EXPRESSION OF STRESS RELATED GENES OF *Penaeus varnamei* IN BIOFLOC CLOSED BREEDING SYSTEM

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FACULTY OF SCIENCE
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EXPRESSION OF STRESS RELATED GENES OF *Penaeus varnamei* IN BIOFLOC CLOSED BREEDING SYSTEM

ABSTRACT

The Pacific shrimp *Penaeus vannamei*, which is also known as the whiteleg shrimp, is fast becoming a key species of shrimp farming worldwide since this species has been cultured widely in many countries, such as Mexico, America, especially in Southeast Asian countries including Thailand, Vietnam, and Malaysia. In addition, the shrimp aquaculture industry has applied many new cultural techniques, especially biofloc technology – which is an eco-friendly culture technique and has many advantages over other traditional methods, such as high productivity, the stable environment of the culture, and low levels of feed conversion. Unfortunately, for so many years, shrimp farming has been constantly hounded by stress, which is caused by several factors of the culture environment including the temperature of the water, dissolved oxygen, salinity, and pH. Stress can give negative effects on cultured shrimps, such as slow growth or high mortality, subsequently economic losses. Therefore, it is important to identify whether shrimp farming using biofloc technology causes any stresses to the Pacific white shrimp. It has been investigated that when the shrimps being stressed, they can generate various physiological as well as genomic reactions to modify the mechanism of compensation or adaptation, thus allowing the mitigation of stressors as well as the maintenance of their hemostasis that help them to survive under the pressure of stress. Therefore, environmental stressors can be determined by detecting those genes associated with the stress response. In the current study, three different sets of primers were used for the amplification of catalase, Na^+-K^+ATPase α-subunit, and heat shock protein 70 – these are the genes that respond to hypoxia stress, salinity stress, and heat shock stress, respectively. Sequences of those primers that were used for the amplification of catalase and Na^+-K^+ATPase α-subunit were designed according to the published mRNA

sequences of *L. vannamemi* available, while sequences of the primers used for amplification of heat shock protein 70 were retrieved from literature. qPCR was then applied for determining the expression of those three genes in the shrimps which were reared in four different biofloc culture conditions over a six-week period. The four conditions included microbubble aeration, a mixture of microbubble and macro bubble, macro bubble with bioballs, and macro bubble without bioballs. The up-regulated of catalase expression was found in the condition of microbubble and the condition of macro bubble with bioballs during the last two weeks of the cultivation and in the condition of macro bubble without bioballs in the 3rd, 4th, and 5th week of the cultivation. Meanwhile, the expression of Na^+-K^+-ATPase α-subunit was up-regulated in the condition of microbubble during the last two weeks of the cultivation, and in the condition of macro bubble with bioballs at the end of the cultivation. Lastly, the up-regulated of hsp70 was found in all four conditions throughout the cultivation. A negative correlation was found between temperature and hsp70 ($n = 3$; $R^2 = 0.454$; $p < 0.05$). These findings make catalase, Na^+-K^+-ATPase α-subunit, and hsp70 genes a suitable predictive biomarker for penaeid shrimps.

Keywords: Stresses, Catalase, Na^+-K^+-ATPase α-subunit, Hsp70, White shrimp.

UNGKAPAN GEN BERKAIT TEKANAN PADA *Penaeus varnamei* DALAM SISTEM PEMBIAKBAKAAN TERTUTUP *BIOFLOC*

ABSTRAK

Udang Pasifik *Penaeus vannamei*, yang juga dikenali sebagai udang putih, dengan pantas menjadi salah satu spesies terpenting dalam penternakan udang di seluruh dunia. Spesies ini telah diternak di pelbagai negara, contohnya Mexico, Amerika, dan terutamanya di negara-negara Asia Tenggara termasuk Thailand, Vietnam, dan Malaysia. Tambahan lagi, pelbagai teknik baru telah digunakan dalam industri akuakultur udang, termasuk teknologi *biofloc* – teknik penternakan yang mesra alam dan mempunyai pelbagai kelebihan berbanding kaedah tradisional yang lain. Antara kelebihannya adalah produktiviti yang tinggi, persekitaran penternakan yang stabil, dan nisbah penukaran makanan yang rendah. Malangnya, penternakan udang telah lama berhadapan dengan masalah tekanan yang disebabkan oleh faktor-faktor persekitaran seperti suhu air, kadar oksigen terlarut, kadar saliniti, dan pH. Tekanan boleh memberi kesan negatif kepada penternakan udang yang mengakibatkan pertumbuhan terbantut dan kadar mortaliti yang tinggi, dan seterusnya kerugian ekonomi. Oleh itu, adalah penting bagi mengenal pasti sama ada penggunaan teknologi *biofloc* dalam penternakan udang dapat memberi sebarang tekanan atau tidak kepada udang putih Pasifik. Kajian telah membuktikan bahawa apabila udang berada dalam tekanan, udang akan menunjukkan pelbagai reaksi fisiologi dan juga reaksi genomik untuk mengubahsuai mekanisma kompensatori atau adaptif, justeru membolehkan tekanan tersebut dapat dikurangkan dan juga memastikan kadar hemostasis dapat dikekalkan bagi membantu mereka untuk bertahan di bawah keadaan tekanan. Oleh itu, tekanan persekitaran boleh ditentukan dengan mengesan gen-gen yang berkait dengan gerak balas tekanan. Dalam kajian ini, tiga set pencetus yang berbeza telah digunakan untuk mengamplifikasi *catalase*, *Na^+-K^+-ATPase α-subunit*, dan *heat shock protein 70* – gen-gen ini masing-masing adalah gen yang bergerak balas

dengan tekanan hipoksia, tekanan saliniti, dan tekanan kejutan suhu. Jujukan bagi pencetus-pencetus yang digunakan untuk mengamplifikasi *catalase* dan *Na^+-K^+-ATPase α-subunit* telah dibangunkan berdasarkan jujukan mRNA udang putih yang telah sedia ada diterbitkan, manakala jujukan bagi pencetus yang digunakan untuk mengamplifikasi *heat shock protein 70* diperoleh melalui dapatan literatur dari kajian terdahulu. qPCR kemudiannya digunakan untuk menentukan kadar ungkapan ketiga-tiga gen tersebut pada udang putih yang diternak dalam sistem *biofloc* dengan empat keadaan berbeza dalam tempoh enam minggu. Empat keadaan tersebut terdiri daripada pengudaraan gelembung mikro, campuran gelembung mikro dan gelembung makro, gelembung makro dengan *bioballs*, dan gelembung makro tanpa *bioballs*. Ungkapan *catalase* pengawalaturan tinggi ditunjukkan dalam keadaan pengudaraan gelembung mikro dan keadaan gelembung makro dengan *bioballs* pada dua minggu terakhir penternakan, dan dalam keadaan gelembung makro tanpa *bioballs* pada minggu ke-3, ke-4, dan ke-5 penternakan. Sementara itu, ungkapan *Na^+-K^+-ATPase α-subunit* mengalami pengawalaturan tinggi dalam keadaan pengudaraan gelembung mikro pada dua minggu terakhir penternakan, dan dalam keadaan gelembung makro dengan *bioballs* pada akhir penternakan. Akhir sekali, pengawalaturan tinggi pada ungkapan *hsp70* dilihat dalam kesemua empat keadaan sepanjang penternakan. Hubungan korelasi negatif ditunjukkan antara suhu air dan ungkapan *hsp70* ($n = 3$; $R^2 = 0.454$; $p < 0.05$). Hasil kajian ini membuktikan gen *catalase*, *Na^+-K^+-ATPase α-subunit*, dan *hsp70* sesuai dijadikan penanda biologi ramalan bagi udang penaeid.

Kata kunci: Tekanan, *Catalase*, *Na^+-K^+-ATPase α-subunit*, *Hsp70*, Udang putih.

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Thank you

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%	:	Percent
^{o}C	:	Degree Celsius
α	:	Alpha
µg	:	Microgram
µl	:	Microliter
µM	:	Micromolar
≤	:	Less than or Equal to
®	:	Registered Sign
A_{260}/A_{280}	:	The ratio of UV Absorbance at 260 nm and 280 nm
BFT		Biofloc Technology
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base Pairs
CAT	:	Catalase
cDNA	:	Complementary DNA
DNA	:	Deoxyribonucleic Acid
dNTP	:	Deoxyribonucleoside Triphosphate
EF-1	:	Elongation Factor 1
FCR		Feed Conversion Rate
gDNA	:	Genomic DNA
GPX	:	Glutathione Peroxidases
GR	:	Glutathione Reductase
GSase	:	Glutamine Synthetase
hsp70	:	Heat Shock Protein 70
L	:	Liter

L. vannamei	:	*Litopenaeus vannamei*
mA	:	Milliampere
mg	:	Milligram
mg L^{-1}	:	Milligrams Per Litre
$MgCl_2$	:	Magnesium Chloride
MI	:	Myo-inositol
min	:	Minute
ml	:	Mililitre
mM	:	Milimolar
mRNA	:	Messenger RNA
NCBI	:	National Center of Biological Information
nm	:	Nanometer
NOS	:	Nitric Oxide Synthase
P. vannamei	:	*Penaeus vannamei*
PCR	:	Polymerase Chain Reaction
pH	:	Power of Hydrogen
ppt	:	Parts Per Thousand
ppt	:	Practical Salinity Units
qPCR	:	Quantitative Polymerase Chain Reaction
RNA	:	Ribonucleic Acid
RNase	:	Ribonuclease
RNS	:	Reactive Nitrogen Species
ROS	:	Reactive Oxygen Species
RT	:	Reverse Transcriptase
s	:	Second
SOD	:	Superoxide Dismutase

TGase	:	Transglutaminase
T_m	:	Melting Temperature
UV	:	Ultraviolet
V	:	Voltage
w/v	:	Weight/Volume
WSSV	:	White Spot Syndrome Virus
xg	:	Gravitational Force

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CHAPTER 1: INTRODUCTION

1.1 Study's Current Background

The human population is growing rapidly. In 2017, the total population of the world was estimated at approximately 7.6 billion, indicating that about one billion people were added to the world over the last dozen years. In addition, the prediction of the global population suggests that, by 2030, the total number of the population of the word will exceed eight and a half billion and will continue to increase to approximately 10 billion by 2050 (United Nations, 2017).

The expansion of the global population has led to an increase in human food consumption. Particularly, over the past few decades, the global consumption of seafood has significantly increased. According to previous reports, in 2013, the average consumption of seafood globally was estimated at approximately 19.7 kg per capita (Gephart, 2019). This significant increase in global seafood demand has led to rapid growth in aquaculture, especially in marine shrimp farming. Shrimps have been investigated to be a high-value seafood commodity. Therefore, regarding the production value, shrimp farming has become one of the main and significant commercial activities in aquaculture (Khatoon et al., 2016). The increase in the cultural production of shrimp during the past decade is noticeable. The global production of shrimp farming has increased by 25 percent since 1994 (Ackefors, 2009). In particular, the production of farmed shrimp worldwide in 2017 was estimated at about 2.9 to 3.5 million tonnes, according to the FAO report (FAO, 2018).

There are less than 300 species of shrimp which are of economic interest worldwide (Gillett, 2008). In addition, a numerous number of tropical shrimp species in the family Penaeidae have been investigated to be now on the world market in most countries. Many

species in this family, including *Penaeus monodon* – the black tiger shrimp; *P. chinensis* – the fleshly shrimp and *P. vannamei* – the Pacific white shrimp, is the dominant farmed species and are cultivated in large quantities globally (Ackefors, 2009).

Among these various types of shrimps, *Penaeus vannamei* (synonym: *Litopenaeus vannamei*), which is also referred to as the whiteleg shrimp or the Pacific white shrimp and is naturally spread across America's Pacific coast (Gao et al., 2016), has become one of the most significant farmed shrimp species in the world. *P. vannamei* has been cultured widely in many countries, such as Ecuador, Mexico (Cuzon et al., 2004), America, China, Thailand (Liu et al., 2016). This is because this species has several typical characteristics that can create value in the farmed shrimp industry. Firstly, it can grow rapidly. Secondly, it has a high rate of survival. Lastly, it has the ability to tolerate a wide range of diseases that occur commonly in farmed shrimp (Khatoon et al., 2016).

However, the development of aquaculture in general and of shrimp farming, in particular, has caused several problems and has impacted the environment. The release of wastewater and the feed cost of shrimp are some of the major challenges. Culture environment's quality in conventional shrimp farming is measured by the continuous exchange of water in the ponds during the shrimp-fattening period (Santaella et al., 2018). The continuous renewal of freshwater will help to adjust the level of salinity and nitrogen in the culture water. In the shrimp farming of many Asian countries, the amount of freshwater required for the control of water quality is estimated at 5.3 million gallons per acre per year. In addition, each acre produces 3 tons of solid waste per year (Cheal et al., 2017). Studies have shown that outflowing water – which was generated in the harvesting process – contains a great number of nutrients, suspended solids, organic matter, salts, microorganisms, and other chemical substances. Releasing this wastewater into the surrounding waters without any prior treatments will significantly damage the

environment since it causes water pollution, including eutrophication, oxygen depletion, and sedimentation. Meanwhile, the economic viability of shrimp farming is affected by feed cost. It has been investigated that the use of feeds accounts for up to 50% of the total cost of production. Therefore, finding an ideal technique that can solve both problems – a negative impact on the environment and economic concerns – is very important (Santaella et al., 2018). An ideal alternative is not only one that helps in enhancing water quality but also one that helps in reducing the cost for sustainable farming (Khatoon et al., 2016).

Closed aquaculture systems have been proven to have several advantages over current conventional systems such as extensive and semi-intensive systems. These advantages are biosecurity, environmental, and marketing advantages. This causes an increase in interest in this aquaculture system. There have been lots of studies regarding the reuse of water culture. The results suggested that the reuse of water will help to reduce or even eliminate a wide range of risks, including the introduction of pathogens, the escape of exotic species, and the release of wastewater. In addition, since the productivity is high, and the use of water is reduced, there is a possibility of using inland areas to raise aquatic animals (Emerenciano et al., 2013).

Studies have shown that biofloc technology can be used as an efficient alternative solution to the above problems since organic matter and nutrients that present in culture water are converted into bioflocs and can be consumed back by the shrimps. This continuously recycled and reused of nutrient (Emerenciano et al., 2013) will help to reduce or totally replace the use of commercial feeds (Santaella et al., 2018). Therefore, recently, biofloc technology – which is an eco-friendly culture technique – has become more popular and widely used in the farming of shrimp, especially in Pacific white shrimp farming. This technique produces a wide range of benefits. The first benefit is the high

levels of productivity. The second benefit is the low levels of feed conversion. And lastly, the ability to maintain a steady environment for the culture (Taw, 2010).

On the other hand, one of the major issues that are commonly found in shrimp farming is stress. Some common stresses are hypoxia stress, salinity stress, pH stress, heat stress, and oxidative stress. They are caused by several variables of the environment, including levels of dissolved oxygen, pH, salinity, and temperature of water (de la Vega et al., 2007). Stresses can give negative effects on shrimp farming, including slow growth, high mortality and all of these will result in economic losses (Gao et al., 2016).

1.2 Problem Statement

Biofloc technology has become a popular technology and has been widely applied in the culture of the whiteleg shrimp *P. vannamei* due to its advantages. One of the most important advantages is that it helps to improve the quality of water. In addition, it also helps to reduce feed costs. However, it has been investigated that shrimps are susceptible to the negative effects of stress. These stresses are caused by many factors, especially unfavorable environmental conditions, thus produce negative results such as slow growth, slow survival rate or high mortality rate, and lead to economic losses, as a result. Therefore, it is important to identify whether *P. vannamei* farming using biofloc technology exhibits detrimental stress in shrimps.

This study tested two different hypotheses. The first hypothesis is that the expression data of environmental stress-related genes are positively correlated with the environmental conditions of the culture tanks in shrimp farming. The second hypothesis is that different environmental conditions that were applied to the culture tanks during the cultivation, including types of aeration used and time points of cultivation, are differently

correlated with the relative expression of the stress-related gene, which in this case is catalase.

1.3 The Study's Objectives

The major objectives of this present study:

- To determine the expression of three stress-related genes (catalase, Na^+-K^+-ATPase α-subunit, and hsp70) through qPCR during the cultivation of *Penaeus varnamei* in biofloc closed breeding system over a six-week period.

- To identify correlations between the expressions of the three stress-related genes in *Penaeus varnamei* and its association with dissolved oxygen, salinity, and temperature in the biofloc culture system.

