Pathogenicity of *Vibrio alginoliticus* on Giant Freshwater Prawn, *Macrobrachium rosenbergii* Post-Larvae

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DECLARATION

I hereby declare that the work embodied in here is the result of my own research except for the excerpt as cited in the references.

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Signature

Student's Name :

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Date :

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Supervisor Signature

Supervisor's Name :

Stamp :

Date :

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In the name of “Allah”, the most beneficent and merciful who gave me strength and knowledge to complete this final year project. This final year project is a part of this course to complete the bachelor of degree in Husbandry Science. This has proved to be a great experience for me.

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ABSTRACT

In recent years, aquaculture sector including prawn industry has developed rapidly in Malaysia. Malaysia government has pay high attention on this sector due to the static fisheries production and the potential of aquaculture to serve as national food. However, aquaculture sector has also facing issue and challenges including low quality of seed, depleted of wild stock population and also disease infection. In prawn industry, microbial disease are reported affects its production. Usually, diseases in prawn in ponds are caused by microorganism such as viruses, fungi, protozoan and bacteria. Vibrio bacteria are one of the primary pathogens reported to cause serious mortality in prawn hatcheries in Malaysia. In this study, the post larvae *Macrobrachium rosenbergii* were conducted with *Vibrio alginoliticus* to test the pathogenicity of bacteria in this prawn and to determine the median lethal concentration (LC 50) of the bacteria. The present study showed that *Vibrio alginoliticus* were isolated from the fish diseases and was immersed to post larvae *Macrobrachium rosenbergii* at four different concentration $1.0x10^4$, $1.0x10^5$, $1.0x10^6$ and $1.0x10^7$ CFU/ml. Most of the infected post larvae *Macrobrachium rosenbergii* were died within 4 days. Based on pathogenicity test result, LC 50 was determined as $1.0x10^3$ CFU/mL, while $1.0x10^4$, $1.0x10^5$, $1.0x10^6$ and $1.0x10^7$ CFU/ml. were started to convinced 100% mortality in the immersion challenge .The clinical sign were observed during the experiment including hepatopancreas and gross appearance of post larvae *Macrobrachium rosenbergii* such as lethargy, anorexia and slow growth development.

Keywords: *Macrobrachium rosenbergii*, post larvae, *Vibrio alginoliticus*, Lethal concentrations, giant freshwater prawn

Patogenik bakteria *Vibrio alginoliticus* dalam udang gergasi air tawar,

***Macrobrachium rosenbergii* terhadap Larva pos**

ABSTRAK

Kebelakangan tahun ini, sektor akuakultur industri termasuk industri udang telah berkembang pesat di Malaysia. Kerajaan Malaysia telah memberi perhatian yang tinggi terhadap sektor ini kerana pengeluaran ikan statik dan potensi akuakultur untuk berfungsi sebagai makanan kebangsaan. Walau bagaimanapun, sektor akuakultur industri juga menghadapi masalah dan cabaran termasuk kualiti benih yang rendah, kekurangan populasi stok induk dan juga jangkitan penyakit. Dalam industri udang, penyakit mikrob dikenal pasti sebagai penyakit yang boleh mempengaruhi pengeluaran stok udang. Kebiasaannya, penyakit dalam udang di kolam adalah disebabkan oleh mikroorganisme seperti virus, kulat, protozoan dan bakteria.Contohnya, bakteria Vibrio adalah salah satu daripada patogen utama yang boleh menyebabkan kematian yang serius di kalangan populasi udang di Malaysia. Dalam kajian ini, larva pos daripada *Macrobrachium rosenbergii* dibuat kajian dengan bakteria *Vibrio alginoliticus* untuk menguji patogenik bakteria dalam udang ini dan menentukan kepekatan maut median (LC 50) bakteria dalam eksperimen ini. Kajian ini juga menunjukkan bahawa *Vibrio alginoliticus* telah diambil daripada satu penyakit ikan dan digunakan untuk menyerang larva pos *Macrobrachium rosenbergii* pada empat kepekatan yang berbeza iaitu $1.0x10^4$, $1.0x10^5$, $1.0x10^6$ dan $1.0x10^7$CFU / ml. Kebanyakan larva pos yang dijangkiti *Macrobrachium rosenbergii* telah mati dalam masa 4 hari. Berdasarkan hasil uji patogenik terhadap bakteria, LC 50 telah ditentukan sebagai $1.0x10^3$ CFU / mL, manakala $1.0x10^4$, $1.0x10^5$, $1.0x10^6$ dan $1.0x10^7$ CFU / ml telah mula menjadikan kematian 100% dalam larva pos. Antata tanda klinikal yang diperhatikan semasa eksperimen termasuklah melihat hepatopancreas secara menyeluruh dan permehatian mata kasar terhadap larva pos *Macrobrachium rosenbergii* seperti keletihan, anoreksia dan pertumbuhan tumbesaran semakin perlahan.

Kata kunci : *Macrobrachium rosenbergii*, larva pos, *Vibrio alginoliticus*, kepekatan median, udang gergasi air tawar

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LIST OF ABBREVIATIONS AND SYMBOL

NO	
CFU	Colony Forming Unit
mL	Millilitre
LC 50	Lethal concentration 50

CHAPTER 1

INTRODUCTION

1.1 Research Background

The aquaculture industry is developing very quickly in recent years and has significantly role in the economic development of the country that contributes food supply to residents (D.V. Lightner & Redman, 1998). The developing countries involved two aquaculture system which are small scale system and large scale system that contribute to the food supply and increase the food security. The giant freshwater prawn, *Macrobrachium rosenbergii* is one of the most cultivated freshwater prawns in the world and has been introduced more than 40 countries such as Thailand, Vietnam and Malaysia. In recent years, *Macrobrachium rosenbergii* has a great export market worldwide. In 2017, the production of global farmed shrimp was estimated between 2.9-3.5 million tonnes (FAO, 2017). However, the production of white shrimp in Malaysia continue to decline because the farmers has returned to black tiger shrimp because of the Early Mortality Syndrome problem in giant freshwater prawn. Early Mortality Syndrome (EMS) or Hepatopancreatic necrosis disease (AHPND) is a sort of disease that affects the shrimp. The disease was first reported in Southern China in 2011 and subsequently in Vietnam, Thailand and Malaysia (FAO, 2013). Typically, EMS

diseases affects the PL shrimp that frequently causes up to 100% mortality after stocking density around 20-30 days.

Generally, most probably of EMS is caused by the presence of activity of Vibrio. After stocking of post larvae shrimp in the aquarium tanks, the luminescent Vibriosis occurred during the first 10-45 days. The disease outbreak was found to be pre-existing by the increasing number of opportunistic Vibriosis during immersion challenge (Jayasree, Janakiram, & Madhavi, 2006). Furthermore, the pathogenic factors such as Vibrio bacteria can influence the rate of mortality that effect on economic development of aquaculture industry.

Disease prevention can be reached by increased the hygiene management to prevent of the transmission of pathogens by quarantine and provide good hygiene (disinfection of culture tanks, water and eggs) (Brock & Bullis, 2001). Other than that, the pathogens of aquaculture are opportunistic because host organism of bacteria is physically stressed (Alderman & Hastings, 1998).This infection can reduce by giving the water treatment in order to improve water quality (Crab, Defoirdt, Bossier, & Verstraete, 2012) and avoiding the stress factors such as high stocking density, handling, temperature and salinity changes (Brock & Bullis, 2001). The fundamental that need to determine the survivability of *M. rosenbergii* post larvae towards the bacterial infection such as Vibriosis by preparing the different concentration of Vibrio bacteria using the immersion method. This information can serve as a guide when practicing routine sanitary procedures to reduce the bacterial load in the aquarium tank. For example, avoid the concentration of bacterial that build up to the 10^3 CFU/ml level because this level leads to significant mortalities within 48 hours.

