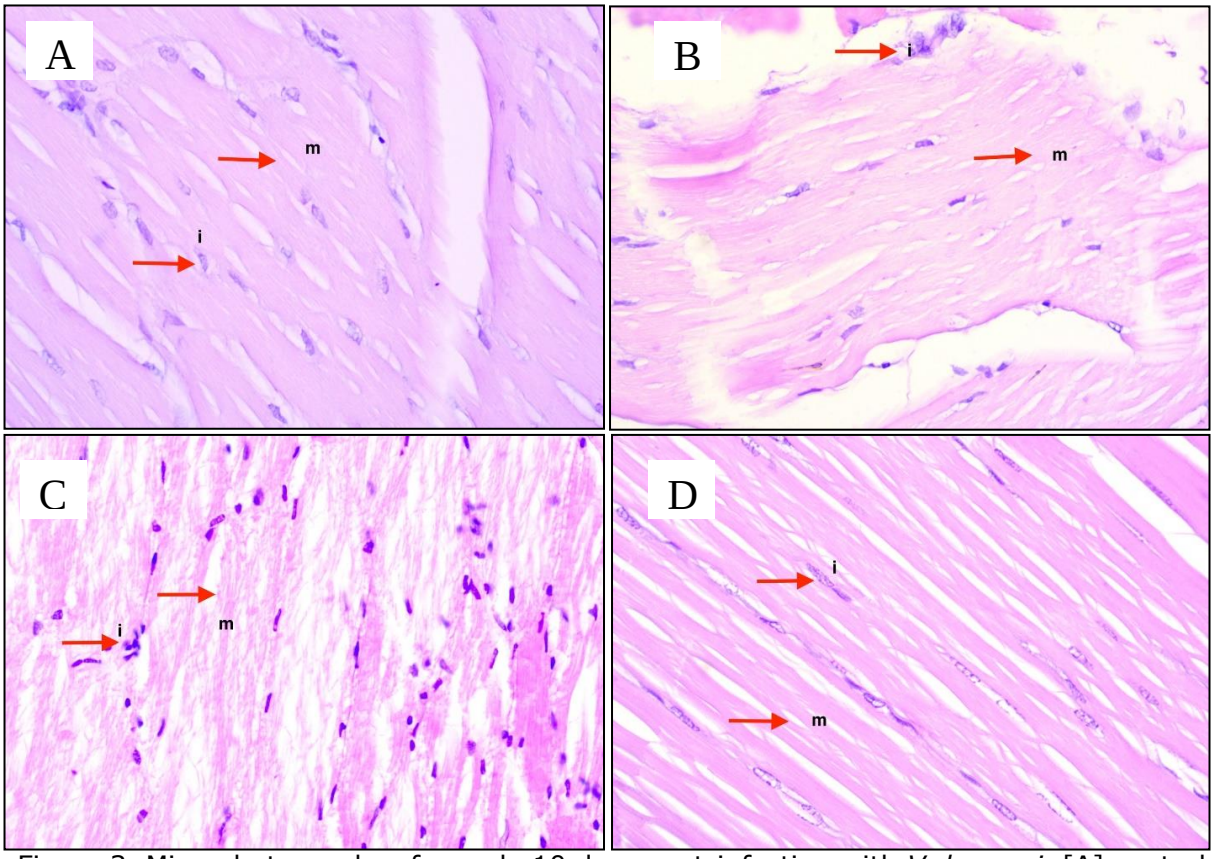

Figure 3. Microphotographs of muscle 10 days post-infection with *V. harveyi*. [A] control without challenge test (C1): score 0 means no change (normal), cell nucleus normal (i) and muscle fibers normal (m). [B] control with challenge test (C2): score 2 means moderate damage, apoptosis of the cell nucleus (i) and moderately necrotic muscle fibers (m). [C] treatment T4, polyculture shrimp-seaweed (400 g): score 3 means high damage, nucleus under apoptosis (i), muscle fibers completely necrotic (m). [D] treatment T8, polyculture shrimp-seaweed (800 g): score 0 means normal cell nucleus (i), normal muscle fibers (m).

Histopathological analysis in hepatopancreas. A histopathological analysis was performed on hepatopancreas in all treatments and controls. The results showed that there is a difference between shrimps reared with and without seaweed. The observation result for the hepatopancreas can be seen in Figure 4.