CHAPTER 2: LITERATURE REVIEW

2.1 Biofloc Technology (BFT)

In the intensive system in aquaculture, the amount of dry weight feed which is needed for the production of 1 kg live weight of the cultured animal is estimated at between 1 to 3 kg (if assumes a ratio of 1:3 as the ratio of the conversion of food) (Crab et al., 2007). However, only 20–30% of the nitrogen input is converted into harvestable products (de Schryver et al., 2009), while approximately 75% of the total amount of the feed P and N that are unused and continue to exist in the environment as waste (Crab et al., 2007). In particular, studies regarding shrimp farming have shown that only 15–30% of the nitrogen added in the feed in the culture environment is assimilated by the shrimps (Rajkumar et al., 2016). These remainders then typically accumulate inorganic nitrogen within the closed systems, consequently increasing the concentrations of toxic to marine culture species (de Schryver et al., 2009).

To cope with the accumulation of toxic nitrogen species, Biofloc Technology (BFT), an aquatic system that is friendly to the environment, was developed (de Schryver et al., 2009). Particularly, in the early 1970s, this technology was first created by Aquacop at Ifremer-COP with a wide range of penaeid species, these species include *L. stylirostris*, *L. vannamei*, and *P. monodon*. In 1999, Avnimelech improved this technique by providing a supplement of carbohydrates to the system's environment (Emerenciano et al., 2013; Liu et al., 2019).

During the past few decades, many studies were conducted to understand and explain the relationship among different aspects, for instance, the relationship between water and bacterial community that presents in water (Emerenciano et al., 2013). Cardona et al. (2016) identified the bacterial diversity in water from the culture of the shrimp *L.*

stylirostris using biofloc technology. By applying RNA next-generation sequencing, a high-throughput sequencing technique, the presence of many different bacteria groups in the cultured water had been figured out, including *Proteobacteria*, *Bacteroidetes*, and *Cyanobacteria* - the three most dominant phyla. In addition, the study also developed a correlation between microbial communities and environmental parameters measured in the system. Indeed, the evolution of *Bacteroidetes Saprospiraceae* was positively correlated with NH_4^+-N concentration ($p > 0.0001$), while the quantities of *Leucotrix* were positively correlated with the concentration of nitrite (p-value < 0.0001). In addition, such studies also investigated the physiology of the nutrition of the shrimp (Emerenciano et al., 2013). It has been investigated that shrimps are not able to synthesize all the amino acids; therefore, the cultured shrimps need to be supplied essential amino acids through the feeds in order to maintain their normal state of physiology and metabolism. However, it is important to identify the optimal level of dietary protein for the supply, since high and low levels both negatively affect the culture environment and the cultured animals. Lee et al. (2018) carried out a study to find out the optimal level of dietary protein required for the supply of Pacific white shrimp cultivation. The results showed that the level of dietary protein which is optimal for maximum growth of the Pacific white shrimp can be affected by differences in many aspects such as shrimp species, size of shrimp, the density of stocks, type of culture system and sources of dietary protein. Indeed, the optimum level of dietary crude protein for small-sized Pacific white shrimp was 34.5%, while the optimum level for medium-sized shrimp was 35.6%, and 32.2% for larger sized shrimp. The findings of such studies play an important role in the establishment as well as the improvement of a biofloc technology comprehension approach (Emerenciano et al., 2013) since they investigated various important knowledge. For instance, they found out that the water harbored a remarkable diversity of microbial communities as well as demonstrated the influence of these communities on the cultured animals (Cardona et al.,

2016); they demonstrated the optimal dietary protein for the growth optima of the cultured shrimps (Lee et al., 2018).

The benefits of applying biofloc technology over conventional practices in farmed shrimp have been successfully investigated. One of the most important benefits of biofloc technology is the enhancing of quality of water due to the balance of carbon and nitrogen in the environment (Crab et al., 2012). Improvement in water quality reduces the need for additional water, thus the farmed shrimps that applying biofloc technology system require minimal or no water exchange (Lara et al., 2017). This will help to minimize the release of culture water that contains waste such as organic matter and nutrients, as well as pathogens and escaped livestock into the surroundings. Thus, it also will lead to a reduction or even elimination of the introduction of pathogens, the escape of exotic species, and releasing of pollution (Emerenciano et al., 2013). In addition, another advantage of biofloc technology is its ability to produce food that contains high nutrients. This food, which is referred to as flocs, has been investigated to be served as an additional source of food and can be consumed by the reared shrimp. This will help to fulfill the shrimp demand for nutrition, and therefore reduce the need for formulated feed protein, subsequently reduce the cost of feed (Khanjani et al., 2016). Furthermore, shrimps can grow more rapidly due to the essential and higher quality of nutrition that bioflocs provide (Manan et al., 2016).

Shrimp aquaculture using BFT has been proven to be more environmentally friendly and more economical than traditional techniques (Lara et al., 2017). Currently, the global shrimp industry has applied this technology and has successfully obtained great achievements, especially in ASEAN countries and other countries in including Brazil, South Korea, the USA, and China (Emerenciano et al., 2013).

2.1.1 What Is Biofloc Technology (BFT)?

The build-up of toxic nitrogen waste is one of the main concerns in the intensive culture system. Previous studies investigated that in most aquatic organisms, the primary product of protein catabolism is ammonia. It occurs in water in two different forms, including ionized (NH_4^+- ammonium) and unionized (NH_3 - ammonia) (de Lourdes Cobo et al., 2012). These forms depending on the temperature and the pH, are in equilibrium. In addition, the sum of them is referred to as total ammonium nitrogen (TAN). Both ammonia and ammonium are toxic to marine organisms, however, NH_3 is toxic more than NH_4^+. This is because ammonia is uncharged and has the ability to dissolve lipid, this will help it to traverse the biological membranes more quickly and more easily than the ammonium ion which is charged and is hydrated (Crab et al., 2007). If the pH of water increases, the toxicity of ammonia will increase as well, since the relative proportion of unionized ammonia increases. Studies have shown that ammonia harmfully affects marine organisms since it can cause tremendous disruption to internal organs' normal function, for instance, it can cause damage to the gill epithelium, as well as disrupt the normal metabolic function of kidneys and liver (Wang et al., 2015b). Thus, lots of studies have been developed to identify how to control the release of ammonia as well as protect aquatic organisms. It has been investigated that if the level of carbon and level of nitrogen in a solution is well balanced, ammonium and waste of organic nitrogen can be converted and produce bacterial biomass. In addition, if carbohydrates are then added to the culture, this will help to stimulate the growth of heterotrophic bacteria, and therefore the uptake of nitrogen through microbial proteins' production can happen (Crab et al., 2012). Therefore, biofloc can be defined as a system where, after a carbon source is added to the culture environment, and the aeration is maintained to be constant, the bacterial communities presenting in the culture will maintain the quality of the water during the culture of the shrimp (Pérez-Rostro et al., 2014).

2.1.2 Composition of Bioflocs

The biofloc is naturally formed in the culture water as aggregates of nitrifying bacteria, protozoans, suspended algae, inorganic flocculants, and other types of organic material including uneaten feed and feces (Hargreaves, 2013; Pérez-Rostro et al., 2014). Each floc is combined in a mucus' loose matrix which is released by bacteria and bound by filamentous microorganisms. In addition, animals that are grazers of flocs also present in the biofloc community, including some zooplankton and nematodes (Hargreaves, 2013).

Typical flocs have an irregular shape and have a wide particle size distribution (Crab et al., 2012). Most bioflocs are microscopic, however, the large bioflocs can be viewed by the human naked eye without aids of the microscope (Hargreaves, 2013). Bioflocs have several typical characteristics, such as they are fine, can be compressed easily, extremely porous (porosity of up to 99%), and are permeable to fluids (Crab et al., 2012).

2.1.3 Vital Role of Microorganisms in Biofloc Technology Systems

Microorganisms play an important role in biofloc technology systems. Firstly, they help to enhance the quality of water by taking in the compounds of nitrogen and producing microbial protein. Secondly, they produce nutrition for the cultured animals, this will help to reduce the ratio of feed conversion, and therefore help to decrease the cost of feed, subsequently increase the feasibility of culture (Emerenciano et al., 2013).

The major groups of microorganisms present in biofloc include (i) chemoautotrophic bacteria; (ii) heterotrophic bacteria and (iii) microalgae. The latter two groups tend to be the most abundant within the biofloc community (Ray et al., 2014; Pacheco-Vega et al., 2018).

In intensive closed aquaculture systems that have low rates of water exchange or zero water exchange, the levels of nitrite and ammonia in the environment are low. This is because these compounds are removed by the microorganisms that present in the culture environment (Piérri et al., 2015). In biofloc systems, the remediation of ammonia has three pathways, including (i) assimilation of algae; (ii) oxidation of chemoautotrophic bacteria; and (iii) assimilation of heterotrophic bacteria. However, it has been investigated that the dominant species in the intensive biofloc systems are bacteria since algae are limited in the number of nutrients that they can assimilate (Ray et al., 2014).

Chemoautotrophic bacteria play an important role in BFT systems as they can perform nitrification. During this process, ammonia can be oxidized to produce nitrite, which is less toxic than ammonia. Then nitrite can be oxidized to produce nitrate, which is less toxic than either of ammonia and nitrite. However, if the concentrations of nitrate are high, it will affect the cultured shrimps in a negative way, consequently leading to the reduction of growth and survival of the shrimps. Once the nitrification process is established, it often functions reliably, however, establishment sometime is inconsistent especially with respect to nitrite oxidation. Chemoautotrophic nitrification has an advantage over heterotrophic processes, as this process requires a low amount of oxygen than the heterotrophic processes (Ray et al., 2014).

Meanwhile, the function of heterotrophic bacteria is the utilization of organic carbon as an energy source and the assimilation of nitrogen to build cellular proteins. The addition of carbohydrates through different carbon sources stimulates the growth of heterotrophic bacteria, leading to heterotrophic assimilation, thus ammonia can be rapidly removed from the culture environment. This process has been suggested to contribute to better growth of shrimp and efficiency of feed conversion (Ray et al., 2014).

Studies have shown that the dominant community of microorganisms could be shifted from autotrophic to heterotrophic if the ratio of C/N is high in the culture. This shift helps to promote the fast growth of bacteria as well as the uptake of nitrogen into bacterial biomass. Therefore, the flocs which are formed from this bacterial biomass become more visible to cultured shrimps (Liu et al., 2019).

On the other hand, a photoautotrophic community such as microalgae also plays a vital role in the BFT systems. The main role of microalgae is the production of biomass by assimilating mainly nitrate and ammonia. In addition, microalgae can produce oxygen through the consumption of carbon dioxide (Emerenciano et al., 2017). Furthermore, microalgae are effectively used for live food enrichment of the cultured shrimps due to their highly nutritive. Similarly, several species of bacteria are also high in nutrients and can be used as nutrient-rich sources for shrimps' consumption (Pacheco-Vega et al., 2018).

The use of bioflocs as a supplemental food source by the cultured shrimps helps to increase the efficiency of feed utilization. In addition, feed residues and fecal excrements can be recycled to replace a significant fraction of the demand for nutrition. Therefore, this not only enhances the growth performance of the cultured shrimps but also reduces feed costs. More importantly, it has been investigated that microorganisms that present in bioflocs can support the activity of the shrimp's digestive enzymes. This will help to increase the number of digestive enzymes that present in the digestive tissues, subsequently leading to higher digestion rates as well as improved absorption of the feed. As a result, this will help to improve the shrimp's growth performance as well as the shrimp's utilization of feed (Khatoon et al., 2016).

2.2 Overview of *Penaeus vannamei*

2.2.1 Taxonomy

The Pacific whiteleg shrimp, *Penaeus vannamei* belongs to the phylum Arthropoda (Dugassa et al., 2018). Many authors described the taxonomic classification of *P. vannamei*. The taxonomy is shown below:

Domain: Eukarya

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Subclass: Eumalacostraca

Superorder: Eucarida

Order: Decapoda

Suborder: Dendrobranchiata

Super family: Penaeoidea

Family: Penaeidae

Genus: Penaeus

Species: *Penaeus vannamei*

2.2.2 Habitat and Biology of *P. vannamei*

The indigenous origin of the whiteleg shrimp, *P. vannamei* is the Pacific coast of South America and Central America, which is referred to as a coastline extends along Northern Peru to Mexico (FAO, 2006). This species likes areas where the temperature of the water is often at above 25°C, most of the time. Therefore, this species mostly lives in tropical

marine environments. The adults of this species live and spawn in the Ocean. Meanwhile, the larvae and juveniles are found in inshore water areas including lagoons, mangrove regions, and coastal estuaries. It has been investigated that the female shrimps of this species grow faster than the male shrimps. In addition, a mature female shrimp – which weighs 30-45 g – can spawn a large number of eggs, vary from 100 000 to 250 000 eggs (Dugassa et al., 2018).

2.2.3 Morphology of *P. vannamei*

The body of penaeid shrimp contains 19 pairs of segments, and the body is divided into three parts, including cephalon, thorax, and abdomen. The cephalon part is made up of the first five pairs of segments. Meanwhile, the next eight pairs of segments make up the thorax part. Lastly, the last six pairs of segments are located in the abdomen. The cephalothorax consists of the head fused with the thorax. The gills are covered by the exoskeleton of the cephalothorax – which also protects the gill chamber. Penaeid shrimp's abdomen consists of six pairs of segments. The first five pairs are the swimming legs, while the last pair is the tail fan. This segment contains 2 pairs of uropods and the telsons. In the case of hazardous, they can help the shrimps to jump backward quickly (Dugassa et al., 2018).

2.3 *P. vannamei* Culture Using Biofloc Technology (BFT)

Among various commercial shrimp species, the shrimp *Penaeus vannamei* is a significant vital species which is being cultured worldwide (Xu et al., 2017), especially in tropical regions (Krummenauer et al., 2011). This is due to this species' typical characteristics. One of the most important characteristics is that this species can tolerate and survive in a large salinity range (Khanjani et al., 2016), since they can grow at salinities as low as 0.5 ppt or as high as 45 ppt (Briggs et al., 2004). It has been

investigated that this species can comfortably grow at salinities of 7 ppt to 34 ppt, however, they grow particularly well at low salinity levels, at around 10 ppt to 15 ppt (where the blood and the environment are isosmotic) (Briggs et al., 2004). In addition, this species has the ability to tolerate various diseases (Khanjani et al., 2016). Despite the fact that *P. vannamei* is highly susceptible to WSSV and TSV, to date, Asia's shrimp farming industry has not experienced major WSSV epidemic with this species, although this virus presents in the environment (Briggs et al., 2004). Furthermore, they can grow rapidly; have a suitable survival, and can be cultured in a high density (Khanjani et al., 2016). Studies have shown that the cultured *P. vannamei* in intensive systems can rapidly grow up to the maximum size of 20 with a high survival rate at 80% to 90%. This species has the potential to be cultured at great densities of stocks at up to 150/m^2 in pond culture; in controlled recirculated systems like tank culture, stocking densities could be as high as 400/m^2 (Briggs et al., 2004). More importantly, *P. vannamei* has the ability to tolerate a wide temperature range. They can grow best at a temperature of around 23-30°C. In particular, the temperature at 30°C is optimal for the best growth small-sized shrimps; while 27°C is optimal for larger sized shrimps (Briggs et al., 2004). In temperate and subtropical climates (Krummenauer et al., 2011), *P. vannamei* can tolerate low temperatures down to 15°C (Briggs et al., 2004), however, such low temperatures will strongly affect the cultured Pacific white shrimps by limit the growth of them, and even cause mortality during the coldest months (Krummenauer et al., 2011). In addition, *P. vannamei* can also tolerate high temperatures up to 33 °C without any issues, however, the growth rates will be reduced (Briggs et al., 2004).

Recently, BFT has become an alternative technique that has zero water exchange and has been widely applied in the culture of whiteleg shrimp. The bioflocs produced in this system not only help to enhance the quality of water by removing toxic nitrogen

compounds including nitrite and ammonia but also help to improve the utilization of feed and healthy shrimp growth from natural productivity (Xu et al., 2017).

2.4 Water Quality in *P. vannamei* Culture Using Biofloc Technology (BFT)

It has been determined that the temperature of the water that is optimum for the culture of *P. vannamei* is between 23-30°C (Briggs et al., 2004). Temperature can have an influence on several aspects of the shrimp culture, including the metabolism of shrimp, the rate of feeding, the consumption of oxygen, survival, and tolerance against toxic compounds. More importantly, the low temperature can affect the shrimp's pathogen or shrimp's disease. For instance, studies have proven that the sudden appearance of the White Spot Syndrome Virus (WSSV) in shrimp is influenced by low temperature. This is because the immune system of shrimp is affected by the sudden change in temperature (Kasan et al., 2018).