1.2 Problem statement

Data from Department of Fisheries (DOF) (FAO, 2013) reported a low survivability of PL *Macrobrachium rosenbergii* due to several causes such as disease infection, water quality and etc. Major pathogen that could cause disease in *M. rosenbergii* is virus, bacteria as well as parasites. Most of the diseases are caused by virus infection but bacterial infection is also cannot be ruled out. Among disease reported in giant freshwater prawn industry is due to the disease caused by bacteria infection including Vibriosis. Vibriosis could cause a luminous disease that can cause high mortality of *Macrobrachium rosenbergii* Post larvae.

1.3 Significance of study

Information on the survivability of *Macrobrachium rosenbergii* Post larvae towards *Vibrio alginoliticus* infection will help in the management of health in the giant freshwater prawn by improving water quality and avoiding stress factors that can increase the opportunistic of aquaculture pathogens.

1.4 Objective

This research study aims to determine the LC 50 of Post larvae *Macrobrachium rosenbergii* exposed to different concentration of *Vibrio alginoliticus.*

1.5 Hypothesis

H_0 : Different concentrations of *Vibrio alginoliticus* will have no significant effect on the post larvae of *Macrobrachium rosenbergii.*

H_1: Different concentrations of *Vibrio alginoliticus* will have significant effect on the post larvae of *Macrobrachium rosenbergii.*

1.6 Limitations of study

In this study, the major limitations of this research were the weather, electricity and limited space that provided by Universiti Malaysia Kelantan. Other than that, the study is limited by the lack of information about the proper LC 50 value that leading to the highest mortality in *Macrobrachium rosenbergii* post larvae after 48 hours exposure of *Vibrio alginoliticus.* Furthermore, only some information of the pathogenicity of *Vibrio alginoliticus* on giant freshwater prawn, *Macrobrachium rosenbergii* Post-Larvae that can determined the LC 50 value that have been published. But, the available published are not the latest articles, which was more than 10 years aged.

CHAPTER 2

LITERATURE REVIEW

2.1 *Macrobrachium rosenbergii*

The giant freshwater prawn, *Macrobrachium rosenbergii* (de man) is a common native habitat in rivers and estuaries in tropical regions of the world (Michael Bernard New, 2005). The giant freshwater prawn, *M. rosenbergii* usually known as 'udang galah,' is grouping from north-west India to Vietnam, Philippines, Papua New Guinea and Northern Australia. Besides, the giant freshwater prawn, *M. rosenbergii* commonly called 'scampi' due to highly growth rate, wide range of temperature (15-35˚C) and tolerance salinity of water .Thus, *M. rosenbergii* is belongs to the genus of the family Palaemonidae. However, the giant freshwater prawn is distributed in most inland freshwater areas, including lakes, rivers, swamps, estuarine areas, ponds, canals as well as in irrigation duct (Michale Bernard New, 2002). Generally, the male of giant freshwater prawn will grow up to 320 mm size and weighing over 200 g thus this species is known as the biggest freshwater prawn in the world. This giant freshwater prawn has been evaluated an important aquaculture species of aquaculture industry and become an important way of increasing the food security and to stimulate economic activity. The giant freshwater prawn is being cultured by the farmers in many regions because of the fast growth, demand in high market and tolerance to environmental

conditions ,and considered profitable freshwater aquaculture species (Michael B. New, 1990)(Ranjeet & Kurup, 2002).The global aquaculture of giant freshwater prawn farming has presented great increase in production in a few years (Michael B. New & Nair, 2012). The *M. rosenbergii* has a great production in global industry from 130,689 to 202,089 tons during 2000-2011 (FAO, 2013). (Banu & Christianus, 2016) stated that *Macrobrachium* (Bate) had reached 458,000 tons of production in 2012; with Giant River prawn (*M.rosenbergii*) provided nearly 48% normal productions. However, it is clear trend of decreasing the production of giant freshwater farming in India since 2005 primarily due to the disease problems. This is supported by (Fisheries & No, 2006) stated that global production of farmed shrimp in 2017 was estimated between 2.9-3.5 million tonnes. In Asia-pacific, around 75 to 80 percent production of farmed shrimp originated come from this particular area. Foremost, In Asia-pacific, around 75 to 80 % production of farmed shrimp originated come from this particular area. However, the production of giant freshwater prawn is facing a serious disease threat that particularly from Vibrio species that lead to the mortality.

In 1977, the giant freshwater was first introduced has resulted in variation in some characteristics, and this characteristics has develop about taxonomic status of the species. The two subspecies that identified by the researcher were eastern *M. rosenbergii rosenbergii* and the western *M. rosenbergii dacqueti* (Lindenfelser, 1984). According to De Bruyn, Wilson, & Mather (2004), which figure out the mitochondrial rRNA 16s gene, concluded that these two forms represent phylogenetically specific species. These two species can be easily differentiate by the basic of a few simple diagnostic traits (Wowor & Ng, 2007).

Macrobrachium rosenbergii is one of the commercial aquaculture species that being high desired production as food consumption products. According to Baticados, Lavilla-Pitogo, Cruz-Lacierda, de la Pena, & Sunaz (1990), Vibriosis is responsible to huge mortality of cultured penaeids worldwide because of the prominently disease in the aquaculture industry. Previous studies have reported that Vibrio act as opportunistic or secondary pathogens that have been related with infections in humans and marine animals penaeids worldwide because of the prominently disease in the aquaculture industry (Austin & Austin, 2012). This shows that Vibrio species are the viral pathogens that live in the giant freshwater prawn environment that can cause serious loss in prawn farming of aquaculture industry.(D.V. Lightner et al., 2006) also found that Vibriosis frequently affect a wide range of fish and shellfish organism such as crustacean species. This ideal was supported by (Alday-Sanz, Roque, & Turnbull, 2002), who stated that the shrimp will be suffered more numerous and earlier pathological changes with the immersion challenge with Vibrio compare to the exposure of the ammonia directly.

2.2 Vibrio bacteria

Recently, Malaysia has experienced the shrimp disease that involved Vibriosis in brood stock, larvae and post larvae. Predominantly, Vibriosis has cause serious loss in prawn hatcheries because of the luminous disease. (Ruangpan & Kitao, 1991) reported that Vibrio bacteria are one of the pathogenic factors of the diseases outbreak that can promote high mortality among economically of important species of farmed marine fish and shrimp in Thailand.

Bacteria are one of the ecological agents that develop diseases such as *Vibrionaceae* that serve the most important group of pathogens for both juvenile shrimp and larvae. For example, Vibrio species are pathogens that frequently effect the hepatopancreas. Hepatopancreas is a sensitive organ and responsible to injury by pesticides and other water pollutants (Saravana Bhavan & Geraldine, 2000). According to Ramesh, Loganathan, & Venkateswaran (1990) can be categorized as Gram-negative, facultative anaerobes, comma shaped rod and halophilic bacteria that begin found abundantly in freshwater and marine ecosystem. Usually, the 'Vibriosis' diseases occur when the giant freshwater prawn are infected by *Vibrio alginoliticus* that come from rearing water. Thus, *Macrobrachium rosenbergii* usually shown discomfort appearance from Vibriosis diseases that be allowed black colour on the carapace, with red discolouration of the shell exoskeleton and loss of physical ability in six days, leading to an mortality rate (Samantaray, Khuntia, & Das, 2010).