The hepatopancreas is a crucial organ, related to the immune response and to the heat stress (Sun et al 2014). The hepatopancreas is an organ that can be damaged due to an infection with *V. parahaemolyticus* (Khimmakthong & Sukkarun 2017). Also, one of the target organ of *V. harveyi* bacteria in shrimp is the hepatopancreas (Robertson et al 1998). The presence of bacterial infection can be seen from the organ damage, according

to the result of histopathological examination. Histopathology result of hepatopancreas showed different conditions between treatments and control. Control 1 (C1) was given a score 0 that means no change (normal), with normal cell nucleus and organ spacing. The difference from Control 2 (C2) was the tissue change. C2 was given a score 2 that means a moderate damage: inclusion bodies, proliferating cells and wide organs spacing. The tissue damage observed from cell changes was related to the *V. harveyi* infection. The histology examination of the shrimp hepatopancreas infected with *V. harveyi* showed necrosis and vacuolization (Utami et al 2017).

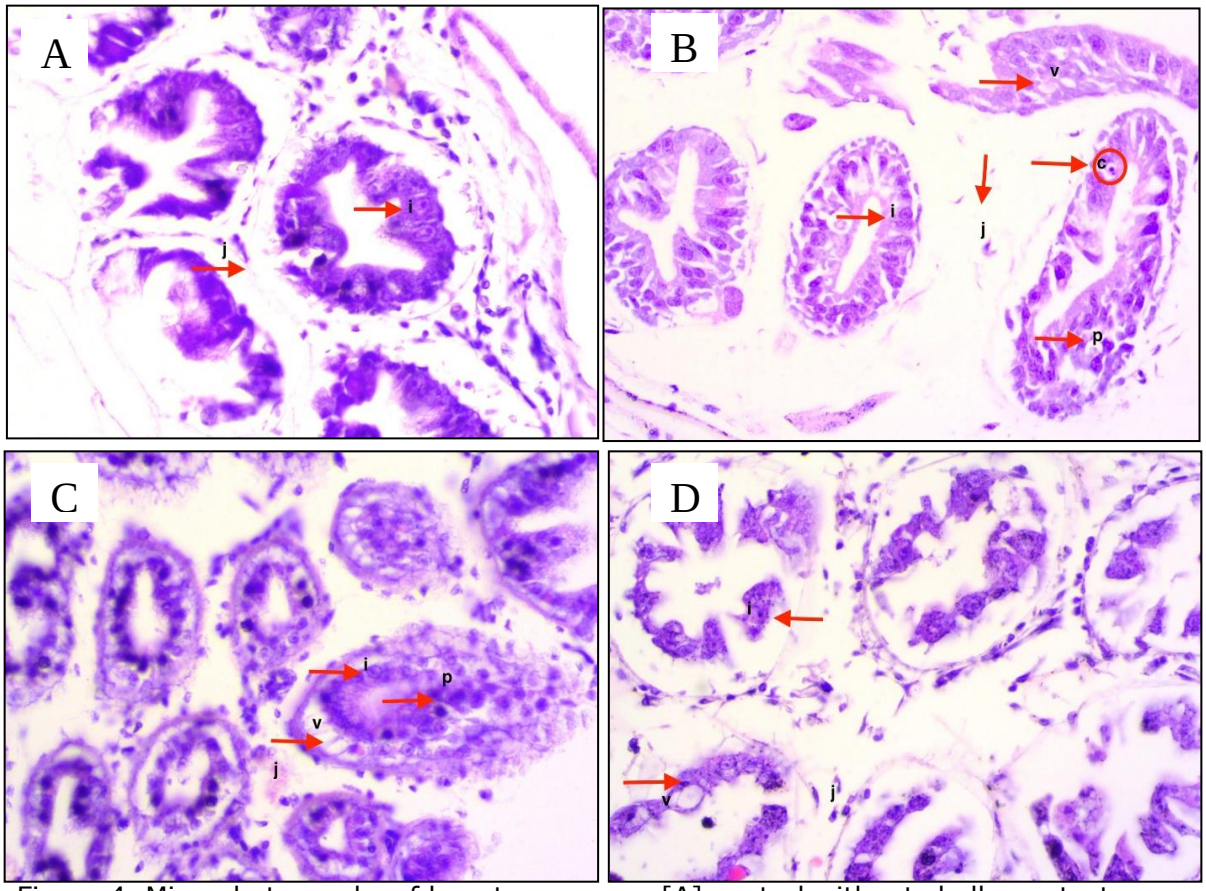

Figure 4. Microphotographs of hepatopancreas. [A] control without challenge test: score 0 means a normal cell nucleus (i), a normal organ spacing (j). [B] control with challenge test: score 2 means a moderate damage, inclusion bodies (c), proliferating cells (p), wide organ spacing (j). [C] polyculture shrimps with 400 g of seaweed: score 2 means moderate damage, dilated vacuoles (v), cell hyperplasia (p), non-dilated spacing (j). [D] polyculture shrimps with 800 g of seaweed: score 0 means normal cell, no change, normal cell nucleus(i), but the hepatopancreas shape was not normal, vacuoles were still present (v), the organ spacing was narrow.

The tissue damage, caused by inclusion bodies and proliferating cells, from C2 is also found in Treatment (T4), which was given a score 2 that means moderate damage, dilated vacuoles, cell hyperplasia and non-dilated spacing. Abnormal tissue caused by *V. harveyi* infection was similar in the histological examinations of shrimps with Acute Hepatopancreatic Necrosis Disease (AHPND), that indicated a massive sloughing of the hepatopancreas tubules, haemocyte infiltrations and proximal-to-distal lesions (Muthukrishnan et al 2019). Salema fish histological results showed hyperplasia, vacuolar degeneration and hematopoietic tissue (Turgay et al 2018). Treatment (T8) was given a score 0 that means normal cell, no change, normal nucleus cell, but the shape of the hepatopancreas organ was not normal, the vacuoles were still present, the organ spacing was not wide. Normality of the hepatopancreas tissue, according to microphotographs of shrimp reared with seaweed 800 g 100 L^{-1}, indicates the positive effect of seaweed on