The level of salinity that is optimum for the culture of *P. vannamei* is at between 15 ppt to 25 ppt, however, this species is well cultured at low salinities (Maia et al., 2011). Studies have shown that salinity is dependent on the rainfall quantity and evaporation rate (Kasan et al., 2018). The salinity will be at high values during the summer season due to the low amount of rainfall and high rate of evaporation; during the monsoon season, salinity will be low since it was moderately reduced by the rainfall and the freshwater inflow from the land (Perumal et al., 2009). In the shrimp pond, high salinity is contributed by a high rate of evaporation as well as less amount of rainfall. Particularly, high salinity can be achieved in hot weather or on sunny days. However, studies have investigated that the whiteleg shrimp has the ability to tolerate a wide range of salinity as low as 1 ppt to as high as 40 ppt (Kasan et al., 2018).

pH is referred to as an alkalinity degree or acidity degree in water. pH is extremely crucial for the growth and survival of the cultured shrimps. This is because pH can have an effect on metabolism and other physiological processes of shrimps. The optimal range of pH is determined between 6.8 and 8.7. However, in biofloc systems, the optimal range of pH for the production of bioflocs is examined at a range from 7.0 to 8.5 (Kasan et al., 2018). Negative effects of low pH on shrimp growth and survival rate have already been determined. When the pH of water in a *P. vannamei* culture using BFT falls under 7.0, the growth of *P. vannamei* decreases, and the feed conversion rate (FCR) increases (Krummenauer et al., 2011). In addition, there have been reports that low pH in shrimp culture is a cause for the infection of WSSV in shrimps (Kasan et al., 2018).

Meanwhile, dissolved oxygen (DO) is an essential variable that has an influence on aquatic organism growth. It has been investigated that, in aquatic culture systems, DO should not be below the levels of 5 mg/L. However, a competition of oxygen demand can readily happen in a BFT system since algae and bacteria which form biofloc both require oxygen, therefore, to prevent the competition of oxygen demand, the range of DO levels - which is optimal for the maintenance of biofloc system - is determined at between 7 and 8 mg/L (Kasan et al., 2018). Studies have shown that the drop in DO below the recommended value for optimum *L. vannamei* growth may have an effect on the shrimp's growth. However, the DO values do not have an effect on the survival of the shrimps (Krummenauer et al., 2011).

Khanjani et al. (2016) examined how different levels of feed can affect the quality of water, growth performance, rate of survival, and composition of the body of the whiteleg shrimp *P. vannamei* in zero water exchange system. No significant differences in water parameters between different treatments were found, such parameters include temperature, pH, and dissolved oxygen. Besides, significant differences between different

treatments in some indices were found, including ammonia, nitrite, nitrate, and salinity. However, values of some parameters such as dissolved oxygen, pH, salinity, and temperature of water still remained within the optimum ranges for the whiteleg shrimp farming.

Lara et al. (2017) analyzed the effects of different types of aeration that commonly used in the market on the quality of water, the growth of shrimps, the formation of biofloc as well as the microorganisms that present in the biofloc. Treatments were conducted with three types of aerations, including propeller aspirator pump aerator, vertical pump aerator, and diffused air blower. The results showed that the temperature among the three treatments did not significantly different and varied between 28 to 30^{o}C, which is suggested as the optimal ranges of temperature for the shrimp growth. Similar results were observed in dissolved oxygen, pH, and salinity levels in the three treatments since the level of these parameters did not significantly different among all treatments. However, the mean oxygen concentrations recorded in all treatments varied from 0.82 to 6.7 mg L^{-1} and remained below the recommended dissolved oxygen concentrations in shrimp culture – which should be higher than 5 mg DO L^{-1}. Therefore, shrimp growth might be affected by these low oxygen concentrations. In addition, the temperature in one of the experimental tanks was recorded at a high degree. This high temperature coupled with low DO concentrations contributed to the low survival of the reared shrimps. Meanwhile, the level of pH and alkalinity were decreased throughout the experiment and were remained within the recommended value for *L. vannamei*. This is mainly due to the inorganic carbon consumption by the nitrifying bacteria and heterotrophic bacteria that present in the culture.

Krummenauer et al. (2011) carried out a study to investigate how different densities of stocking can affect the whiteleg shrimp in the BFT system. The water quality

parameters were measured throughout the experiment. According to the results, throughout the culture, levels of DO decreased with the lowest value was recorded at 1.30 mg/L; while other parameters such as temperature, salinity did not significantly different.

Kasan et al. (2018) carried out an experiment to study the effect of BFT on the quality of water, the health of the shrimps, and the productivity of the pond which applying BFT in *P. vannamei* culture. In the study, water quality parameters were measured prior to each time of sample collection. These parameters include dissolved oxygen, salinity, temperature, pH, ammonia, and nitrite. The results showed that the average temperature throughout each culturing day was varied from 27.6°C to 30.5°C, these values still remained within the recommended range that is optimal for shrimp culture, since the optimum range is suggested at between 25°C to 31°C. Meanwhile, the average level of salinity for each day of culture was varied between 20.0 ppt and 37.0 ppt, exceeding the optimum range of salinity for shrimp culture – which is suggested at between 15 ppt to 25 ppt. Lastly, the DO values throughout each day of culture varied from 4.80 to 7.0 mg/L, below the recommended optimum DO level range for the aquatic culture that applying BFT. In fact, the optimum DO level in general aquatic systems has been suggested to not less than 5 mg/L. However, there have been studies showed that in biofloc systems, a competition between microorganisms that form biofloc (such as bacteria and algae) and the cultured animals can easily occur. This is because all of these organisms require oxygen for their growth. Therefore, the optimum DO level range for aquatic animals in the biofloc systems has been suggested to vary from 7 to 8 mg/L to prevent the oxygen demand competition.

It has been investigated that the low dissolved oxygen concentrations influence the presence of cyanobacteria in the culture. The presence of these organisms in the culture has been investigated to affect the shrimp performance. This is because some

cyanobacteria produce substances that may be toxic to the cultured animals (Lara et al., 2017).

These findings help to assess the impact of biofloc technology on the quality of culture water and the health of cultured shrimps since they provided a status of water quality in biofloc systems (Kasan et al., 2018). These studies proved that environmental parameters including temperature, salinity, pH, and dissolved oxygen are not stable and will be varied throughout the culture. According to Pontinha et al. (2018), abrupt changes in water quality parameters will strongly affect the growth of cultured shrimps, as well as their survival and their ability to resist diseases.

2.5 Stress in Aquatic Farming

2.5.1 Definition of Stress

It is hard to give an accurate definition of the word "stress" since there have been several, different definitions of this word that were defined by numerous authors. According to Brett (as cited in Schreck et al., 2016), stress can be defined as a state produced by an environment or other factors, in which, the adaptive responses are extended and gone beyond the normal range. Or it also can be defined as a condition, in which, the demand for an environmental factor exceeds the natural regulatory capacity of an organism, according to Koolhaas (as cited in Schreck et al., 2016). Another definition is given by Wedemeyer and McLeay (as cited in Schreck et al., 2016) is that stress is a total of all physiological responses which occur when the animal is attempting to maintain or re-establish homeostasis after being exposed to a challenge which is beyond the normal range. In summary, a general definition that runs through all of the above explanations is that stress is the physiological response to a stressor. The stressor is defined as an environmental variable or other factors that cause the response, therefore, the stress in

shrimp can be defined as a physiological response of the shrimp to a stressor (Schreck et al., 2016).

2.5.2 Stress and Its Responses in Aquatic Animals

In aquaculture, the stress of aquatic organisms is caused by any physiological or physical disturbances in the environment of the culture. In addition, stress also can be produced in the system which is transpired due to several factors, including crowding, handling, transportation, and changes of chemical factors as well as changes in physical factors. Several negative effects of stress in aquaculture have been identified, especially negative economic impact. Stress negatively affects the health of aquatic animals, reducing productivity as well as final products' quality, thus impairing aquatic and aquaculture systems, and consequently leading to economic loss. In order to prevent or even eliminate the occurrence of stress in an aquatic system, numerous studies have been conducted (Gao et al., 2016; Li et al., 2016; Liu et al., 2016). Results have shown that there are some regulatory systems have played an important role in stress response. These systems include immune systems, endocrine, and neural. It has been investigated that when being stressed, aquatic animals like fish and shrimp can exhibit multiple physiological responses as well as genomic responses in order to adjust the compensatory or adaptive mechanism, and therefore mitigating the stressors, as a result, will help them to keep their hemostasis and subsequently can survive under the pressure. Consequently, the processes of stress response involve highly significant genes in many different tissues and organs (Eissa et al., 2014).

Previously, the stress in aquatic animals was determined by using traditional stress biomarkers. The principal of these biomarkers is based on measurement of levels of hormones, hydro-mineral parameters as well as hematological parameters. However, it

has been investigated that both extrinsic factors and intrinsic factors can control the response of stress, this consequently causes difficulty in interpreting results in some circumstances. Thus, several studies have been conducted to develop an alternative that can overcome this issue. The genomic tool promises to be one of the most efficient tools in stressor determination to examine the potential genes or the candidate genes which have an association with the response of stress are often unique signatures or unique imprints of specific stressors, therefore stressors can be determined at the initial indication. Consequently, it is possible to use these candidate genes as molecular biomarkers for the diagnosis of stress in aquatic animals, and therefore leading to an improvement of stress tolerance, a reduction of diseases, as well as an enhancement of stocks in aquaculture activities (Eissa et al., 2014).

There have been studies to investigate and understand the physiological response mechanisms of shrimps when adapting to stress caused by environmental stressors. Qiu et al. (2018) carried out research to study changes in the activity of the ammonia-metabolizing enzyme and the expression of genes in *L. vannamei* when exposing to ammonia stress. The results indicated that there was a correlation between enzyme activities and stress. According to the results of the study, a high level of ammonia concentrations led to an increase in activities of glutamine synthetase (GSase), while inhibiting activities of transglutaminase (TGase). Such correlations indicate that catabolic enzymes such as GSase, and TGase can be used as biomarkers to detect ammonia stress in *L. vannamei*.

2.6 Common Stresses and Stressors in Shrimp Culture

Shrimp is a poikilothermic animal with open hemolymph systems. It has been investigated that the hemolymph flows directly into the body cavity of shrimps. In

addition, external substances including ammonia and nitrogen are extracted from the water around through the gills. Both biological factors and non-biological factors present in the water around have been investigated to give effect on shrimps. The diverse microbial communities presenting in water are preferred to as biological factors. Many of these microorganisms have been investigated to be potentially pathogenic since, under certain conditions, they can infect shrimp and cause disease (Chen et al., 2019b). Indeed, vibrios are commonly found in the shrimp culture, since they are ubiquitous and abundant in the aquatic environment (Thompson et al., 2004), from which some species including *Vibrio harveyi*, *V. alginolyticus* and *V. parahaemolyticus* are most frequently isolated. These vibrios have been defined as opportunistic pathogens; during or soon after some stressful events such as sudden changes in salinity or sudden changes in temperature, outbreaks of these pathogens negatively affect the cultured shrimps, resulting in the high rate of mortalities (Zhou et al., 2012). Meanwhile, the complex and changeable physical parameters of the water around are referred to as non-biological factors, such factors can directly affect the physiological processes in shrimp. In addition, it has been investigated that biological and non-biological factors in aquatic systems can regulate each other. This is because physio-chemical factors can dictate the growth of many microorganisms, while these microbial communities can give an effect on non-biological aquatic factors including DO. Thus, it can be concluded that, in shrimp as well as in other aquatic organisms, the environmental stress is closely related to the immune responses (Chen et al., 2019b).

Numerous studies regarding the shrimp culture in the semi-intensive system have investigated several environmental variations that affect the culture. Such environmental factors include fluctuations of dissolved oxygen, temperature, or accumulation of compounds that are potentially toxic such as ammonia or nitrite. In addition, it has been investigated that these environmental factors' variations sometimes do not affect the

culture independently. In fact, these environmental variations could occur at the same time, thus causing a combined effect that may be stressful for the cultured shrimps. Consequently, this will reduce the immune defense of the shrimps as well as will increase susceptibility to being infected by pathogens (Mugnier et al., 2008). There have been several studies to evaluate the combined effect of several environmental factors on shrimp culture (Mugnier et al., 2008). Results showed that when *L. stylirostris* were submitted independently to ammonia or hypoxia, the rate of mortality was low. Meanwhile, the combination of these two factors gave a synergic effect on mortality. In addition, the combined effect of ammonia and hypoxia led to a stronger physiological response than this observed for ammonia or hypoxia independent.

It has been investigated that changes in the expression of the genes, which include qualitative, quantitative, and reaction coefficients' changes, are related to a wide range of stressors. Currently, several different types of genomic tools, including gene expression profiling and microarrays, are commonly applied to study the responses of crustaceans such as fish and shrimp to many different types of stressors. The principal of cDNA microarrays is the ability to enable a large number of genes that can be screened at exactly the same time to identify differentially expressed genes that are in relation to biochemical pathways which involve in a range of responses. Meanwhile, gene expression has been used to investigate the relationship between acute stressors and the expression of those genes related to the stress response. Thus, this helps to develop a knowledge of the influence of the environmental factors on the expression of the gene. Some of the most common stressors that have been studied include hypoxia, temperature, and toxicants (Sopinka et al., 2016).

2.6.1 Hypoxia

In aquatic systems, hypoxia is one of the most common water quality issues which can seriously impact the system. Hypoxia refers to dissolved oxygen levels of 2 mg L^{-1} or lower. It can also be defined as the limited oxygen concentration in the media, which usually occurs when the amount of consumed oxygen by the organisms, which are living in the environment, is greater than the amount of supplied oxygen that has been provided into the environment. In aquatic environments, hypoxia can occur naturally, but it can also be a symptom of degraded water quality – a result of several anthropogenic activities such as eutrophication or nutrient pollution. In recent years, there have been increases in the severity, occurrence frequency, and hypoxia duration. Numerous negative effects caused by hypoxia have been investigated and this environmental stressor is now recognized as one of the most serious threats to global aquaculture activities. Hypoxia or reoxygenation that follows hypoxia has been investigated to be in association with the diseases' appearance, this negatively affects the growth and development of aquatic animals, or even results in the death of these organisms (O'Connor et al., 2007; Trasviña-Arenas et al., 2013; Li et al., 2016).

It has been investigated that hypoxia is a cause of a series of cellular responses, these responses include generation of reactive nitrogen species (RNS) and generation of reactive oxygen species (ROS), as well as increases of nitric oxide synthase (NOS, an RNS regulating enzyme) concentrations (Rahman et al., 2017).

ROS are defined as highly reactive molecules, these are hydroxyl radical (OH), superoxide radical (O_2^-), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). These molecules can damage the membranes of the cells, proteins, enzymes, and DNA. These damages will consequently lead to various pathological changes in tissues and

dysfunction of organs. It has been investigated that ROS can be produced by various processes such as oxygenases, electron transport chain, or specific systems such as NADPH-oxidase. During respiration, about 0.1 to 0.2% of the oxygen consumed by aerobic cells is converted to ROS which is important for cells' normal functions including pathogen resistance and redox signaling. However, an excess of ROS will cause oxidative damage. Therefore, a stable state of ROS level is maintained by the balance between generation and elimination of ROS, in order to prevent the excess of ROS. In aquatic animals, the elimination of ROS is produced by antioxidant defense systems, which include enzymatic and non-enzymatic components. Some of the antioxidant enzymes that play the main role in eliminating ROS are catalase (CAT), glutathione reductase (GR), glutathione peroxidases (GPX), and superoxide dismutase (SOD). However, studies have investigated that the ROS balance can be broken by various environmental factors including dissolved oxygen, salinity, temperature, ammonia, pH, heavy metals, and virus. Consequently, this unbalanced ROS will result in oxidative damage (Li et al., 2016).

It has been investigated that under hypoxic conditions, accumulated electrons are available for ROS formation using whatever oxygen remains, subsequently leading to an increase of ROS production. In addition, the assessment of the antioxidant system has been investigated to be used as a potential indicator of oxidative stress in aquatic animals when they are under hypoxia as well as other environmental stresses (Li et al., 2016). In shrimps, some antioxidant enzymes that involve in this regulation have been characterized, including catalase, glutathione, and peroxidase (Trasviña-Arenas et al., 2013).