Vibrio species have been reported as the causative agents for numerous disease outbreak (Alavandi et al., 2004),(Jayaprakash, Rejish Kumar, Philip, & Bright Singh, 2006), (Kennedy, Venugopal, Karunasagar, & Karunasagar, 2006). According to Jayaprakash et al. (2006) reported that presence of vibrio species has positive relation

with the genus *Macrobrachium rosenbergii* in a culture water. The bacterial infections can cause vibriosis and chitinolytic bacterial shell diseases. Furthermore, culture water is one of the main potential ways for introducing pathogenic bacteria into shrimp hatcheries. Hoa, Oanh, & Phuong, (2000) stated that *M. rosenbergii* PL can influenced the serious mortality when they were challenged by immersion with Vibriosis species at the concentration 10^4-10^8 cells mL^{-1}.This diseases can give the symbolic economic losses, increased mortality, reduced growth rates, decreased product quality and increased hatcheries management costs. Any mortality in prawns relies on the pathogens that have different concentrations of bacterial cell for depending on the species to resist stress influence by their nutritional and physiological factors. Other than that, the *M. rosenbergii* become inactive in nature constantly and mortality happen at the bottom of the tanks after 48 hours when exposed to *V. alginoliticus* species during immersion challenge. This infection of *Vibrio alginoliticus* can be noticed after 4 days based on the symptoms of *M. rosenbergii.*

In additional, Vibriosis also has been described as the serious illness that can generated by a group of bacteria called Vibrio (Chen, Liu, & Lee, 2000). Vibrio have been reported as one of the prominently agents of diseases in aquatic organisms such as *V. alginoliticus* that have been used as probiotics for shrimp productions (Vandenberghe, Thompson, Gomez-Gil, & Swings, 2003). Aguirre-Guzmán, Ruíz, & Ascencio (2004) reported that the clinical signs of Vibriosis disease towards *Macrobrachium rosenbergii* similarly showed such as legathy, tissue and appendage necrosis, slow growth, slow metamorphosis, body malformation, bioluminescence, muscle opacity and melanisation. . Moreover, Vibriosis can be described as opportunistic pathogens because the host organisms is stress and immune suppressed to the more intensive culture and adverse environmental condition (Guillemot,

1999).Researchers stated that *Vibrio parahaemolyticus* in Mexico acts as primary pathogen for shrimp that can causes acute hepatopancreatic necrosis disease (AHPND) (Soto-Rodriguez, Gomez-Gil, Lozano-Olvera, Betancourt-Lozano, & Morales-Covarrubias, 2015). This showed that Vibriosis is an extremely disease problem in aquaculture industry that give economic damage to the farmers in production department.

Vibriosis are also universal and primary component of prawn culture environment that consists of a main part of the normal flora crustacean (D.V. Lightner & Redman, 1998;Ruangpan, Danayadol, Direkbusarakom, Siurairatana, & Flegel, 1999) that can cause serious diseases. Culture water is a dominant potential route for introducing pathogenic bacteria into shrimp hatcheries. Despite that, the sterility of rearing water is very crucial to achieve in laboratory environment and commercial shrimp hatcheries. Numerous of methods that can reduce the number of potential pathogens occurring in hatchery such as water supplies, antibiotic and drug resistance agents. Mainly, this disease caused 100% mortality within 2 days or days in freshwater prawn hatcheries and nursery pond in different parts of India (Sahul Hameed, Yoganandhan, Sri Widada, & Bonami, 2004).The grow-out farm production has been increase losses because of poor survival of the post larvae with a low-level asymptomatic infection that give severe economic losses.

The production cycle of giant freshwater prawn rely on disease causative agents, factor of environment and management practices of the hatchery operation. The major diseases of causative agents in infectious diseases of *M. rosenbergii* are fungi, bacteria, viruses and protozoans. However, the occurrence of viral and bacterial effect has been stated by Anderson, Shariff, & Nash (1990) showed that serious mortalities in *Macrobrachium rosenbergii* hatcheries caused by *Vibrio spp.* that can caused

luminescent bacterial disease. Thus, the pathogen can penetrate the hatchery system in various ways such as feed, brood stock, equipment, water and unhygienic handlings of workers. According to Olafsen (2001), a pathogen that related with the hatchery systems due to interaction pathogen on post larvae health development and disease outbreak. Furthermore, Vibrio spp. is the opportunistic pathogens that creating stresses disease in post larval population. Thus, the luminescent bacterial disease on post larval stages of *M. rosenbergii* found to be infected on body surface and internal organs. Outbreaks may exist when environment factors generate the rapid reproduction of bacteria already tolerated at low levels in shrimp blood (Davis & Sizemore, 1982). Besides, the strategy for disease management in prawn aquaculture is developing to find out the causative agents from diseased host.

The shrimp in a past few years has been great crisis problem because of the largely increase in virulence pathogens, especially bacteria diseases due to *Vibrio spp.* that caused white spot viruses. The virulent pathogens usually start appear when having the interactions of microbes, animals and their environment under the stress of commercial production, and the use of antimicrobial chemical (D.V. Lightner, 1996). Thus, one of the factors of the death causes for the mass mortality in the hatcheries from the infections caused by *Vibrio spp.* that lead to vibriosis disease. Other than that, luminous vibriosis was clearly observed in most of penaeid hatcheries that infected post larvae which was glow in the dark when infected (Karunasagar, Pai, Malathi, & Karunasagar, 1994). In shrimp industry, the contamination of bacteria that spread all the way from hatching to larval rearing tanks lead to serious problem for shrimp farmers. Generally, the lethal concentration that was calculated by the researchers at concentration of 10^2 cells/mL (D.V. Lightner, 1996).The other agents that causing

vibriosis in shrimp included *V. anguillarum, V. splendidas, V. parahaemolyticus, V. fluvialis, V. vulnificus and V. alginolyticus* (Mohney, Lightner, & Bell, 1994).

Moreover, the identification of Vibrio infection in shrimp can be examined on the merging of the clinical symptoms such as histological examination and bacteria culture (Flegel, 2012). According to Collins (2010), histological examination known as good implement to observe the proportion of tissue changes because of stress that changes due to the structural pathology that infected with disease. In additional, the rapid diagnostic technique for identification disease is known as immunohistochemistry. The immunohistochemistry technique enables to identify of tissue constituents by means of antigen-antibody reactions through changes of colour in prawn (Noraini et al., 2013). The both techniques are prominently in investigation of prawn disease to prevent the outbreak of disease in aquaculture industry. This technique can determine the distinct antigen of *V. alginoliticus* that able to identify using Davidson's fixative paraffin. This technique was reported by Alday-Sanz et al., (2002) that showed that the use of immunoperoxidase technique can determine the appearance of antigens *Vibrio Vulnificus* in the tissue of black tiger shrimp (*Penaeus monodon*). The used of immunohistochemistry can give a valuable understanding of the disease with the accurate result of diagnosis in giant freshwater prawns for effective disease control and efficient treatments for this opportunistic pathogens, *V. alginoliticus.*

2.3 Practices to reduce the Vibriosis disease

The giant freshwater prawn hatchery, the post larvae, water, brood stocks should were disinfected, and all tanks and equipment were sterilized to reduce this causative agents of disease. Moreover, Vibriosis is controlled by proper water management and sanitations to prevent the access of vibrio in culture water and to reduce stress on the prawns (D.V. Lightner, 1996).Other than that, antibiotics such as drug resistance showed high antimicrobial activities against the bacteria pathogen, *Vibrio alginoliticus* and could be used as an effective bacterial treatment in prawn cultures

CHAPTER 3

METHODOLOGY

Materials and methods

3.1.1 Material

Materials that used in this study were *Macrobrachium rosenbergii* post larvae (1cm-3cm), anti-chlorine, tap water, giant freshwater commercial prawn feed.