the shrimp's health. Moderate damage on microphotographs of shrimp reared with 400 g 100 L^{-1} seaweed was thought to be due the small influence of the seaweed, still allowing infections during the maintenance period.

The moving habits of the shrimp on the bottom of the aquarium seemed disturbed in the seaweed polyculture. However, using bags to accommodate seaweed can be an alternative to consider in further research. The water quality measurement showed that the values of temperature, salinity, pH and DO were still within the tolerance limits of the shrimp, namely a temperature of 27-29^{o}C, a salinity of 31-35 ppt, a DO of 5.2-7.3 ppm and a pH of 7.5-7.9. This study shows that polyculture of seaweed and vannamei shrimp has the potential to improve the health status of shrimp.

Conclusions. The results of this research showed that the seaweed and shrimp polyculture has an effect on the bacterial growth and shrimp health. The bacterial population in the seaweed and shrimp polyculture was significantly lower than in the control (without seaweed). Microphotographs of muscle and hepatopancreas showed a better condition of the shrimps reared with 800 g of seaweed 100 L^{-1} (T8) than without seaweed (control). T8 has a score 0, meaning a normal tissue. The control, with experimental infection with *V. harveyi* has a score 2, meaning a moderate damage of the tissue.

Acknowledgements. The authors would like to thank the Takalar Brackish Water Center and the Maros Veterinary Center for their willingness to help carrying out the research. This research was partially supported by the Fisheries and Marine Education Center, BRSDM KP, Ministry of Marine Affairs and Fisheries (2019).

Conflict of interest. The authors declare no conflict of interest.

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*** FAO, 2020 The state of world fisheries and aquaculture. Sustainability in action. https://doi.org/10.4060/ca9229en.

Received: 13 July 2021. Accepted: 01 October 2021. Published online: 13 October 2021.
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How to cite this article:
Kurniaji A., Anton, Yunarty, 2021 Histopathological analysis of pacific white shrimp (*Litopenaeus vannamei*) experimentally infected with *Vibrio harveyi* in a laboratory scale polyculture with seaweed (*Gracilaria verrucosa*). AACL Bioflux 14(5):2826-2836.