2.6.1.1 Whiteleg Shrimp *P. vannamei* Catalase

Catalase (EC 1.11.1.6) is an oxidoreductase enzyme that can catalyze the conversion of two molecules of hydrogen peroxide ($2H_2O_2$) to two molecules of water ($2H_2O$) and one of oxygen (O_2). Catalase plays a vital role in the redox equilibrium of the cell, and in the regulation of hydrogen peroxide (H_2O_2) concentration, which is referred to as a harmful reactive oxygen species (ROS) and is produced during hypoxia. The catalase activity in response to hypoxia in aquatic animals as well as *P. vannamei* has been investigated (Trasviña-Arenas et al., 2013).

Tavares-Sa´nchez et al. (2004) investigated a cDNA that contains the complete coding sequence for catalase in hepatopancreas and gills of the whiteleg shrimp *P. vannamei*. The complete sequence is 1692 bp long. In addition, the mRNA catalase level obtained in hepatopancreas was higher than the level obtained in gills. However, in muscle, catalase mRNA could not be detected. The findings of this study provided important knowledge of the adaptive response of this commercially valuable species to oxidative stress conditions and toxicity of the environment; particularly the expression of catalase in the responses of this species to conditions of stress that commonly found in the shrimp culture.

Trasviña-Arenas et al. (2013) carried out research to determine the catalase gene' structure in the shrimp *P. vannamei*, examined the expression of it as well as the activity of the enzyme under short term hypoxia. Fragments of the catalase gene were produced by PCR using many different primers, which had been developed according to the sequence of cDNA. The complete coding sequence is 2974 bp long and has four introns of 821, 223, 114, and 298 bp, from 5' to 3', respectively. In addition, the study's results indicated that the expression of catalase in gills significantly increased in response to

hypoxia. The study's findings provided important knowledge of the physiological and metabolic responses to certain environmental stresses in *P. vannamei*, especially the vital role of catalase in the responses of this species to short term hypoxia and subsequent reoxygenation.

2.6.2 Salinity

Crustaceans including shrimps live in various environments with different salinities from normal water to seawater. Salinity can be defined as the measure of the total amount of dissolved salts in a sample of water. This is commonly expressed as parts per thousand (or ppt). In terms of aquaculture, salinity is one of the vital factors of the environment since salinity can change osmoregulation, and therefore causing significant stress on cultured animals. Such stress will affect the survival, growth, and distribution of the aquatic animals and will lead to high economic loss, as a result (Wang et al., 2015a; Gao et al., 2016).

The whiteleg shrimp *L. vannamei* has been investigated to have a high tolerance to a wide range of salinity from between 0.5 to 45 ppt. This species not only can grow in a high salinity environment such as in seawater but also can grow in the regions that have a medium and low level of salinity such as brackish water and freshwater, respectively. However, the level of salinity in the environment is not stable. In fact, the salinity level is affected by changes in conditions of the environment. These changes include excessive freshwater, rainstorms, water pollution, high evaporation, and disappearance of the dominant species of plankton. This continuous change of salinity will give significant effects on the ecophysiological performance of the shrimps. Therefore, it is important to investigate the adaption of whiteleg shrimp to the fluctuation of salinity, as well as examine the effects of changes in salinity on the shrimps (Hu et al., 2015).

For over decades, lots of researchers have studied how salinity can affect the growth performance as well as determine genes which involve in the response of the whiteleg shrimp *L. vannamei* to salinity stress. For instance, Wang et al. (2015a) carried out research to study the molecular pathway and gene responses of the whiteleg shrimp when it is exposed to acute low salinity stress. The study's results showed that there are 991 genes that had different expressions in the gill of those shrimps that were reared in low levels of salinity. In detail, among these 991 genes, 468 genes were up-regulated while 523 were down-regulated. Furthermore, most of these genes were regulated between 2-fold to 5-fold.

Hu et al. (2015) carried out a study to investigate the molecular response of shrimps to salinity stress by detecting the transcriptomic responses in the gills of the whiteleg shrimp under low salinity stress. The results showed that there are 585 genes that were differentially expressed under low salinity challenge. In detail, among these 585 genes, 292 genes were up-regulated, while 293 genes were down-regulated. In addition, the study also found out several genes that have significant differential expressed and have a relation to osmotic regulation. These genes then divided into three types, based on their functions. These three types include ion transport enzymes; ion transporters; and genes that participate in intracellular signaling pathways.

2.6.2.1 Whiteleg Shrimp *P. vannamei* Na^+-K^+-ATPase α-subunit

It has been investigated that some genes that relate to the metabolic, such as carbonic anhydrase (CA), Na^+-K^+-ATPase α-subunit in gills, trypsin, and chymotrypsin in hepatopancreas, have undergone significant changes when some crustaceans including shrimps under salinity stress. Particularly, studies have shown that the α-subunit gene transcriptions or the α-subunit expression increased when some crustaceans like shrimp

had been exposed to dilute media. In addition, such increases in the α-subunit expression and the α-subunit gene transcription have been investigated to be attached to the stimulation of Na^+-K^+-ATPase activity. Thus, this can give a probable explanation for the role of Na^+-K^+-ATPase α-subunit when shrimp being exposed to salinity stress. Salinity changes will affect the expression of Na^+-K^+-ATPase α-subunit, as well as regulate Na^+-K^+-ATPase activity. Subsequently, Na^+-K^+-ATPase can lead to the flux of cell ions to maintain the balance of osmotic pressure (Hu et al., 2015).

Gao et al. (2016) carried out a study to examine how long-term low salinity stress affects the *L. vannamei* shrimp growth performance. In addition, the study also evaluated the expression of the genes related to osmolarity and metabolism during the low salinity challenge of the shrimp. The genes that related to osmolarity and metabolism include Na^+-K^+-ATPase α-subunit in gills, chymotrypsin, and trypsin in hepatopancreas. The results showed that low salinity not only could significantly reduce the growth performance and survival of the shrimps but also could influence transcript levels of several metabolism-related genes. In detail, the levels of mRNA expression of carbonic anhydrase and Na^+-K^+-ATPase α-subunit were significantly increased in those shrimps that were reared in low levels of salinity such as 2 ppt and 10 ppt. Meanwhile, at a salinity of 2 ppt and 10 ppt, mRNA expression levels of chymotrypsin and trypsin were decreased significantly.

Sun et al. (2011) studied the Na^+-K^+-ATPase α-subunit mRNA expression in the whiteleg shrimp in response to salinity stress. In the study, the gene of the partial Na^+-K^+-ATPase α-subunit was examined in the gills and hepatopancreas of those shrimps that had been exposed to salinity stress. The results indicated a fragment of approximately 707 bp long which was amplified from gills and hepatopancreas. In addition, results of the study indicated that the relative mRNA expression levels of Na^+-K^+-ATPase in gills and

in hepatopancreas here were increased after the experimental shrimps had been exposed to salinity stress. These results indicated that the expression of Na^+-K^+-ATPase of *L. vannamei* is stimulated when those shrimps expose to salinity stress.

2.6.3 Heat Shock Protein

In shrimp farming, heat shock stress is referred to as a sudden increase in temperature. It has been investigated that the shrimps have to overcome the heat shock stress by inducing sets of proteins including heat shock protein (HSPs). This process is referred to as a heat shock stress response (Rungrassamee et al., 2010).

HSPs also referred to as stress proteins, are a group of highly conserved, broadly distributed proteins in nature and can be found in all organisms. HSPs serve as regulators of the normal function of the cell. Moreover, stress proteins play a key role as molecular chaperones in heat tolerance due to their ability to repair and refold denatured proteins. In addition to heat shock stress, HSPs also have the ability to respond to other stresses including oxidative stress and heavy metals stress; and respond to other factors including pathogen infection. In particular, it has been investigated that, in a living organism, the expressions of HSP genes increase in response to various stresses as well as in response to Vibrio infection. In eukaryotes, HSPs are found in many different forms and are generally divided into different families according to the molecular weights of them. There are six major families of HSPs, including small HSPs, HSP60, HSP70, HSP90, HSP100, and HSP110. In eukaryotes, the HSP70 family has been extensively characterized as a primary family of heat shock proteins. This family consists of stress-inducible (Hsp) and constitutively expressed (Hsc) forms that have different structures and different expression levels under heat shock stress (Rungrassamee et al., 2010; Qian et al., 2012).

In cells, HSPs are main stress proteins that help to minimize histological, physiological, and biochemical alterations of the host which were caused by various changes in the environment. Thus, this helps HSPs become vital factors for homeostasis maintenance across environmental regimes (Qian et al., 2012). It has been investigated that the effectiveness of these stress proteins against wide cellular stress conditions' sources is mainly due to the chaperoning functions of them. Since HSPs have unique sensitivities to numerous environment changes, they have been used to study the stress response of organisms for decades. In addition, the use of them as biomarkers for the diagnosis of stress in cells has been developed very well and has achieved success in several organisms. Recently, there have been studies on the profile of the expression of HSPs genes in response to wide types of environmental stresses in aquatic animals including the bay scallop, the tilapia, the abalone, the flat oyster and the shrimp (Qian et al., 2012).

2.6.3.1 Whiteleg Shrimp *P. vannamei* HSP70

Up to the present, there have been studies on HSPs from *L. vannamei*. These studies have characterized the full-length cDNA sequences of four major families of HSPs that present in this shrimp species, including the family of hsp60, hsp70, hsc70 and hsp90 (Qian et al., 2012). Furthermore, the expression of *L. vannamei* HSP genes, including hsp60, hsp70, and hsp70, in response to different conditions of heat shock and in different tissues have been investigated (Qian et al., 2012).

Qian et al. (2012) revealed that different HSP genes showed different expressions when the experimental shrimps were exposed to different stressors. Particularly, when the shrimps were under acute thermal stress, significant increases were obtained in the expressions of all of the four HSP genes, especially the hsp70. The levels of transcription

of hsp70 were the most sensitive to the fluctuation of the temperature. This finding suggests that, among various types of HSPs in the whiteleg shrimp *L. vannamei,* hsp70 is the most suitable candidate that can be used as a marker for the determination of thermal stress.

2.7 Real-Time PCR (qPCR)

Conventional polymerase chain reaction (PCR) has been widely used for over decades as this is a powerful technique that helps to increase the number of DNA molecules efficiently due to its ability to amplify specific DNA or cDNA sequences to many thousand-folds or even to a million-fold. However, there have been disadvantages regarding the applying of this technique. One of the issues is time-consuming. This is because after the reaction has completed the last PCR cycle, the amplified PCR products must be detected by post-PCR analysis such as gel electrophoresis or image analysis. In other words, the post-PCR analysis could not be performed if the PCR reaction is still in progress, thus consuming much more time to detect the amplicons. Therefore, finding an alternative that can overcome this issue is an important task. Recently, qPCR has been considered as an ideal alternative technique that able to measure the PCR amplicons at the early stages of the reaction. Therefore, the number of PCR products can be measured although the reaction is still in the exponential phase, consequently reducing the time taken for the process (Invitrogen, 2008; Pestana et al., 2010).

2.7.1 The Principal of Real-Time PCR (qPCR)

qPCR is a technique that was developed based on an improvement of the traditional PCR technique. This technique allows the collection of data after each cycle throughout the PCR process, therefore amplification and detection can be combined into a single step. This is achieved by using various fluorescent markers which can correlate the

concentration of PCR product to the intensity of fluorescence since the increase in fluorescent signal is directly proportional to the amount of generated PCR products (Wong et al., 2005; Invitrogen, 2008). Therefore, specific nucleic acid sequences of DNA, cDNA, or RNA targets can be quantified by determining the cycle where the PCR product can first be detected. This is in contrast with endpoint detection in traditional PCR techniques since traditional PCR could not enable accurate quantification of nucleic acids (Qiagen, 2004).

qPCR has been proven to be an efficient method of detection and quantitative measurement of generated PCR product molecules. This method has several advantages over the traditional PCR method. Firstly, this technique can monitor the progress of the PCR reactions since it occurs in real-time. Secondly, the use of Real-Time PCR provides precise measurements of the number of amplicons generated at each cycle of the process. The third advantage is that the quantification of PCR products does not require post-PCR process since the amplification and the detection are combined into a single step. This will not only help to reduce the time consuming of the reaction but also help to reduce the chances of carryover contamination (Invitrogen, 2008; Pestana et al., 2010).

2.7.2 Types of qPCR

Based on the use of fluorescent chemistries, gene detection, and quantification by qPCR are divided into three common methods, including fluorescent DNA binding dyes, fluorescent primers, and fluorescent probes (Kanmogne, 2014).

Among the three major types of qPCR as mentioned above, qPCR using fluorescent DNA binding dyes is the most common method. The principle of this method is the performance of PCR in the presence of a fluorescent dye that can intercalate and bind to a double-strand DNA but not to single-stranded DNA and will fluoresce when it's so

bound. There are various DNA binding dyes that can be used such as SYBR Green I, LC Green 1, EVA Green, and SYTO 9. Among these dyes, SYBR Green is the most common one that has been widely used since this is a cost-effective dye in comparison with other chemistries, thus this method is cheaper compared to other methods. Moreover, it is easy to use since it allows the initial optimization of primers for any real-time chemistry. When SYBR is free in solution, the level of fluorescence is very low, however, after the addition of double-strand DNA, the fluorescence can increase over 1000-fold. When the accumulation of newly generated PCR amplicons increases with the reaction cycles, the fluorescence intensity also increases since more SYBR Green molecules are binding to the double-strand DNA. This association will help to measure the accumulation of the target product in real-time. Several advantages have been proven, including measuring the number of PCR products generated in real-time is associated with the fluorescence intensity; monitoring the amplification of any double-strand DNA; reducing the set-up time and costs since there is no requirement of the specific probe. However, some disadvantages of qPCR using DNA binding dyes-SYBR Green have been investigated. One of the most serious disadvantages is the generation of false-positive signals due to the formation of primer-dimer or non-specific products since SYBR Green is a non-specific dye and can bind to any double-strand DNA such as mismatched strands, primer dimers, and fragments from non-specific amplification. In addition, this will lead to a requirement of the melting curve analysis at the end of the amplification reaction to identify the presence of primer-dimer or the formation of non-specific products. Therefore, when performing qPCR using the DNA-binding dyes method, there are some important aspects that must be strictly under controlled. Particularly, the first thing that needs to be ensured is the purity of the molecules, no contaminants are presented in the DNA or RNA samples. Secondly, primers used for amplification must be specific. Lastly, PCR conditions such as melting temperature or annealing temperature must be optimal to

ensure that there is no formation of primer-dimer and no sequence of mispriming (Pestana et al., 2010; Kanmogne, 2014; Stephenson, 2016).

2.7.3 A Powerful Method for Quantifying Gene Expression - qPCR

qPCR has been investigated to be an efficient technique for quantification of gene expression. The basic theory of this technique is the correlation between the concentration of the PCR product and the intensity of fluorescence. Reactions are characterized (or PCR cycle) where the target amplification is first detected. This value is also referred to as cycle threshold (Ct), the time at which fluorescence intensity is greater than background fluorescence. Thus, when the amount of target DNA in the starting material is high, the fluorescent signal will quickly appear and significantly increase, resulting in a low value of Ct. Several advantages of qPCR over other techniques for quantifying gene expression have been investigated. The first point is the production of quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude without the requirement of post-amplification manipulation. Secondly, the qPCR technique is more sensitive than other techniques, for instance, in comparison with RNA protection technique and dot blot hybridization, the qPCR technique is 10 000- to 100 000-fold more sensitive and 1000-fold more sensitive than these two techniques, respectively. In addition, an important point is that this technique is even able to detect a single copy of a specific transcript. Moreover, this technique can reliably detect differences in gene expression between samples as small as 23%. Furthermore, this technique has a low coefficient of variation in comparison to other end-point techniques. Lastly, Real-Time PCR requires much less RNA template than other techniques due to its ability to discriminate between messenger RNAs (mRNAs) with almost identical sequences. However, the use of this technique also has some disadvantages. The economic cost is one of the most serious issues since this technique requires equipment and reagents which are extremely expensive. Moreover,

since this technique is an extremely high sensitivity, it is compulsory to develop appropriate experiments and have an in-depth knowledge of normalization techniques in order to accurately analyze and conclude the results (Wong et al., 2005).

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample Collection and Water Quality Parameters Measurement

A total number of 84 live and healthy *P. vannamei* shrimps were collected from a biofloc culturing system – which was conducted at Terra Aqua Laboratory, University of Malaya, Malaysia. The culture was carried out with four different types of biofloc categories, including microbubble aeration, macro bubble aeration with bioballs, a mixture of microbubble and macro bubble aeration, and macro bubble aeration without bioballs. In addition, the culture was carried out in 200 L capacity indoor tanks culture for a period of six weeks; total water culture volume in each tank was 150 L.