3.1.2 Apparatus and equipment

The apparatus and equipment that used were 15 units of aquarium glass tank (60 cm x 30 cm x30 cm) at a stocking density 70 post larvae per tank, 15 aerators, and green net as substrate and extension wire.

3.2 Methods

3.2.1 Preparation of aquarium tanks

The aquarium tanks were cleaned using the standard aseptic cleaning process for the experiment. Firstly, the aquarium tanks were rinsed and filled with the tap water and left overnight. After that, the aquarium tanks were cleaned with the Clorox to kill the bacteria and sprayed with the 70% of ethanol. On the next days, the aquarium tanks were rinsed thoroughly. 2/3 of the aquarium glass tanks were filled with the tap water with additional of anti-chlorine that functions to neutralize chlorine in the tap water. Aeration was supplied to each aquarium tank. Lastly, the green net were put as substrates in each aquarium tanks.

3.2.2 Sample preparation

A total of 1050 of *Macrobrachium rosenbergii* Post larvae were collected from hatchery in Pantai Remis, Setiawan at Perak. After collected, the *M. rosenbergii* PL were transferred in 15 aquarium tanks that prepared in aquaculture laboratory of Universiti Malaysia Kelantan Jeli, Campus. About 70 of *M. rosenbergii* Post larvae were transferred to each aquarium tanks. Every concentration of *Vibrio alginoliticus* were tested and 3 replications of each different treatments concentration. The aquarium tanks were labelled in 4 different concentrations of bacteria with three replications of each concentration such as Control (Control 1,Control 2,Control 3),Treatment 1 (V10

^{4}R1, V10^{4} R2, V10^{4} R3),Treatment 2 (V10^{5} R1, V10^{5} R2, V10^{5} R3),Treatment 3(V10^{6} R1, V10^{6} R2, V10^{6} R3) and Treatment 4 (V10^{7} R1, V10^{7} R2, V10^{7} R3).

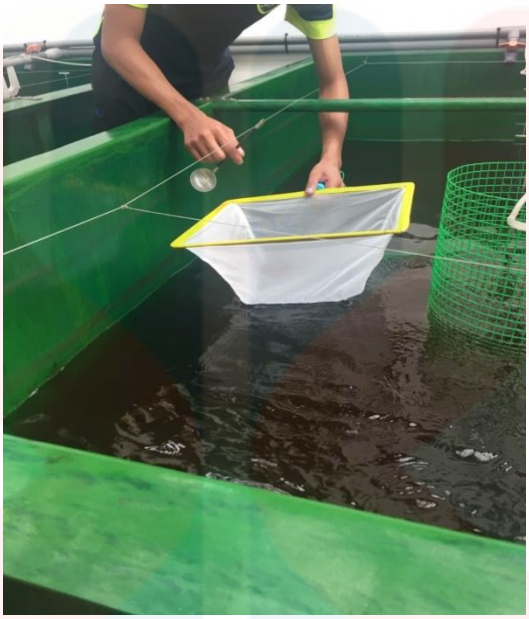

Figure 3.2.2: Process of harvesting the Post larvae of *Macrobrachium rosenbergii* using prawn filter were collected in Perak.

3.2.3 Preparation of bacteria culture in serial dilution

The bacterial stocks of *Vibrio alginoliticus* were collected from Faculty of Veterinary Medicine, Pengkalan Chepa, at UMK from the bacterial infections disease in fish. The *Vibrio alginoliticus* isolated from the fish was used in the pathogenicity test. The bacteria were grown on TSB for 24 hours at 30˚C in incubator shaker for the growth of bacteria. Then, broth cultures were centrifuged at 4000 rpm for 10 minutes at

4˚C and the bacterial pellets were washed twice with the tap water to collect the bacterial suspension. The pellet was resuspended in tap water and adjusted to an OD of 0.1 at 600nm, equivalent to 1.0 X 10^8 CFU/ml, from this concentration of 10^4, 10^5, $10^6$and 10^7 CFU/ml were prepared by serial dilution to conduct the pathogenicity. Bacterial concentration was estimated by optical density and using spectrophotometer. An OD value of 0.1 is known to be to be equivalent to 1.0x 10^8 CFU/ml.

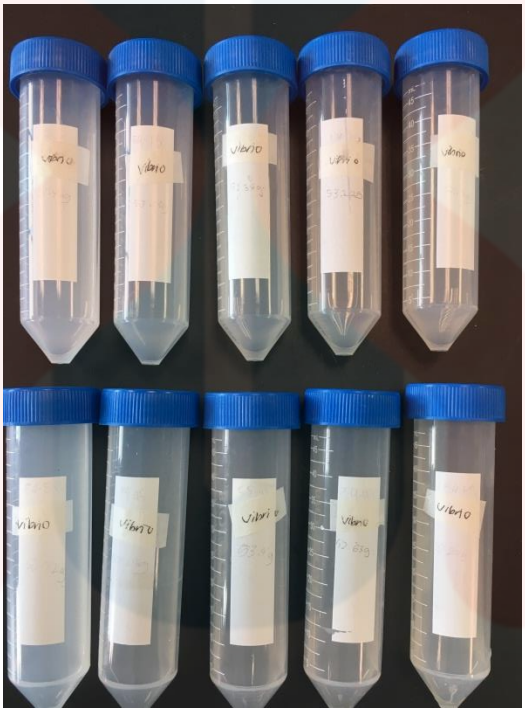

Figure 3.2.3: *Vibrio alginoliticus* bacteria suspended in tap water.

3.2.4 Challenge test

In this experiment, 70 of post larvae of *Macrobrachium rosenbergii* were used for each aquarium tanks that containing aerated, chlorinized and tap water with anti-chlorine. No water exchange during this pathogenicity test. The post larvae of *M. rosenbergii* were immersed in aquarium tanks containing *Vibrio alginoliticus* at the concentration of 10^4, 10^5, $10^6$and 10^7 CFU/ml. The post larvae of *M. rosenbergii* were fed twice a day with commercial freshwater prawn feed. During the experiment period, water temperature, pH, dissolved oxygen and ammonia concentration was maintained. Survival rates were determined during 4 days of post infection challenge. The post larvae *of M. rosenbergii* samples were processed for bacteriological examination after the immersion challenge. The mortality was determined after 3 hours exposure, 6 hours exposure, 12 hours exposure, 36 exposure, 48 hours exposure and 96 hours exposure of *Vibrio alginoliticus.*

Figure 3.2.4: Setting up of experiment with the tanks at different concentrations of *Vibrio alginoliticus*

3.2.5 Isolation and differentiation of bacteria

The dead post larvae of *Macrobrachium rosenbergii* were used to identify the presence of bacteria in the samples. Gram staining and O-F test were performed during the experiment. Gram staining was performed using crystal violet solution and O-F test were performed using O-F basal medium.

3.2.6 Statistical analysis

All the data were subjected to one-way ANOVA using spss V. 16 software (SPSS inc., Chicago, llinois, USA).The means mortality were compared between the concentration of bacteria and to find the difference at 5 % of ($p<0.05$) level of significance. P value of <0.05 was considered as statistically significant for all test at 95% confidence interval.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Clinical signs

The clinical symptoms that observed within the post-challenge infections in the *Macrobrachium rosenbergii* Post-Larvae showed mostly highly whitish muscles in the abdominal region. During this study, post larvae of *M. rosenbergii* colour could be observed, ranging from the normal transparent, faint white band to nearly complete white colouration across the entire abdomen (Fig 4.1).The first sign that showed of post larvae in this study was the poor feeding and lethargy of the giant freshwater prawn during the first 4 days of post infection challenge. Then, a few whitish appearances of the muscles in tail of PL were noticed 2-4 days after metamorphosis. The infected of post larvae *M. rosenbergii* also expressed other important clinical signs such as moulting obstacles, difficulties in eating, weak swimming and sinking to the bottom of the tank, and whitening of the hetopancreas that resulting in higher mortality. Generally, mortality increased day by day and reached up 100% within 4 days of post infection challenge after the presence of milky white in post larvae. This mortality happened because of the higher number of bacteria agents (*Vibrio alginoliticus*) that could cause infections and mortality in giant freshwater prawns. This study has shown that infected post larvae were started dying drastically in day 2-post infection. This bacterial

infection progressively destroyed the abdominal muscles of the post larvae *Macrobrachium rosenbergii* that leading to mortality. However, (D.V. Lightner & Redman, 1998) points out that *Vibrio* act as opportunistic or secondary pathogens that can cause mortality from a few to 100% in affected population under stress. Furthermore, *Vibrio alginoliticus* is primary bacteria agent in shell diseased shrimp that can damages the cells of the hetopancreas and makes them more susceptible to secondary infections. *Vibrio spp.* infections have been shown to be associated with spontaneous mortality among the cultural tiger prawn in Taiwan (Song, Cheng, & Wang, 1993). Furthermore, the disease sign observed due to experimental infection were similar to those of infected prawn in commercial farms that can cause high mortality and could gave losses to the farmers.

Figure 4.1: Infected post larvae of *Macrobrachium rosenbergii* were shown in whittish tails.

4.2 Post-infection challenge experiment

Figure 4.2.1 shows an overview of the time exposure between different treatments concentration of *Vibrio alginoliticus*. ANOVA test were used to analyse the relationship between time exposure and mortality of post larvae *Macrobrachium rosenbergii* in different concentration of *Vibrio alginoliticus*. On challenging the post larvae with *Vibrio alginoliticus* at $1.0x10^4$, $1.0x10^5$, $1.0x10^6$, $1.0x10^7$ CFU/mL in three replicate, the mean mortality of 8.22 (±11.25), 9.89 (±11.01), 9.17 (±12.71) and 10.72 (±12.84), were observed respectively within 96 hours of post infection. In the control group, the corresponding mean mortality was 8.11 (±13.83).The ANOVA test showed that the mean mortality was significantly higher in Treatment 4 ($1.0x10^7$ CFU/mL) compare to other treatments and control group.

However, the findings of the current study do not support the previous research. Previous research stated that the highest mortality usually occurs in the seven days of post infection of Vibrio bacteria which was the mortality rate of this disease ranges from 80% to 90% (Pan et al., 2016). Moreover, this mortality happened due to researchers found that main pathogen in prawn that transmitted to sensitive host by digestive system that located in the gut and produced the toxin that cause declination of hepatopancreas function (Zorriehzahra, 2015).

This present of study showed that the mortality was significantly higher ($p<0.05$) in different treatments and control. What is interesting in this data is that this result was significant at the $p=0.00$ level ($p<0.05$). Based on the observation during the experiment, the *Vibrio alginoliticus* were introduce the signs of post larvae death in *Macrobrachium rosenbergii* such as anorexia, weak swimming and settling to the bottom of the tank. There was a significant difference between the mortality and time of exposure of *Vibrio alginoliticus* bacteria with different concentration of bacteria.

From Figure 4.2.1, obviously, the control group post larvae of *Macrobrachium rosenbergii* that exposed to *Vibrio alginoliticus* displayed a significantly higher mean mortality which was 33.33±4.62 at 36 hours post-exposure. In treatments with $1.0x10^4$, $1.0x10^5$, $1.0x10^6$, $1.0x10^7$ CFU/mL, the mean mortality was 21 (± 8.66),30 (±11.36),27.33 (±2.08), 27.67 (±3.21) respectively. According to Sindermann (1979), the commonly mortality of *Macrobrachium rosenbergii* that happened in the species due to stressful environmental factors such as low oxygen and inconstancy temperature. Therefore, the extraordinary higher stocking density of the post larvae *Macrobrachium rosenbergii* that observed in this study may be dominant risk factors of the higher mortalities. Other than that, mortality recorded among the control groups it was apparently not because of the pathogenicity Vibrio *alginoliticus*, but because of moulting and related cannibalism. This finding was supported Alday-Sanz et al.,(2002) who showed that crustacean, when exposed to ammonia prior to an immersion challenge with Vibrio, suffered more frequent and earlier pathological changes than prawns only exposed with the bacteria only . Besides, ammonia can be found in post larvae feed and become toxic to the post larvae that leading to higher mortality.

However, this figure also showed there is a clear trend of decreasing of the mean mortality of post larvae *Macrobrachium rosenbergii* among the control and other treatments at 48 hours exposure and 96 hours exposure with different concentration *Vibrio alginoliticus* .At 96 hours post-exposure, Treatments 2 ($1.0x10^5$ CFU/mL) were presented a significantly higher mortality which was 5.33 (±4.04) compared other treatments. In treatments with $1.0x10^6$, $1.0x10^7$, $1.0x10^4$ CFU/mL and control, the mean mortality was 5.33 (±4.04), 0.67 (±0.58), 1.67 (±1.15) and 1.00 (± 1.00) respectively. The significantly higher mortality rate observed at 36 hours post exposure of bacteria in

the Treatments 2 ($1.0x10^5$ CFU/mL) that determined the pathogenicity of *Vibrio alginoliticus* to the post larvae *Macrobrachium rosenbergii.*

However, this concentration of *Vibrio alginoliticus* does not significant indicate the main pathogen that responsible for the degeneration of hetopancreas that leading to higher mortalities. Surprisingly, Hameed (1995),Prayitno & Latchford (1995), Harris & Owens (1999) and Saulnier et al., (2000) have reported that virulence of Vibrio species can be correlated with *M. rosenbergii* are the route of infection and specific host factors such as species, age and physiological state. Generally, the infected *M. rosenbergii* showed less feeding, lethargic and initiated opaqueness in the abdomen and leading to a milky white appearance that has been observed during the 4 days post infection.

The relative mortality rates of post larvae *Macrobrachium rosenbergii* is not associated with the time exposure of bacteria that lead to standard error in this experiment. The standard error that happened during the study was natural mortality and climate change. This error was determined by weather such as changes of temperature which could affect the mortality of post larvae *Macrobrachium rosenbergii*. There had been strong thunderstorm and raining heavily at 36 hours of exposure of bacteria. Generally, in most crustacean species, cannibalism becomes a major problem in higher mortality of post larvae *Macrobrachium rosenbergii* to fight the space and food source in the aquarium tank. Lastly, the stocking density may influence the mortality of post larvae *Macrobrachium rosenbergii* due to stress environment in the aquarium tanks.