Samples were collected weekly, in the afternoon. In particular, samples were collected from tank 4 (microbubble aeration), tank 9 (a mixture of microbubble and macro bubble aeration), tank 10 (macro bubble with bioballs), and tank 13 (macro bubble without bioballs) on the 1st, 6th, 13th, 19th, 27th, 34th and 37th day of culture at 2 pm. From each category, three alive shrimps were collected randomly, each individual was then stored in a 2.0 mL sterile microcentrifuge tube, then these tubes were immediately frozen in liquid nitrogen, they then were delivered to the laboratory. After reaching the laboratory, sterile microcentrifuge tubes that contained samples were immediately stored at -80^{o}C for use in future procedures.

The biofloc system used in this current study was conducted, monitored, and maintained by other co-workers (Ezzah et al., unpublished) for the purpose of others' studies. The system was conducted with several culture tanks that applied different types of biofloc categories as described above, including microbubble aeration, macro bubble aeration with bioballs, a mixture of microbubble and macro bubble aeration, and macro bubble aeration without bioballs. In addition, a recirculation system was applied to some

cultured tanks during the daytime, including tank 4 and tank 9. Meanwhile, the setup of other tanks, including tank 10 and tank 13, was the same as the conventional method (it means no recirculation systems were applied). The temperature of the culture area was controlled by air-conditioners in order to measure the temperature of the culture would not exceed 27ºC during the daytime. Besides, water quality parameters were measured daily, using some instruments including HI-98193 Professional Waterproof Dissolved Oxygen and BOD Meter (Hanna Instruments, Malaysia) for DO and temperature measurement; HI-8314 Water-resistant hand-held pH, mV and Temperature Meter for measurement of pH (Hanna Instruments, Malaysia); and HI 96822 Seawater Refractometer for salinity measurement (Hanna Instruments, Malaysia). All measurements were performed following the manufacturer's protocol. In particular, the measurement of DO and temperature was as follows: first, removed the probe's protective cap. Then pressed RANGE to access the DO measure screen. Pressed MODE to change the measuring unit to mg/L. Immersed the tip of the probe into the culture water at the tank's center and hold for approximately 1 minute for the reading to stabilize. The value of DO in mg/L and temperature in ºC were displayed on the screen. Recorded values obtained in a log. Meanwhile, measurement of pH was as follows: first, submerged the electrode tip into the culture water at the tank's center, selected pH mode, then shook briefly and waited for a few minutes for the reading to stabilize. Recorded the pH value showed on the display in a log. Lastly, salinity measurement was as follows: first, collected water sample at the tank's center and kept in a clean bottle. Then wiped off prism surface located at the bottom of the sample well and ensured that the prism and sample well were completely dry. Dripped sample onto the prism surface using a plastic pipette. Filled the well completely and waited approximately 1 minute to allow thermal equilibration between the temperature of the sample and the temperature of the instrument, then pressed the READ key. The salinity value was shown on the display.

Recorded the obtained salinity in ppt unit in a log. After that removed sample from the sample well by absorbing on soft tissue. Rinsed prism and sample well with distilled water using a plastic pipette, lastly wiped dry.

Furthermore, the concentration of ammonia and nitrite was tested every five days. The measurement of ammonia was done using HI 96733C Ammonia High Range ISM (Hanna Instruments, Malaysia) following the manufacturer's protocol. Generally, the measurement took place in two phases, including zeroing of the meter using the unreacted sample, and measurement of reacted sample after the reagents had been added. In particular, the measurement procedure was as follows: first, collected water sample at the tank's center and kept in a clean bottle. After that, turned the meter on by pressing ON/OFF. The meter was ready when the beeper sounded briefly, and the LCD displayed dashes. Filled one cuvette with 1 ml of unreacted sample, by means of the syringe in order to zero the instrument. Then added 9 ml of HI93733B-0 Ammonia Reagent B using the plastic pipette up to the 10 ml mark. Placed the cuvette into the cuvette holder and ensured that the notch on the cap had been positioned securely into the groove. Pressed ZERO/CFM button, after a few seconds, the display showed "-0.0-", indicating that the meter was now zeroed and ready for measurement. Removed the cuvette, opened it, and added 4 drops of HI 93733A-0 Nessler Reagent, then replaced the cap and swirled the cuvette gently to mix. Replaced the cuvette into the cuvette holder and ensured that the notch on the cap had been positioned securely into the groove. Waited for 3 minutes and 30 seconds then pressed the READ/►/TIMER button. The concentration in mg/L of ammonium ion (NH_4^+) was directly displayed on the LCD of the instrument at the end of the measurement. Multiplied by the factor 0.944 to convert the reading to mg/L of ammonia (NH_3). Then converted the newly converted ammonia value in mg/L unit to ppt unit using an online converter tool available at http://www.endmemo.com/sconvert/mg_mlppt.php. Meanwhile, the measurement of

nitrite was done using HI 96708 Nitrite High Range ISM (Hanna Instruments, Malaysia) following the manufacturer's protocol. Similar to the measurement of ammonia, the measurement of nitrite also took place in two phases, including zeroing of the meter using the unreacted sample, and measurement of reacted sample after the reagents had been added. In particular, the measurement procedure was as follows: first, collected water sample at the tank's center and kept in a clean bottle. After that, turned the meter on by pressing ON/OFF. The meter was ready when the beeper sounded briefly, and the LCD displayed dashes. Filled the cuvette with 10 mL of unreacted sample and replaced the cap. Then placed the cuvette into the cuvette holder and ensured that the notch on the cap had been positioned securely into the groove. Pressed ZERO/CFM and waited for a few seconds until the display showed "-0.0-" indicating that the meter was now zeroed and ready for measurement. Removed the cuvette, then added the content of one packet of HI 93708-0 reagent, replaced the cap, and shook gently. Then replaced the cuvette into the cuvette holder and ensured that the notch on the cap had been positioned securely into the groove. Waited for 10 minutes then pressed the READ/►/TIMER button. At the end of the measurement, the instrument directly displayed concentration in mg/L of nitrite on the LCD. Then converted the obtained nitrite value in mg/L unit to ppt unit using an online converter tool available at http://www.endmemo.com/sconvert/mg_mlppt.php.

Last but not least, in order to refill the water loss due to evaporation, new culture water was added whenever the total water of the tank decreased, usually twice per week for tank 4 and 9, and once per week for tank 10 and 13.

Prior to sample collection, the values of water quality parameters, including DO, salinity, pH, temperature, ammonia, and nitrite concentration were noted according to a log provided by the co-workers. Besides, all the collected samples were labeled according to the time of collection and the name of the tank where the samples were collected.

In addition, a total number of the shrimp stocks (14-day old larvae) that had been transferred at the beginning of the culture, and the total number of the survived shrimps at the end of the culture were also noted according to the provided record. Furthermore, the survival rate was determined using the formula (3.1) below (Susilowati et al., 2014):

$$SR = \frac{N_t}{N_0} \times 100\ \% \qquad (3.1)$$

Where:

SR = the survival rate of shrimp (%)

N_t = the number of shrimps that live at the end of the culture

N_o = the number of shrimps that live at the beginning of the culture

3.2 RNA Extraction and RNA Quality Analysis

A total number of 60 samples collected from week 2 to week 6 were used for the isolation of RNA since the quantification of mRNA expression later would use samples collected between week 2 to week 6 only. Meanwhile, 24 samples remaining were stored at -80ºC as laboratory's library stock. Total RNA of each sample was extracted from hepatopancreas and gills using TransZol Up Plus RNA Kit (TransGen Biotech, Beijing) following the manufacturer's protocol.

Prior to the isolation procedure, working surfaces and other needed stuff such as micropipettes were treated with 10% chlorox, 70% ethanol, and RNase Zap in order to promote an RNase-free environment. In addition, all the necessary equipment such as mortars, pestles, forceps, surgical scissors, and stainless-steel lab spoons – which had

been autoclaved – were prechilled by placing them in a Laboratory chest freezer for 1 hour.

According to the instruction, 100 mg of hepatopancreatic tissue and gill tissue were removed from the frozen sample and transferred quickly into a sterilized and pre-chilled mortar that had been added an appropriate amount of liquid nitrogen (≈15 ml), then carefully ground the pooled of hepatopancreatic and gill tissues to complete powder. Next, transferred the sample powder to a microcentrifuge tube, and 1ml of TransZol Up (TransGen Biotech, Beijing) was added to it. The tissue samples were homogenized and repeatedly pipetted up and down. It was then incubated for 5 minutes at room temperature. Then, added 0.2 ml of chloroform, and shacked vigorously for 30 seconds, then it was incubated for 3 minutes, at room temperature. The sample was then centrifuged at 10,000×g at 4ºC for 15 minutes. The mixture separated into a lower pink organic phase, interphase, and a colorless upper aqueous phase which contained the RNA. Transferred the colorless, upper phase containing the RNA to a fresh RNase-free tube and added an equal volume (≈ 600 µl) of absolute ethanol. Next, inverted the tube to mix this mixture gently. Then, transferred the resulting solution to a spin column and centrifuged at 12,000×g at room temperature, for 30 seconds. Discarded the flow-through and added to the spin column 500 µl of Clean Buffer 9 (CB9) and centrifuged at 12,000×g at room temperature for 30 seconds. Discarded the flow-through. Then, repeated this step once more time. After that 500 µl of Wash Buffer 9 (WB9) was added to the spin column and was centrifuged at 12,000×g at room temperature for 30 seconds. Discarded the flow-through then performed this step once more time. Then, the spin column was centrifuged at 12,000×g at room temperature for 2 minutes in order to completely remove the remaining ethanol. The column matrix was then air-dried for several minutes. After that, the spin column was placed into a clean 1.5 ml of RNase free tube. Two hundred µl of RNase-free water was added into the spin column matrix and incubated for 1 minute at

room temperature. Lastly, centrifuged the tube at 12,000×g for 1 minute, at room temperature, to elute RNA. The isolated RNA was then stored at -80°C.

The next step was the assessment of the integrity of the extracted RNA by performing gel electrophoresis on 1% agarose gel. In addition, the RNA purity and RNA concentration and purity were verified by using a Nanodrop 2000 Spectrometer (Thermo Scientific, USA) to measure the absorbance at 260 and 280 nm.

3.3 Reverse Transcription and First Strand cDNA Synthesis

Synthesized cDNA for each sample from an equal amount of total RNA (~500 ng) using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix kit following the manufacturer's protocol (TransGen Biotech, Beijing). Moreover, random primers were used for initiating cDNA synthesis. Due to their random binding (i.e., no template specificity), random primers can be used for the synthesis of the first-strand cDNA from all species of RNA molecules present in the sample. Subsequently, a population of cDNA products primed internally along the entire RNA sequence can be produced from the cDNA synthesis that using random primers.

According to the instruction, for higher efficiency, mRNA, random primer, and RNase-free water were mixed first in a microcentrifuge tube. Then incubated the mixture at 65°C for 5 minutes – a PCR cycler was used for this incubation step. The mixture was then placed ice for 2 minutes. Other components such as 2×TS Reaction Mix, TransScript® RT/RI Enzyme Mix and gDNA Remover was added in. The volume needed for each component is shown in Table 3.1 below. The tube with the mixture was incubated at 25°C for 10 minutes followed by incubation at 42 °C for 15 minutes. Lastly, it was incubated 85°C for 5 seconds for the inactivation of the reverse transcription enzyme. These temperature steps were conveniently set up by using Bio-Rad Thermal Cycler. The

cycling protocol is described in Table 3.2. Then cDNA was stored at -20 ºC for further use.

Table 3.1: First-strand cDNA synthesis reaction components and their corresponding volume.

Component	Volume
Total RNA/mRNA	4 µl
Random Primer (0.1 µg/ µl)	1 µl
2×TS Reaction Mix	10 µl
TransScript® RT/RI Enzyme Mix	1 µl
gDNA Remover	1 µl
RNase-free Water	3 µl
Total volume	**20 µl**

Table 3.2: RT temperature protocol.

Step	Time	Temperature	
Incubation	5 min	65ºC	
	Pause cycle	25ºC	Remove samples, place on ice, added other RT components.
Reverse-transcription reaction			After adding RT components, placed samples in the cycle again and continue
Annealing	10 min	25ºC	
Reverse-transcription step	15 min	42ºC	
Inactivation of reaction	5s	85ºC	

3.4 Primers Optimization

The primers used for the amplification of catalase and Na^+-K^+ATPase α-subunit were designed according to the published mRNA sequences of *L. vannamemi* available at the GenBank (https://www.ncbi.nlm.nih.gov), including catalase (GenBank accession

number AY518322.1) and Na^+-K^+ ATPase α-subunit (GenBank accession number KF765670.1) using publicly available PrimerQuest Tool (Integrated DNA Technologies, Inc.). Meanwhile, sequences of the primers used for amplification of heat shock protein 70 were retrieved from literature (Qian et al., 2012). All the primers were produced by Bio Basic Canada Inc. Primer optimization was done prior to using in qPCR by identifying the optimal annealing temperature (Ta) of the primers. A PCR gradient was carried out using Bio-Rad Thermal Cycler. In addition, *L. vannamei* cDNA library was used as a template. The range of temperature was started from 5 ºC below the calculated temperature of the primer melting point (Tm) to 5 ºC above the calculated temperature of the primer melting point (Tm). Primers sequences, their melting point (Tm), and the expected size of their products are given in Table 3.3.

GoTaq® Flexi DNA Polymerase components were used for all PCR reactions (Promega, USA). The components and their corresponding volumes are given in Table 3.4. In addition, Table 3.5 shows PCR thermal cycling conditions. After the amplification process has done, obtained the PCR products and then performed gel electrophoresis on 2% agarose gel using 1X TAE buffer, the voltage at 80 V, current of 180 mA, in 35 minutes. Only the temperatures that had given a single band with approximate expected size without primer dimer were chosen to be used as annealing temperature. After that, the PCR products with respective annealing temperature – which had been chosen previously – were sent to sequencing service for sequencing (MyTACG Bioscience Enterprise, Malaysia). According to the service provider, the method of sequencing used was Sanger sequencing, while the primers used for sequencing were as same as the primers used for amplification (Table 3.3). Then after these PCR products had been sequenced, a BLAST check was conducted against the nucleotide database at NCBI in order to confirm the obtained sequence.

Table 3.3: Oligonucleotide primers used in the present study.

Target gene	GeneBank accession No.	Primer name	Type	Sequence (5′–3′)	Tm	Length (bp)	Pro-duct size (bp)	Reference
Litopenaeus vannamei catalase (Cat) mRNA	AY518322.1	CatGenFw	Forward	GCGTGCGATTGACAAGATAAAG	62	22	113	Self-designed
		CatGenRv	Reverse	CAACCAACACCTTCCACATCTA (*)	62	22		
Litopenaeus vannamei Na,K-ATPase alpha subunit mRNA	KF765670.1	NaKGenFw	Forward	GGTCCTAGAGGGCAACATTAAG	62	22	103	Self-designed
		NaKGenRv	Reverse	CTCAAACCAGCACCAACAATC (*)	62	21		
Litopenaeus vannamei heat shock protein 70 gene	AY645906.1	hsp70 F	Forward	CTCCTGCGTGGGTGTGTT	58.60	18	120	Qian et al. (2012)
		hsp70 R	Reverse	GCGGCGTCACCAATCAGA	58.89	18		

(*): Primers were designed using the PrimerQuest Tool

Table 3.4: Components of PCR reaction for amplification of *L. vannamei* catalase, Na^+/K^+ ATPase α-subunit, and hsp 70.

Reagent	Final concentration	Volume per reaction
5X Green GoTaq® Flexi Buffer	1X	5 µl
$MgCl_2$ (25 mM)	1.5 mM	1.5 µl
dNTPs (10 mM)	0.2 mM each dNTP	0.5 µl
Forward primer (10 µM)	0.2 µM	0.5 µl
Reverse primer (10 µM)	0.2 µM	0.5 µl
GoTaq® Flexi DNA polymerase (5 u/ µl)	1.25 u	0.25
Template		1.2 µl

Table 3.4, continued.

Nuclease-free water	15.55 µl
Total volume	**25 µl**

Table 3.5: Thermal cycling conditions for PCR amplification.