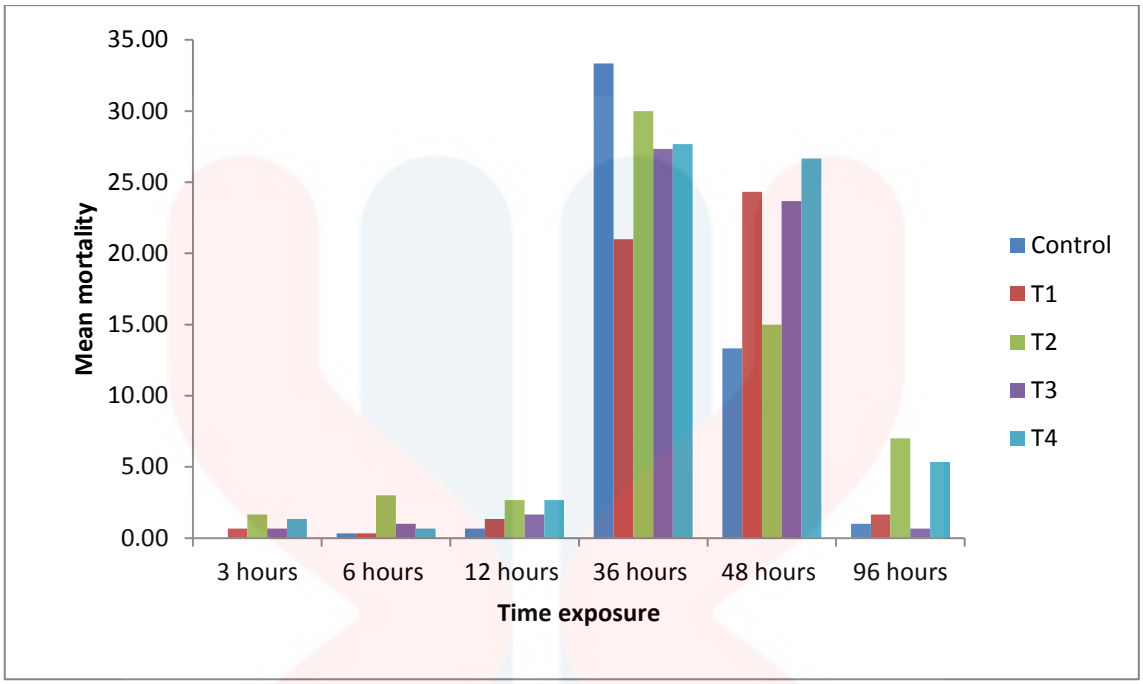

Figure 4.2.1: The relationship between the time exposure and mean mortality with different treatments.

As shown in Table 4.2.2, there was a significant positive correlation between the different concentrations of *Vibrio alginoliticus* in post larvae *Macrobrachium rosenbergii*. As the results of laboratory test revealed that the pathogenicity *Vibrio alginoliticus* towards *M. rosenbergii* post larvae during 4 hours post infection could give the higher mortality. Interestingly, positive correlation was found between Control and Treatment 1,Treatment 2,Treatment 3 ,Treatment 4 showed that this result highly significant at the ($p<0.001$). From this data in Table 4.2.2, it summarised that positive correlation was found between Treatment 1 and Treatment2,Treatment 3,Treatment 4 which was highly significant at the ($p<0.001$). Furthermore, it presented that positive correlation was determined between Treatment 2 with Treatment 3 and Treatment 4 was high significant at the ($p<0.001$).Lastly, it analysed that positive correlation happened between Treatment 3 and Treatment 4 in highly significant at the ($p<0.001$).

Table 4.2.2: Correlation analysis to reveal the relationship between different concentrations of *Vibrio alginoliticus* .

	Control	*T1*	*T2*	*T3*	*T4*
Control	1.00				
T1	0.72 ***	1.00			
T2	0.88 ***	0.78 ***	1.00		
T3	0.88 ***	0.94 ***	0.81 ***	1.00	
T4	0.86 ***	0.88 ***	0.75 ***	0.96 ***	1.00

*p <0.05, ** p<0.01, ***p<0.001

T1: $1.0x10^4$ CFU/mL, T2: $1.0x10^5$ CFU/mL, T3: $1.0x10^6$ CFU/mL, T4: $1.0x10^7$ CFU/ML

4.3 LC 50

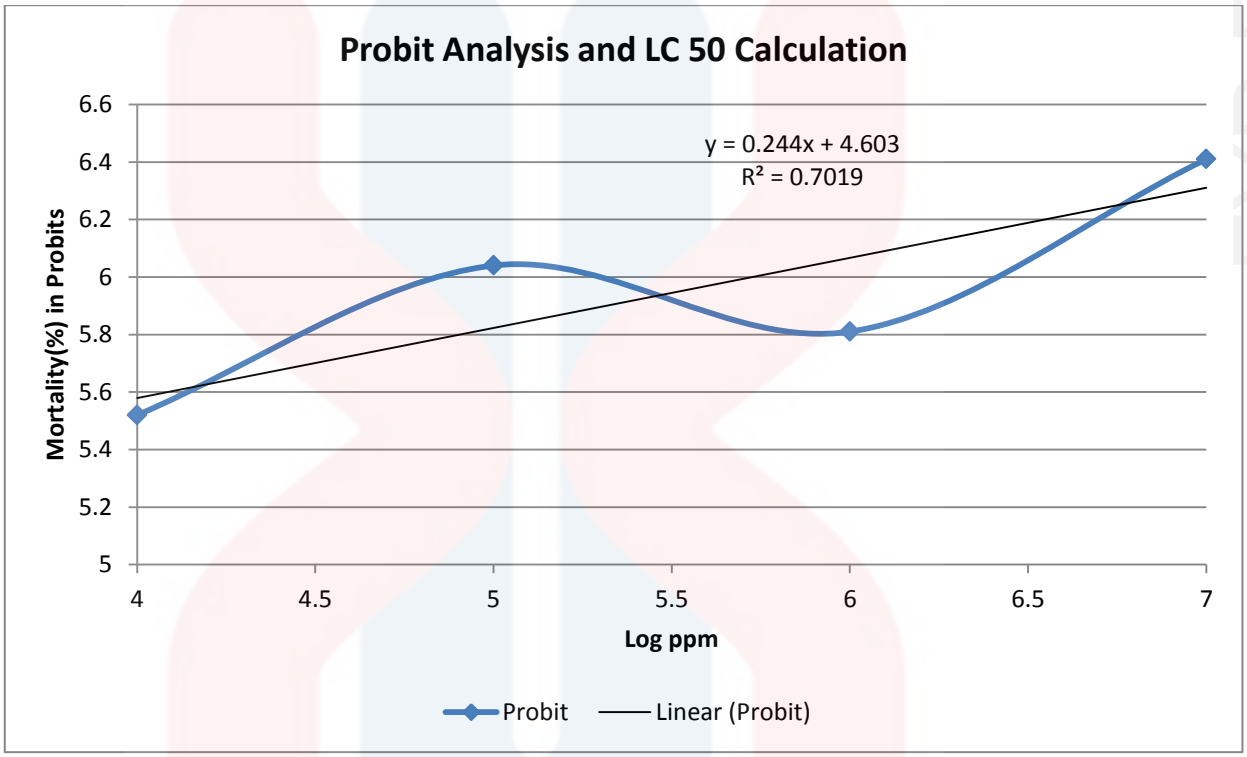

Figure 4.3: Determination of LC 50 using Probit analysis with different concentration of bacteria without control

	Coefficients
Intercept	4.603
log(ppm)	0.244

Equation y=ax+b

Y=0.24x+(4.60)

5=0.24x+4.60

5-4.60=0.24x

x=(5-4.60)/0.24

x=1.67

LC 50=antilog x

LC 50=antilog

1.67 46.77351413

LC 50=46.77 0.47

LC 50 =1.0 x10^3 CFU/ML

The Probit analysis determines an LC 50 value in this experiment was 1.0x10^3 CFU/mL (Figure 4.2) that give 50% of mortality of *Macrobrachium rosenbergii* Post larvae. The LC 50 value was found in the lower concentration because the *Vibrio alginoliticus* infection occurred very quickly in immersion methods. The virulence of a pathogen can be estimated from experimental studies of the LC 50 (median concentration) which is the amount of pathogens required to kill 50% at uniformly susceptible animal inoculated with the pathogens. The present of study showed that the information on LC 50 of a pathogen to a specific *M. rosenbergii* was very important for any type of research related to the interactions of that pathogen in *M. rosenbergiii* post larvae. However, this result has not previously been described by previous research.

This is because the previous research commonly used lethal dose (LD 50) to test the pathogenicity of the bacteria. For example, Abdolnabi, Ina-Salwany, Daud, Mariana, & Abdelhadi (2015) were determined to be $1.0x10^6$ CFU/50µl that lead to 50% mortality in the experiment and $1.0x10^7$ CFU/50µl that lead to 100% mortality in the experimentally injected prawn. According to Islam, Mostafa, & Rashid (2013),LD 50 was calculated to be $9.6x10^6$ CFU/fish. However, mortality was found with the doses between 10^6 and 10^8 CFU/fish.

CHAPTER 5

CONCLUSION

5.1 Conclusion

Based on results of this experiment, it is concluded that the different concentration of *Vibrio alginoliticus* was highly significantly among the mortality of post larvae of *Macrobrachium rosenbergii*. There was a significant positive correlation between different concentrations of *Vibrio alginoliticus* and mortality *Macrobrachium rosenbergii* post larvae. This study has found that generally Treatment 4 (1.0×10^7 CFU/mL) showed the highest mortality that exceed LC 50 value which was required 50 % of mortality in post larvae *Macrobrachium rosenbergii* which was at the concentration (1.0×10^3 CFU/mL) of *Vibrio alginoliticus*. The evidence from this study suggest that the lower concentration of bacteria *Macrobrachium rosenbergii* which was (1.0×10^3 CFU/mL) give the best value of LC 50 to the mortality of post larvae *Macrobrachium rosenbergii* in 4 days of post infection challenge.

5.2 Recommendation

This is the preliminary study focused on the mortality in post larvae of *Macrobrachium rosenbergii* at different concentration of *Vibrio alginoliticus* bacteria at concentrations of $1.0x10^4$, $1.0x10^5$, $1.0x10^6$, $1.0x10^7$ CFU/mL. Future research might explore the relationship between different types of bacteria in same group species such as *Vibrio cholera, Vibrio carchariae and Vibrio mimicus* at different concentration of bacteria that give mortalities of post larvae *Macrobrachium rosenbergii*. These types of bacteria will directly affect the *M. rosenbergii* such as anorexia and slow growth. Furthermore, future research needs to examine more closely the links between the time exposure of post infection of bacteria and mortality of post larvae *Macrobrachium rosenbergii* at different concentration bacteria *Vibrio alginoliticus*. To improve this research, the lower concentrations of bacteria ($1.0 \text{ x}10^1$ CFU/mL) must be prepared to know the accurate value of LC 50 and pathogenicity of *Vibrio alginoliticus* on *Macrobrachium rosenbergii* post larvae. Future research might investigate the methods of isolation, identification and characterization of *Vibrio alginoliticus* from the infected giant freshwater prawns in this experiment to verify and evaluate the characteristics of these bacteria such as O-F test, 0/129 antibiotics sensitivity test and oxidase test.

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APPENDIX A

Table A.1: Mortality of *Macrobrachium rosenbergii* postlarvae (PL) that challenged by isolates prepared bacteria *Vibrio alginoliticus*.

Experimental groups(PL) (n=70)	Hours of post infection						Cumulative mortality (%)
	3 hours exposure	6 hours exposure	12 hours exposure	36 hours exposure	48 hours exposure	96 hours exposure	
Control 1	0	1	2	28	19	2	52
Control 2	0	0	0	36	7	0	43
Control 3	0	0	0	36	14	1	51
T1 R1 (1.0x10^4 CFU/mL	0	0	1	26	21	1	49
T1 R2	1	0	1	11	32	1	46
T1 R3	1	1	2	26	20	3	53
T2 R1 (1.0x10^5 CFU/mL)	3	8	3	22	19	7	62
T2 R2	1	0	3	25	22	6	57
T2 R3	1	1	2	43	4	8	59
T3 R1 (1.0x10^6 CFU/mL)	1	1	2	28	20	1	53
T3 R2	0	2	2	25	25	0	54
T3 R3	1	0	1	29	26	1	58
T4 R1 (1.0x10^7 CFU/mL)	1	1	3	29	28	1	63
T4 R2	2	0	1	30	23	9	65
T4 R3	1	1	4	24	29	6	65

Table A.2: Mean mortality (± SD) of post larvae *Macrobrachium rosenbergii* that exposed different concentration of treatments *Vibrio alginoliticus* in 4 days of post infection.

Time exposure	Treatment				
	Control	T1	T2	T3	T4
3 hours exposure	0 ± 0	0.67 ± 0.58	1.67 ± 1.15	0.67 ± 0.58	1.33 ± 0.58
6 hours exposure	0.33 ± 0.58	0.33 ± 0.58	3.00 ± 4.36	1.00 ± 1.00	0.67 ± 0.58
12 hours exposure	0.67 ± 1.15	1.33 ± 0.58	2.67 ± 0.58	1.67 ± 0.58	2.67 ± 1.53
36 hours exposure	33.33 ± 4.62	21.00 ± 8.66	30.00 ± 11.36	27.33 ± 2.08	27.67 ± 3.21
48 hours exposure	13.33 ± 6.03	24.33 ± 6.66	15.00 ± 9.64	23.67 ± 3.21	26.67 ± 3.21
96 hours exposure	1.00 ± 1.00	1.67 ± 1.15	7.00 ± 1.00	0.67 ± 0.58	5.33 ± 4.04
Average	8.11	8.22	9.89	9.17	10.72
P-value	0.00	0.00	0.00	0.00	0.00

Table A.3: Probit analysis data and LC 50 calculation.

Concentration	Concentration (ppm)	log(ppm)	Probit	% Dead	% Mortality	Total
1.0x10^4	10000	4	5.52	70	148	210
1.0x10^5	100000	5	6.04	85	178	210
1.0x10^6	1000000	6	5.81	79	165	210
1.0x10^7	10000000	7	6.41	92	193	210

Table A.4: Transformation of percentages to probits.

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

APPENDIX B

Figure B.1 : $V10^4$ R1 ($1.0x10^4$CFU/mL)

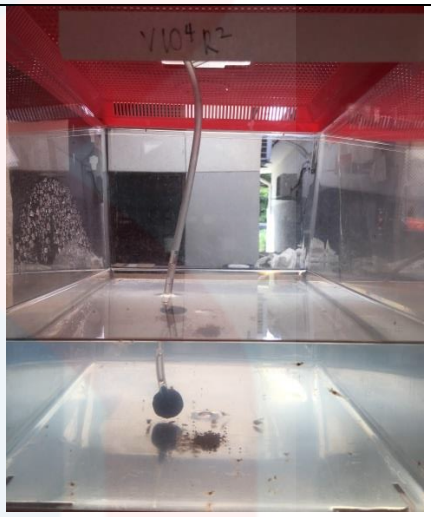

Figure B.2 : $V10^4$ R2 ($1.0x10^4$CFU/mL)

Figure B.3 : $V10^4$ R3 ($1.0x10^4$CFU/mL)

Figure B.4 : $V10^5$ R1 ($1.0x10^5$CFU/mL)

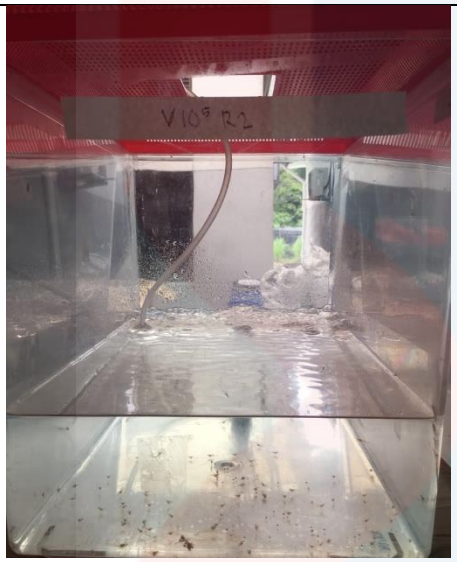

Figure B.5 : $V10^5$ R2 ($1.0x10^5$CFU/mL)