Step	Temperature	Time	Number of cycles
Initial denaturation	95ºC	30s	1 cycle
Denaturation	95ºC	5s	40 cycles
Annealing	Temperature gradient: - 57ºC to 67ºC (for CatGenFw and CatGenRv primers, and NaKGenFw and NaKGenRv primers) - 53ºC to 63ºC (for hsp70 F and hsp70 R primers)	30s	
Extension	72ºC	30s	
Final extension	72ºC	10 min	1 cycle
Hold	4ºC	∞	

3.5 Quantification of mRNA Expression of *L. vannamei* Catalase, Na^+-K^+ ATPase α-subunit and Heat Shock Protein 70 By Real-Time PCR

The expression of the three target genes in hepatopancreas and gills was measured by qPCR using a Real-Time PCR machine (Mx3005P qPCR System, Agilent Technologies, Inc., Santa Clara, CA, USA). SYBR Green I was used as a fluorescent dye for detection and ROX was the reference dye used to normalize fluorescent reporter signal. In addition, the elongation factor – EF-1 was used as an internal control to normalize gene expression. Each target gene along with the reference gene and no template control (primer control) was analyzed in one plate for qRT-PCR. Moreover, each reaction was repeated 3 times.

Promega GoTaq qPCR Master mix components (Promega, USA) were used for all qPCR reactions. The components and their corresponding volumes are shown in Table 3.6. Prior to performing qPCR reactions, the synthesized cDNA was diluted 1:5 in nuclease-free water. Briefly, reaction volumes were 20 µl and included 4 µl of diluted cDNA (approximate concentration was 10 ng/ µl), 0.4 µl of each primer (10 µM), 10 µl of GoTaq® qPCR Master Mix, 0.4 µl of passive reference dye (50x), and 4.8 µl of nuclease-free water. The thermal cycling conditions were as follows: step 1, 95ºC for 3 min; step 2, 40 cycles of 95ºC for 15s and 60ºC for 30s. The combined annealing/extension temperature at 60 ºC was ideal for all primers. Lastly, at the end of the process, performed a melting curve analysis by following the instructions for melting curve analysis which is provided by the manufacturer. Table 3.7 shows the thermal profiles of the SYBR Green Real-Time PCR program.

Table 3.6: Reagents used for the quantification of catalase, Na^+/K^+ ATPase α-subunit and heat shock protein 70 and their corresponding volume.

Reagent	Final concentration	Volume
(10 µM) Forward Primer	200 nM	0.4 µl
(10 µM) Reverse Primer	200 nM	0.4 µl
GoTaq® qPCR Master Mix, 2X		10 µl
Passive Dye (50x)		0.4 µl
RNA –free water		4.8 µl
Template cDNA	≤ 100 ng/reaction	4 µl
Total volume		**20 µl**

Table 3.7: Thermal profiles of the SYBR Green Real-Time PCR.

Step		Temperature	Time	Cycles
PCR initial activation step	Initial activation	95ºC	3 min	1
2-step cycling	Denaturation	95ºC	15s	40
	Combined annealing/extension	60ºC	30s	
Melting curve analysis		95ºC	1 min	1
		55ºC	30s	1
		95ºC	30s	1
Hold		10ºC	∞	

3.6 Statistical Analysis

In qPCR, a quantitative endpoint is the threshold cycle (CT). The CT is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. For quantification of gene expression, the cycle of threshold (Ct) for each gene transcript needs to be determined (Schmittgen et al., 2008).

Ct value of the PCR amplification curve of the target gene is compared with the Ct value of the internal reference gene to obtain ΔCt, which is then used to conduct relative quantitative analysis on the expression level of the target genes by using the comparative CT method (also referred to as the $2^{-\Delta\Delta Ct}$ method) (Schmittgen et al., 2008).

Expression ratio: $2^{-\Delta\Delta Ct}$ = Normalized expression ratio

$$\Delta\Delta Ct = \Delta Ct \text{ (sample A)} - \Delta Ct \text{ calibrator} \quad (3.2)$$

$$\Delta Ct \text{ (sample A)} = Ct \text{ (target gene)} - Ct \text{ (reference gene)}$$

$$\Delta Ct \text{ (calibrator)} = Ct \text{ (target gene)} - Ct \text{ (reference gene)}$$

In this present study, each sample test consisted of three biological replicates, for each biological replicated, three technical replicates were run. In addition, EF-1 was used as an internal reference gene. The data analysis was needed to be undergone different steps in order to obtain the $2^{-\Delta\Delta CT}$ results. Firstly, averaged the cycle threshold (Ct) values which were obtained from three technical replicates of qPCR for each of the biological replicates. Secondly, calculated ΔCt for each biological replicate by using the newly created average Ct values. Since the data obtained from week 2 were used as a calibrator, therefore, averaged the ΔCt obtained from three biological replicates. Next, transformed these ΔCt values to the formula of ΔΔCt, where ΔΔCt = ΔCt Week x – Avg. ΔCt Week 2. In particular, week x is referred to as the data obtained from week 3, week 4, week 5 and week 6; while Avg. ΔCt Week 2 means averaged ΔCt that obtained from week 2. Then, for each sample, averaged the newly calculated ΔΔCt obtained from the three biological replicates. Next, transformed the average ΔΔCt to $2^{-\Delta\Delta CT}$. However, it has been investigated that, sometimes the value of $2^{-\Delta\Delta CT}$ is < 1. This indicates that there has been a reduction in the expression due to treatment. In this case, the fold change reduction in expression is calculated by taking the negative inverse of $2^{-\Delta\Delta CT}$ (Schmittgen et al., 2008). Therefore, the transformation of the normalized expression ratio $2^{-\Delta\Delta CT}$ into fold change was done by looking at the $2^{-\Delta\Delta CT}$ values:

If the value of $2^{-\Delta\Delta CT}$ <1, fold change = $-1/2^{-\Delta\Delta Ct}$

If the value of $2^{-\Delta\Delta CT}$ >1, fold change = $2^{-\Delta\Delta CT}$

All of the calculation was done using Microsoft Excel 2011. Meanwhile, the significance of the differences in expression was determined using one-way ANOVA followed by Tukey post hoc analysis. In addition, the relationship between environmental stressors and relative expression of stress-related genes was developed by using simple linear regression analysis followed by one-way ANOVA. Besides, Bivariate Correlation

was used to determine the correlation between different time points of cultivation and changes in levels of water quality parameters including DO, salinity, pH, and temperature; while the significant differences in levels of these parameters among different categories were done using one-way ANOVA followed by Tukey post hoc analysis. All of the statistical analysis was performed by SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). P values less than 0.05 were considered statistically significant. The bar charts were drawn using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA).

The error bars on the plotted diagrams are given as the standard error of the mean (SEM). Formulas used for the formation of SEM is as below:

$$SD_{\Delta\Delta Ct} = \sqrt{(SD_{\Delta Ct}\ of\ sample\ A\ )^2 + (SD_{\Delta Ct}\ of\ calibrator)^2} \qquad (3.3)$$

$$SEM\ = \frac{SD_{\Delta\Delta Ct}}{\sqrt{n}}$$

Where:

SD = standard deviation

SEM = standard error of the mean

n = number of samples

CHAPTER 4: RESULTS

4.1 Sample Collection, Parameters of Water Quality and Survival of The Cultured Shrimps

All the collected samples were labeled according to the time of collection and the name of the tank where the samples were collected. The record of the label is as Table 4.1. below.

Table 4.1: Label of collected samples.

Time of collection	Tank 4 (Microbubble)	Tank 9 (Mixture)	Tank 10 (Macro-bubble with bioballs)	Tank 13 Macro-bubble without bioballs)
Week 0 (the 1st day of culture)	W0T4	W0T9	W0T10	W0T13
Week 1 (the 6th day of culture)	W1T4	W1T9	W1T10	W1T13
Week 2 (the 13th day of culture)	W2T4	W2T9	W2T10	W2T13
Week 3 (the 19th day of culture)	W3T4	W3T9	W3T10	W3T13
Week 4 (the 27th day of culture)	W4T4	W4T9	W4T10	W4T13
Week 5 (the 34th day of culture)	W5T4	W5T9	W5T10	W5T13
Week 6 (the 37th day of culture)	W6T4	W6T9	W6T10	W6T13

Values of water quality parameters including DO, salinity, pH, and temperature were noted according to the record provided by the co-workers. Variations of these water quality parameters in each culture tank throughout cultivation are shown in Figure 4.1. Theoretically, the values of these parameters should be maintained stable and the differences are not expected to see throughout the culture since the biofloc system is believed to maintain water quality well. If any of these parameters change over the culture time, the changes are expected to be non-significant. As shown in Figure 4.1, values of

DO, salinity, pH, and temperature in all tanks varied throughout the culture time since the values dropped at this time point but increased at the other point. Unfortunately, this current study failed to perform any statistical analyses to investigate if these changes in DO, salinity, pH, and temperature levels were statistically significant differences since the total sample size (n = 1) was not sufficient enough to perform the analysis test. The reason for having a small sample size (n = 1) was due to mistakes made during the measurement of water quality parameters. Each parameter had been measured once only at each measurement time, thus only one value of the parameter was obtained for each time point. However, the sample size required for a successful statistical analysis shall be not less than 3 ($n \geq 3$), this means each parameter must be measured at least 3 times to obtain at least 3 values for each time point. This is also one of the limitations of the study.

Since this current study could not be able to identify whether changes in DO, salinity, pH and temperature levels were statistically significant differences, correlations between different time points and levels of water parameters were established instead to identify if levels of DO, salinity, pH and temperature increase or decrease during the culture. Besides, differences in levels of these parameters among different four culture conditions were also established (Figure 4.2).

DO ranged from 9 - 10.61 (mg/L) in tank 4, in tank 9 was 8.28 to 9.23 (mg/L), in tank 10 varied from 7.24 to 7.67 (mg/L), in tank was at between 7.24 to 7.58 (mg/L). Results showed that there was a negative correlation between time of cultivation and DO levels in tank 4 ($r = -.875$, $p < 0.01$), indicating a decrease of DO levels throughout the culture. Meanwhile, no statistically significant correlation found between time of cultivation and DO levels in tank 9 ($r = -.794$, $p > 0.05$), tank 10 ($r = -.160$, $p > 0.05$), and tank 13 ($r = -.302$, $p > 0.05$). Besides, the differences of DO levels among four different culture

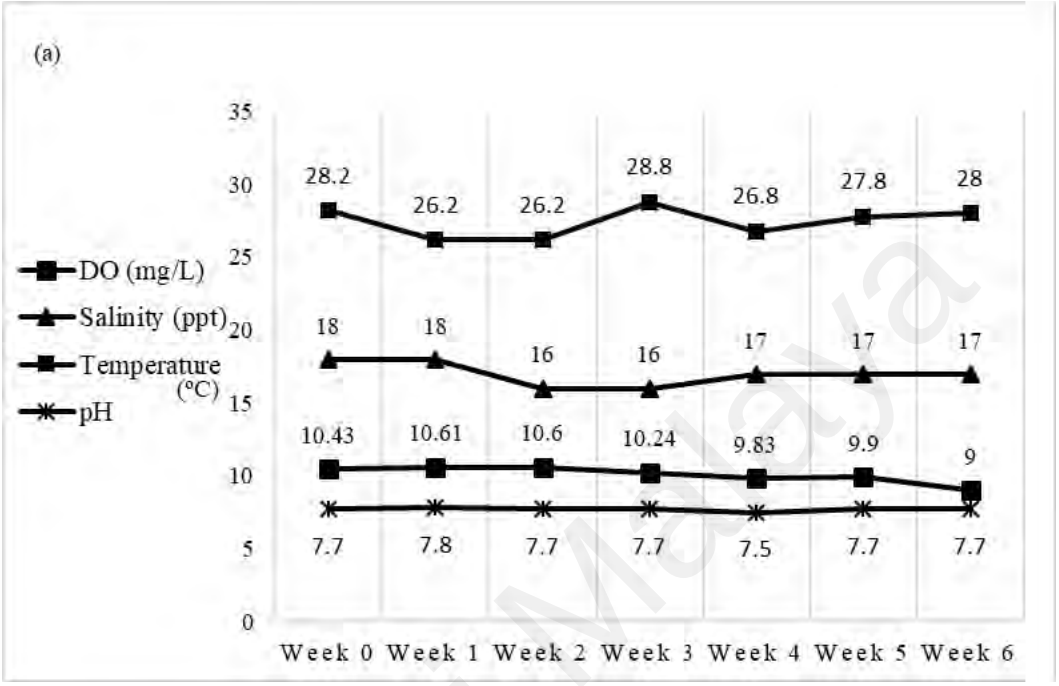
conditions were statistically significant ($p < 0.05$), the highest level was archived in tank 4, and the lowest levels were found in tank 10 and tank 13.

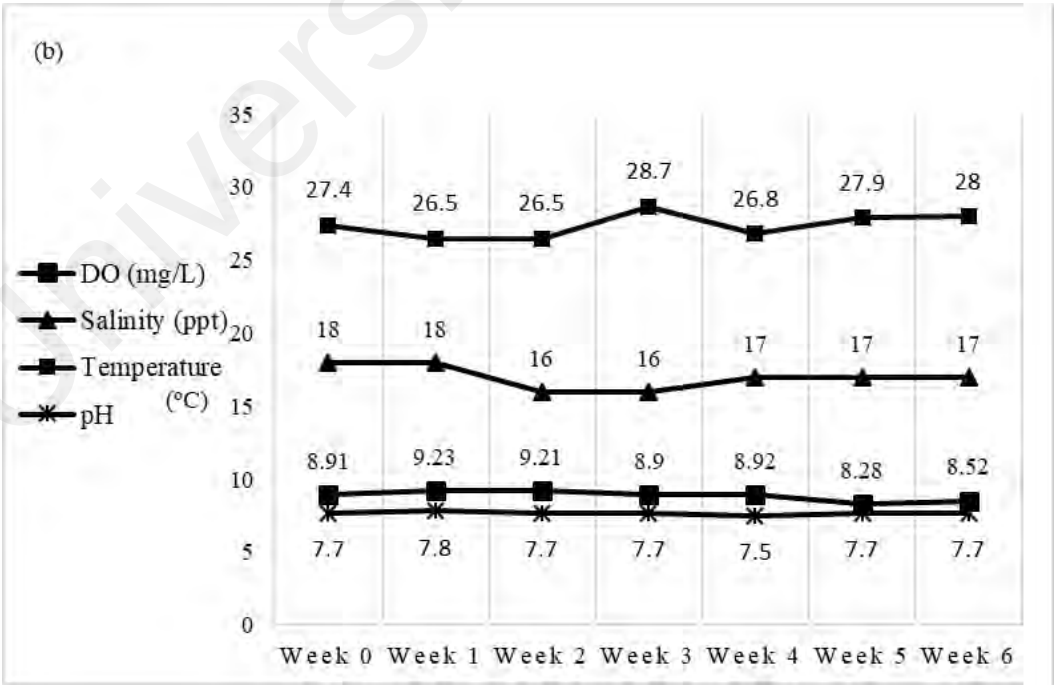
Meanwhile, salinity ranged from 16 to 18 (ppt) in tank 4 and tank 9, and 16 to 20 (ppt) in tank 10 and tank 13. No statistically significant correlation found between time of cultivation and salinity levels in tank 4 and tank 9 ($r = -.378$, $p > 0.05$). Meanwhile, there was a negative correlation between time of cultivation and DO levels in tank 10 and tank 13 ($r = -.926$, $p < 0.01$), indicating a decrease of salinity levels throughout the culture. Furthermore, the differences in salinity levels among four different culture conditions were not statistically significant (p-value > 0.05).

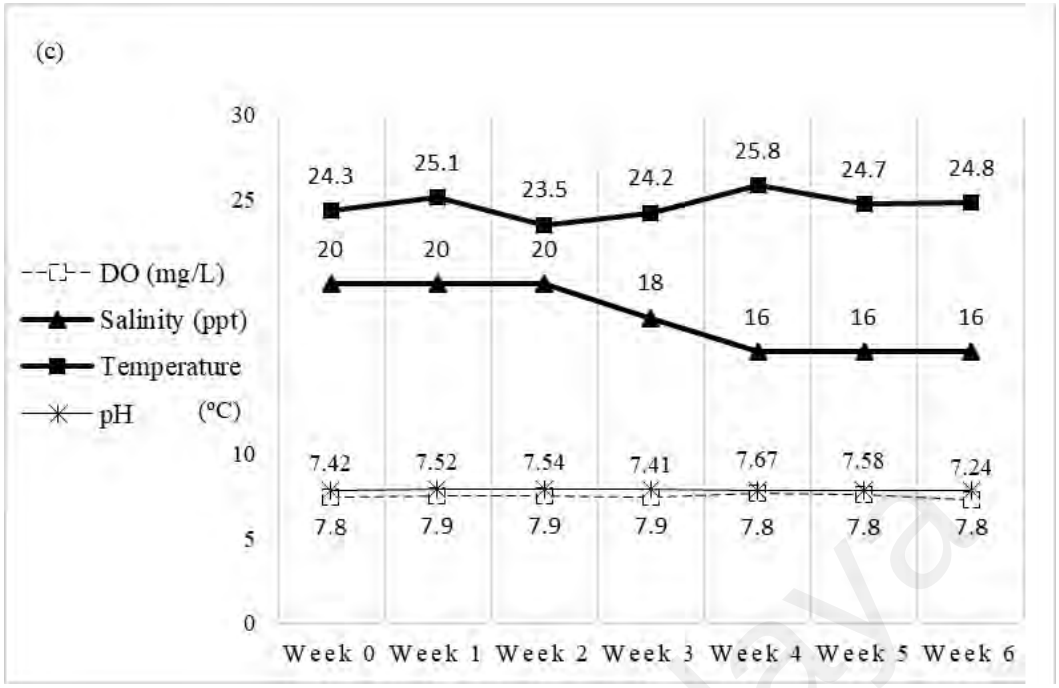
pH ranged from 7.5 to 7.8 in tank 4 and tank 9, and 7.8 to 7.9 in tank 10 and tank 13. No statistically significant correlation found between time of cultivation and pH levels in tank 4 and tank 9 ($r = -.343$, $p > 0.05$), in tank 10 ($r = -.433$, $p > 0.05$). Meanwhile, there was a negative correlation between time of cultivation and pH levels in tank 13 ($r = -.866$, $p < 0.05$), indicating a decrease of pH level throughout the culture. Besides, the differences of pH among four different culture conditions were statistically significant ($p < 0.05$), the highest level was obtained in tank 10 and tank 13, while the lowest level was found in tank 9 and tank 10.

Lastly, temperature in tank 4 ranged from 26.2 to 28.2 (°C), in tank 9 it was 26.5 to 28.7 (°C), in tank 10 it varied from 24.2 to 25.8 (°C), and 24.2 to 25.7 (°C) in tank 13. No statistically significant correlation found between time of cultivation and temperature in tank 4 ($r = .240$, $p > 0.05$), tank 9 ($r = .448$, $p > 0.05$), tank 10 ($r = .317$, $p > 0.05$) and tank 13 ($r = .423$, $p > 0.05$). However, there were statistically significant differences found in temperature values among four different culture conditions ($p < 0.05$), the highest values

were archived in tank 4 and tank 9, while the lowest values were found in tank 10 and tank 13.







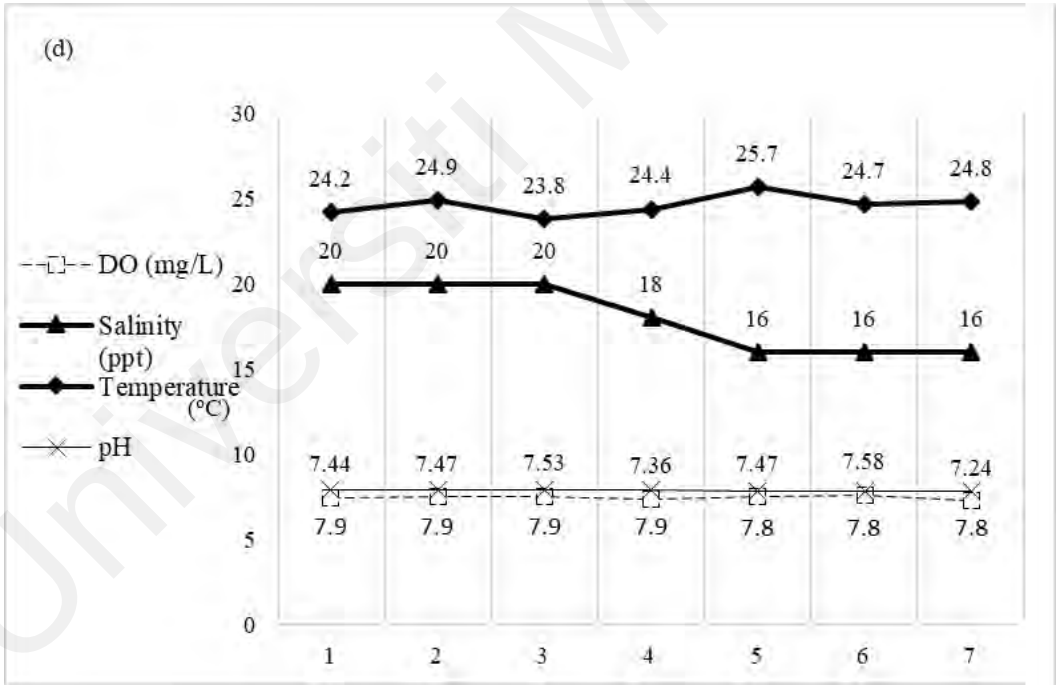
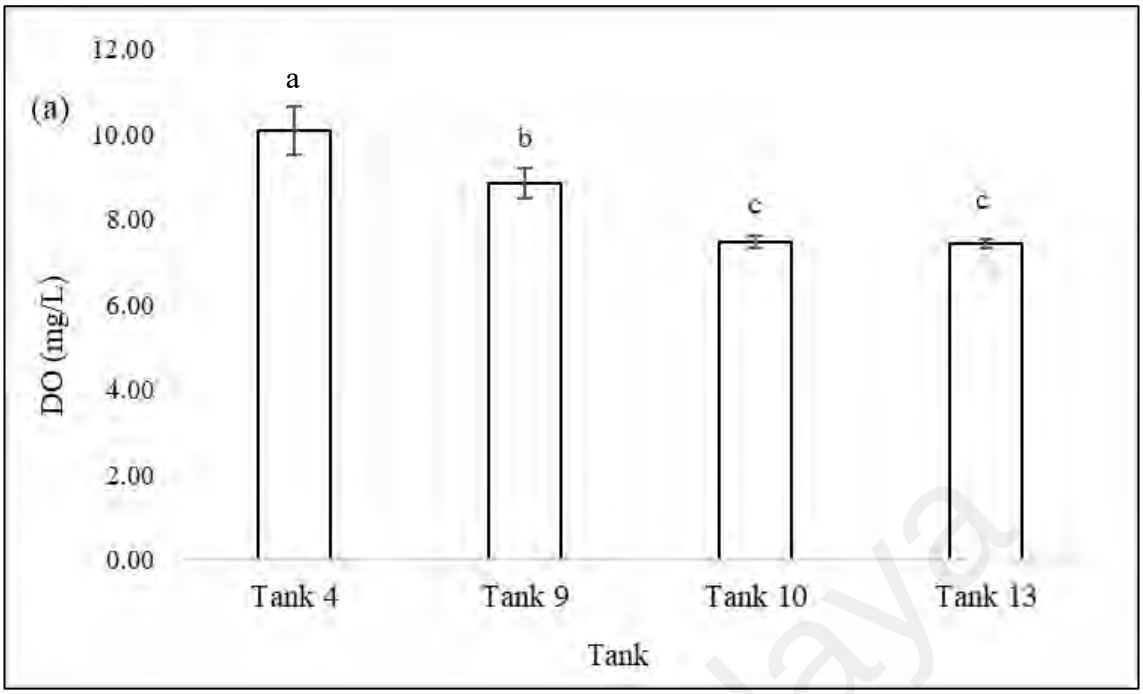
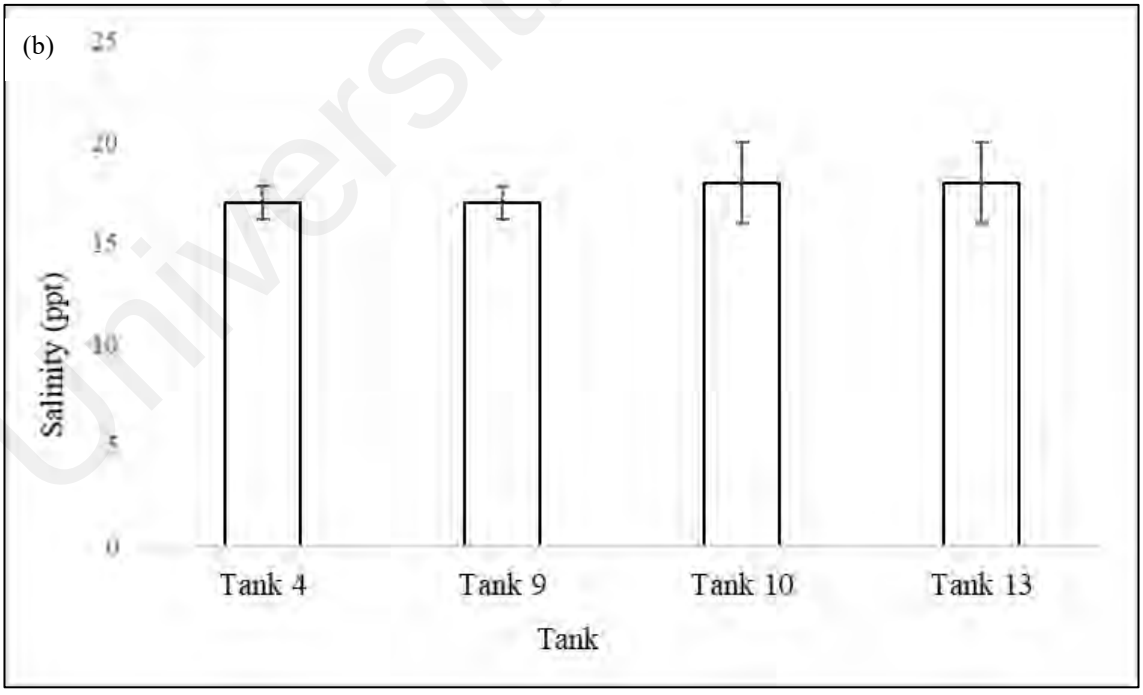
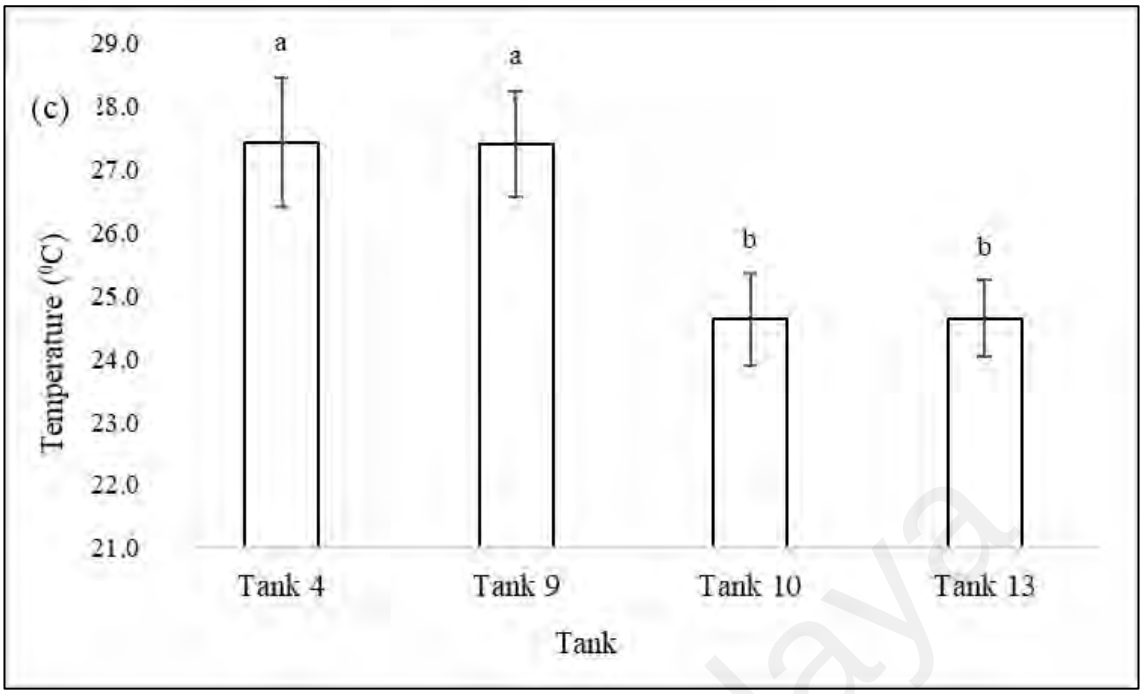


Figure 4.1: Variation of water quality parameters in (a) tank 4, (b) tank 9, (c) tank 10, (d) tank 13 during cultivation.







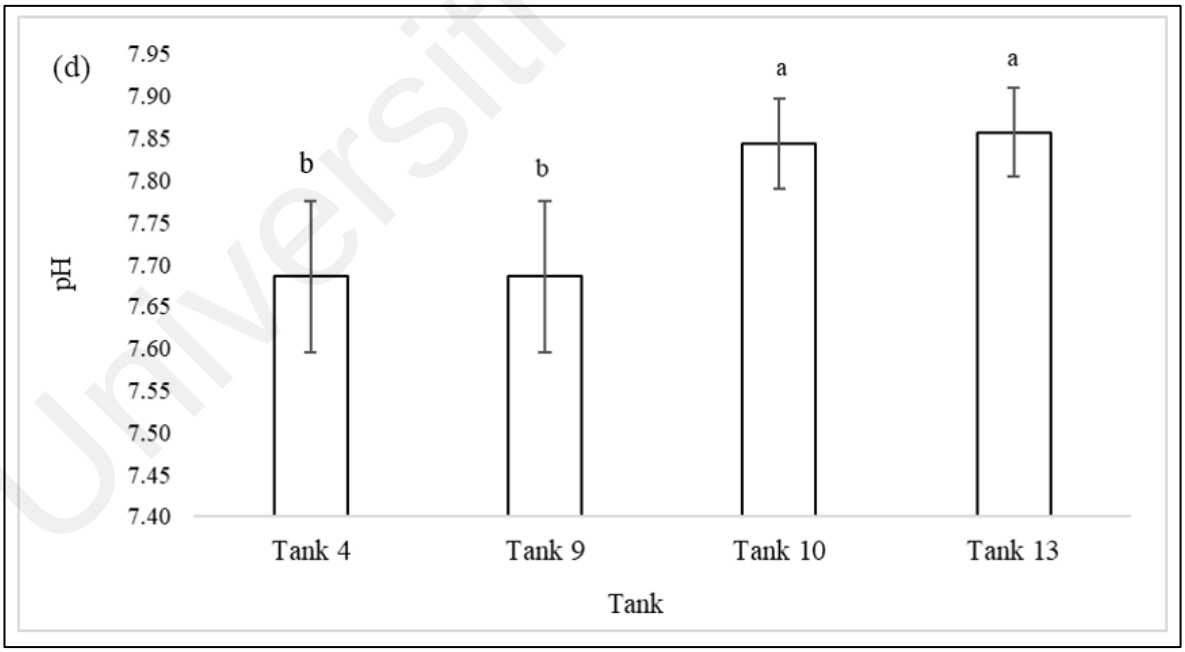


Figure 4.2: Differences in levels of (a) DO, (b) salinity, (c) temperature, and (d) pH among different four culture conditions. Data are presented as means ± SE (standard error). Different lowercase letters indicate statistically significant differences ($p < 0.05$).

The concentration of ammonia and nitrite obtained in each tank throughout the culture is as Table 4.2 below. Overall, ammonia was found in all of the four tanks, meanwhile, nitrite was found in tank 13 during the first three weeks. In particular, ammonia levels were the same in tank 4 and tank 9 with the average value was at 0.21±0.09, in tank 10 it was 0.18±0.12, and tank 13 obtained a lower ammonia level at 0.11±0.13. Besides, no nitrite found in tank 4, 9 and 10 throughout the culture, while in tank 13 it was found at 0.18±0.19.

Table 4.2: Concentration of ammonia and nitrite.

Concentration \ Time		Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Average
Tank 4	Ammonia (ppt)	0.25	0	0.25	0.25	0.25	0.25	0.25	0.21±0.09
	Nitrite (ppt)	0	0	0	0	0	0	0	0
Tank 9	Ammonia (ppt)	0.25	0	0.25	0.25	0.25	0.25	0.25	0.21±0.09
	Nitrite (ppt)	0	0	0	0	0	0	0	0
Tank 10	Ammonia (ppt)	0	0	0.25	0.25	0.25	0.25	0.25	0.18±0.12
	Nitrite (ppt)	0	0	0	0	0	0	0	0
Tank 13	Ammonia (ppt)	0.25	0	0.25	0.25	0	0	0	0.11±0.13
	Nitrite (ppt)	0.5	0.25	0.25	0.25	0	0	0	0.18±0.19

The number of shrimp stocks, the number of shrimps survived, and the survival rate is given in Table 4.3. In tank 4, the survival rate was obtained at 28%, it was at 96.67% in tank 9, while the survival rate in tank 10 was 100%, and 97.50% in tank 13.