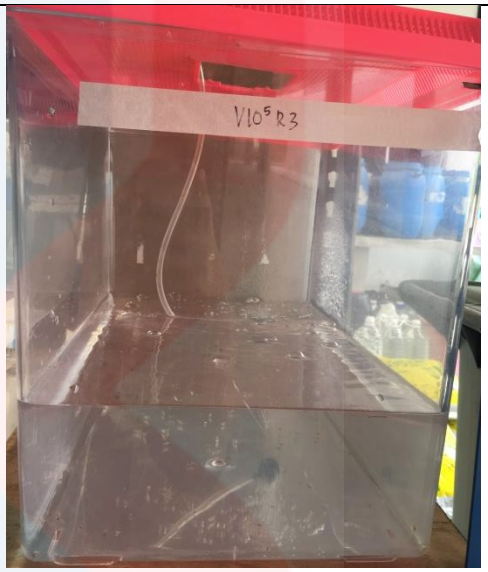

Figure B.6 : $V10^5$ R3 ($1.0x10^5$CFU/mL)

Figure B.7 : $V10^6$ R1 ($1.0x10^6$CFU/mL)

Figure B.8 : $V10^6$ R2 ($1.0x10^6$CFU/mL)

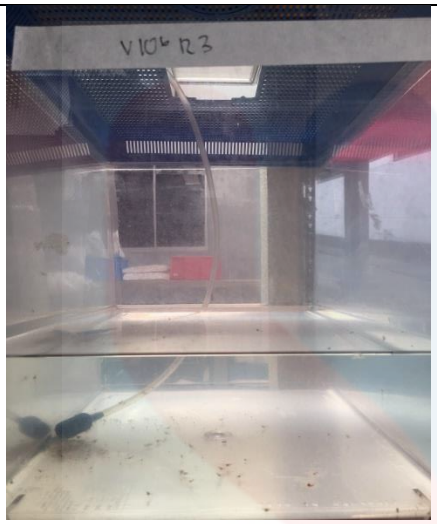

Figure B.9 : $V10^6$ R3 ($1.0x10^6$CFU/mL)

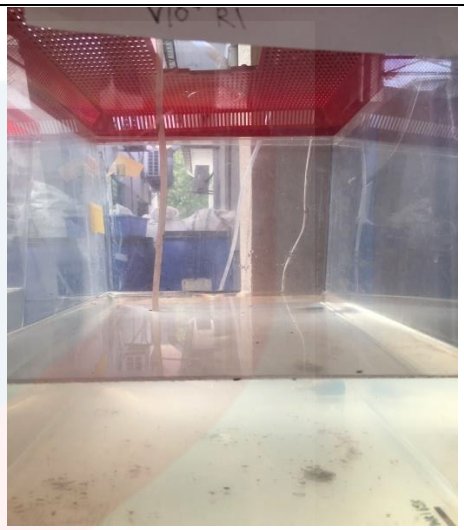

Figure B.10 : $V10^7$ R1 ($1.0x10^7$CFU/mL)

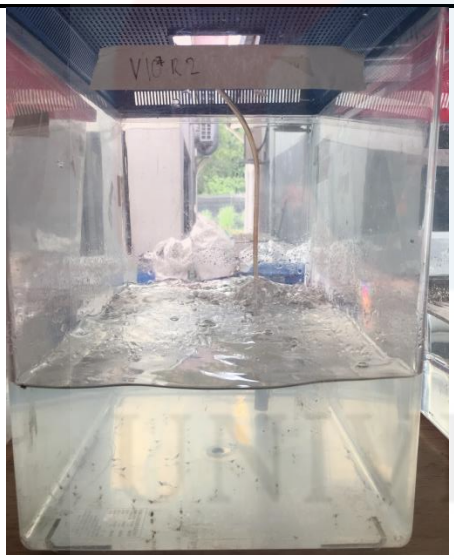

Figure B.11 : $V10^7$ R2 ($1.0x10^7$CFU/mL)

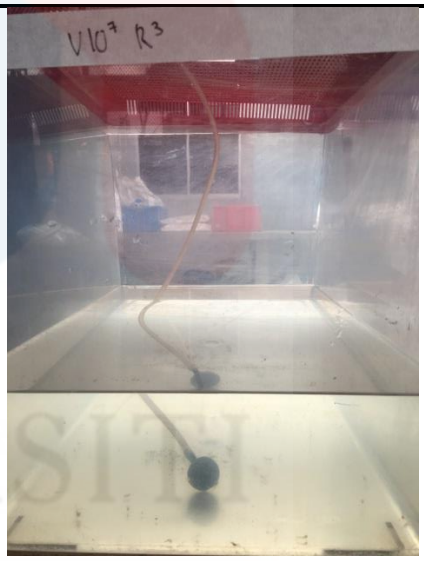

Figure B.12 : $V10^7$ R3 ($1.0x10^7$CFU/mL)

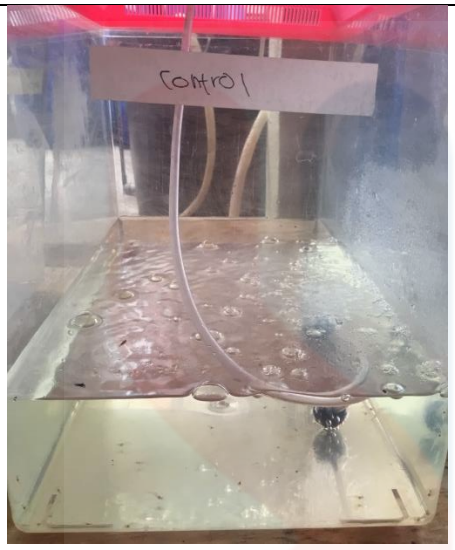

Figure B.13 : Control 1

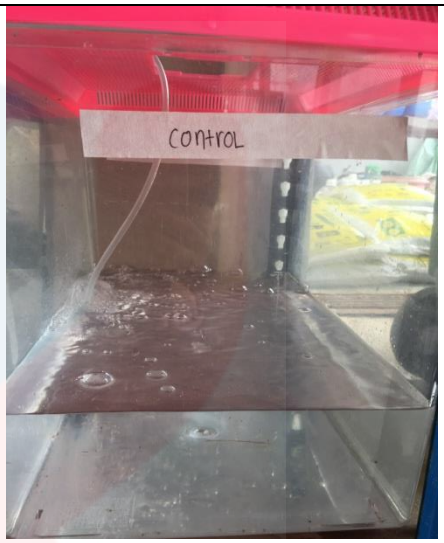

Figure B.14 : Control 2

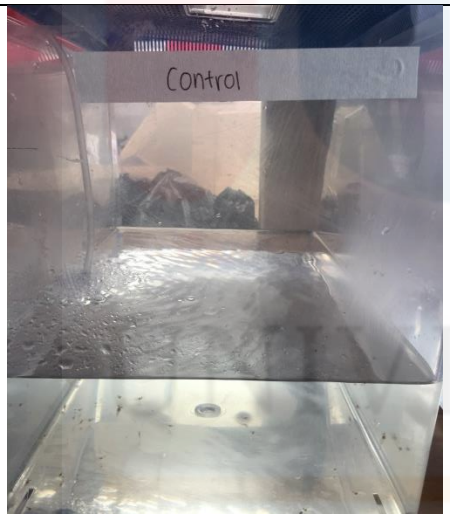

Figure B.15 : Control 3

Figure B.16 : The aquarium tanks