Table 4.3: Survival rate obtained.

Category / Number of shrimps	**Tank 4** (Microbubble)	**Tank 9** (Mixture)	**Tank 10** (Macro bubble with bioballs)	**Tank 13** (Macro bubble without bioballs)
At the beginning of culture	100	120	170	120
At the end of culture	38	116	170	117
Survival rate (%)	38	96.67	100	97.50

4.2 RNA Extraction and Quality Analysis

A ratio of A260-A280 ranging from 1.8 to 2.1 at pH 7.5 is widely accepted since this indicates that the RNA is highly pure. In addition, the ratio A260-A230 is used to examine the purity of nucleic acid. It has been investigated that RNA that is pure usually yields a ratio of A260-A230 at approximately 2 or slightly more than 2, but, so far, there has been no generally accepted values on the acceptable lower limit of this ratio. Moreover, the contribution of contaminants to a low ratio of A260-A230 has not been fully established. Some possible candidates that caused contamination are “salt”, carbohydrates, peptides, and phenol (Silke et al., 2010)

The 260/280-nm absorbance of all 60 samples in this study had desirable 260-280 ratios since their value was from 2.00 to 2.28. This indicates that extracted RNA was adequate pure. Meanwhile, the 260/230-nm absorbance ranged from 0.89 to 2.24. Previous studies have investigated that the increase of absorbance at 230 nm in total RNA is usually caused by the contamination of guanidine thiocyanate. This is a slat that has a strong absorbance at 220–230 nm. Moreover, it usually presents in the extraction reagent or the lysis buffer, which are used in most RNA purification procedures such as TRIzol®, at an extremely high level of concentration. Furthermore, previous studies showed that the A260-A230 ratio of RNA samples will be strongly decreased if there is a presence of

guanidine thiocyanate even at submillimolar concentrations (Silke et al., 2010). Since all the RNA of this study were extracted by the TransZol Up, thus the low value of the 260-230-nm absorbance might due to the contamination of guanidine thiocyanate contained in the TransZol reagent. However, according to studies of Silke et al. (2010), guanidine thiocyanate concentration of up to 100 mM in the RNA samples will not lower the reliability of qPRC. Also, in the study of Kuang et al. (2018), no significant correlations between the amplification efficiency of qPCR and A260-A230 were found. Therefore, although some of the extracted RNA in this study had a low value of the A260-A230 absorbance, as their values of A260-A280 above 2.0 indicate no contamination of DNA genomic, their qualities were thought to be sufficient enough to give reliable and comparable results in downstream applications including qPCR. However, there were three samples that had a ratio of A260-A230 at lower than 1, these samples were re-extracted to avoid any problem or inaccurate result caused during qPCR performance later.

The assessment of the integrity of RNA of all samples then was done on 1% agarose gel. Most of the RNA samples had two sharp bands, this indicates that the RNA was intact (Figure 4.3). While some of them appeared as a lightly smear rather than sharp bands, this indicates that the RNA might be degraded.

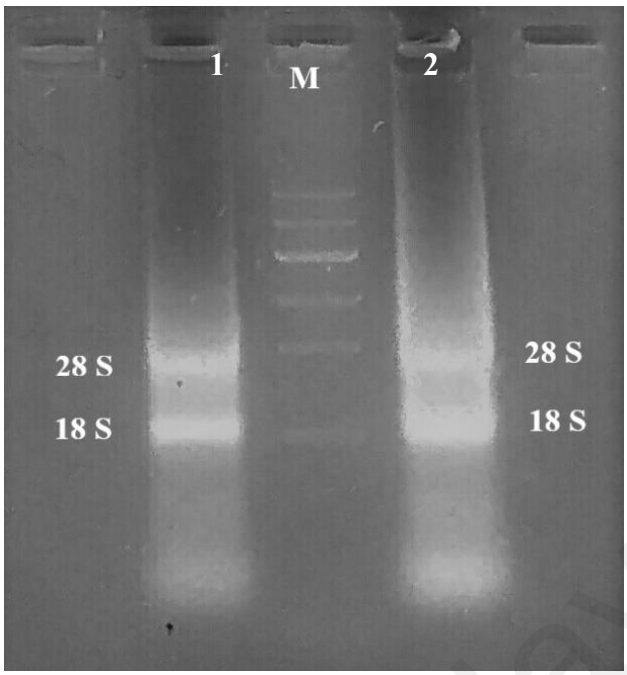


Figure 4.3: Electrophoresis result for the assessment of the integrity of the extracted RNA. Lane M represents 1kb maker. Lane 2 represents extracted RNA from sample W2T4a. Lane 2 represents extracted RNA from sample W2T4b.

4.3 Primers Optimization

The annealing temperature for the amplification of catalase and Na,K-ATPase alpha subunit was investigated at 59 ºC, and 56 ºC for the amplification of hsp70 (Table 4.4). Figure 4.4 presents the gel electrophoresis results for the amplification of target genes at the chosen annealing temperature and their desired products.

Table 4.4: Appropriate annealing temperature for the amplification of desired products.

Target gene	Primer name	Ta	Length (bp)	Product size (bp)
Litopenaeus vannamei catalase (Cat) mRNA	CatGenFw CatGenRv	59 ºC	22 22	113

Table 4.4, continued.

Litopenaeus vannamei Na,K-ATPase alpha subunit mRNA	NaKGenFw NaKGenRv	59 °C	22 21	103
Litopenaeus vannamei heat shock protein 70 gene	hsp70 F hsp70 R	56 °C	18 18	120

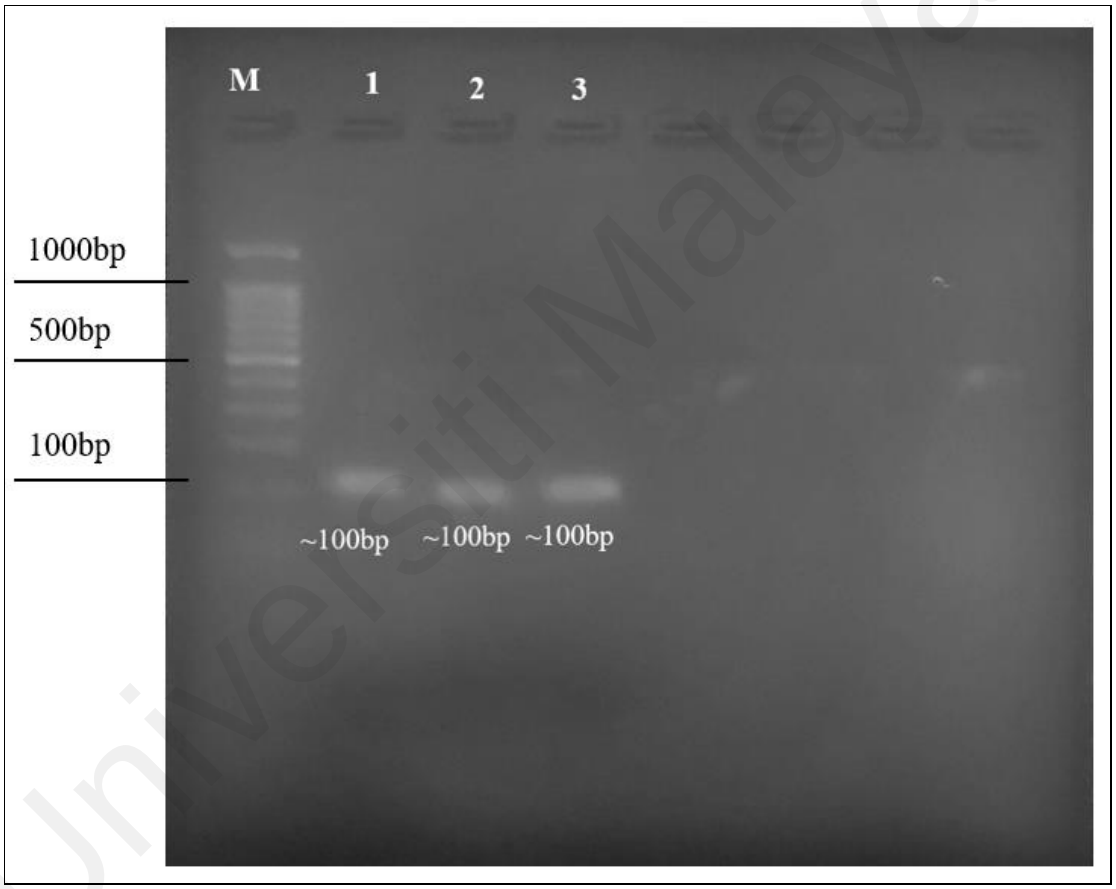


Figure 4.4: Electrophoresis results for the amplification of target genes. From left to right, Lane M represents 100 bp DNA ladder. Lane 1 represents PCR product upon using CatGenFw primer and CatGenRv primer, annealing temperature was at 59°C. Lane 2 represents the PCR product upon using NaKGenFw primer and NaKGenRv primer, annealing temperature was at 59°C. Lane 3 represents PCR product upon using hsp70F primer and hsp70R prime, annealing temperature was at 56°C.

The BLAST result of the catalase sequences gave 100% similarity to the *L. vannamei* catalase (Cat) mRNA, complete cds (Accession number AY518322.1). Meanwhile, the BLAST result of the Na^+-K^+ATPase α-subunit sequences gave 98% similarity to the *L. vannamei* Na,K-ATPase alpha subunit mRNA, complete cds (Accession number KF765670.1). Lastly, the BLAST result of the heat shock protein 70 gave 99% similarity to the *L. vannamei* heat shock protein 70 gene, complete cds (Accession number AY645906.1). The success of PCR amplification and the high similarity in comparison with an available database of the targeted genes indicating that the designed primers are efficient and can be used for the qPCR experiment.

4.4 Quantification of mRNA Expression of *L. vannamei* Catalase, Na^+-K^+ ATPase α-subunit and Heat Shock Protein 70 By Real-Time PCR

In accordance with the $2^{-\Delta\Delta CT}$ method as described in the previous chapter, the samples which were collected at week 2 from each category were used as the calibrator. The reason for the selection of samples from week 2 as a calibrator is to avoid the possibility of false results. According to the study of Mukherjee et al. (2009), when fishes are introduced in a new habitat ecologically different from their initial place of rearing but is still within its range of tolerance, they may undergo a number of cellular and physiological changes which are variable and flexible during the period of acclimatization before they are adapted and grow well in the conditions. Factors that cause physiological changes including changes in water quality, high stocking density, and interactions between individuals. In addition, the time required for fish to acclimatize new conditions depends on the quality of water, especially the concentration of ammonia in the water. Therefore, in the present study, the samples collected at week 0 (after stocks had been transported to the laboratory and then had been transferred into the biofloc system) could not be used as calibrators since the shrimps would be stressed due to transportation, as stress during the

transportation is unavoidable. Meanwhile, the samples collected at week 1 were not appropriate for the use as a calibrator, since the shrimps need time to adapt to the new conditions, usually from 5-10 days, therefore any type of stress occurs within this period is caused by adaptation activities, not by the system itself.

Relative expression of catalase in tank 4 ranged between -3.47 ± 1.41 to 2.02 ± 0.87, it varied from -5.23 ± 0.55 to -1.22 ± 0.92 in tank 9, in tank 10 it was -2.15 ± 0.63 to 1.06 ± 0.65, and from -1.31 ± 1.12 to 3.79 ± 1.04 in tank 13.

Relative expression of Na^+-K^+ATPase α-subunit in tank 4 varied from -2.41 ± 0.58 to 1.93 ± 0.67, it ranged from -2.14 ± 0.93 to -1.04 ± 0.65 in tank 9, in tank 10 it was -1.73 ± 0.75 to 1.31 ± 0.43, and -5.40 ± 0.70 to -1.36 ± 0.41 in tank 13.

Relative expression of hsp70 in tank 4 was 1.03 ± 0.77 to 2.21 ± 0.59, it ranged from -1.11 ± 1.10 to 1.23 ± 0.93 in tank 9, in tank 10 it varied from 1.61 ± 0.99 to 2.81 ± 0.96, and 2.46 ± 1.05 to 3.43 ± 0.99 in tank 13.

4.4.1 Relative Expression of Catalase

The expression profiles of the catalase gene of *P. vannamei* shrimps in the biofloc system are shown in Figure 4.5. Quite different expressions were observed in the catalase level of *P. vannamei* in different categories. At week 3 and week 4 of the culture, the relative expression of catalase of the shrimps that were collected from tank 4, 9 and 10 was down-regulated, while the catalase level of the shrimps which were collected from tank 13 was up-regulated. However, no significant differences were found in these relative expressions of catalase. Starting from week 5 and week 6, quite different expressions of catalase were observed. Particularly, the relative expressions of catalase of the shrimps which were collected from tank 4 and tank 10 were increased and become

up-regulated. Meanwhile, the expression of catalase of the shrimps which were collected from tank 9 at week 5 and week 6 was still down-regulated and no significant differences were found. Lastly, relative expression in the catalase level of the shrimps which were collected from tank 13 at week 5 and 6 was decreased and became down-regulated.

The up-regulated in the relative expression of catalase obtained in tank 4 (week 5 and 6), tank 10 (week 5 and 6), and tank 13 (week 3, 4, and 5) was at the same level.

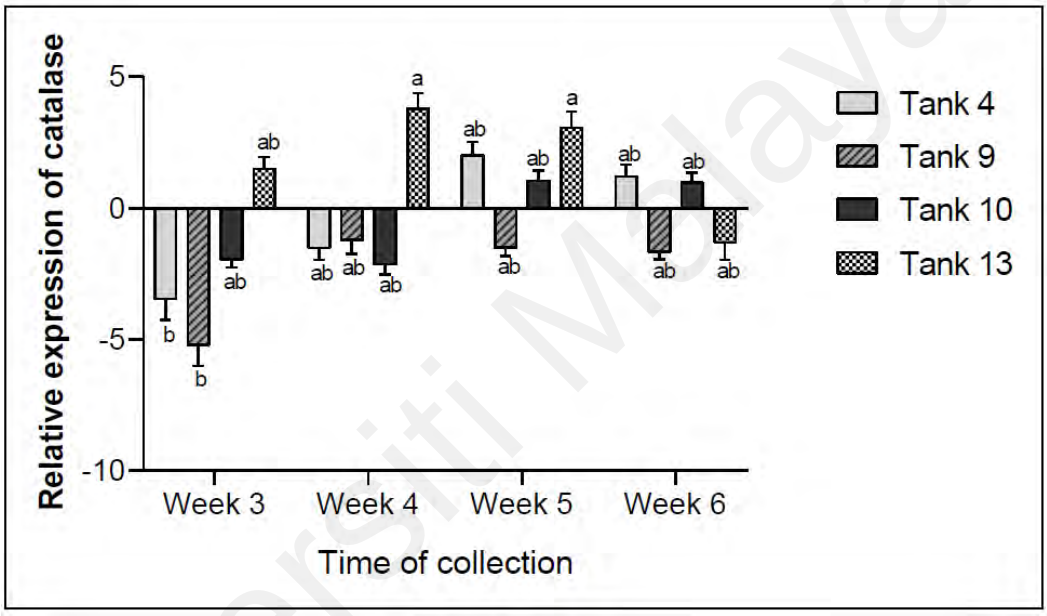


Figure 4.5: Histogram of relative expression of catalase at different times. Shrimp samples were collected at weeks 2, 3, 4, 5 and 6 of the culture. The bars in the graph show the up and down-regulation of catalase. Data are presented as means ± SE (standard error) from three independent biological samples (three shrimps) with three technical replicates each (n=3). Different lowercase letters indicate statistically significant differences ($p < 0.05$) in the relative expression of catalase among different sampling time points.

4.4.2 Relative Expression of Na^+-K^+ ATPase α-subunit

The expression profiles of the Na^+-K^+ ATPase α-subunit gene of *P. vannamei* shrimps in the biofloc system are shown in Figure 4.6. Quite different expressions were observed in Na^+-K^+ ATPase α-subunit level of *P. vannamei* in different categories. At week 3 and week 4 of the culture, all of the relative expression of Na^+-K^+ ATPase α-subunit were

down-regulated. However, no significant differences were found in these relative expressions of Na^+-K^+ ATPase α-subunit. Starting from week 5 and week 6, quite different expressions of Na^+-K^+ ATPase α-subunit were observed. Particularly, the relative expression of Na^+-K^+ ATPase α-subunit in the shrimps collected from tank 4 become up-regulated during week 5 and week 6, while the relative expression of Na^+-K^+ ATPase α-subunit of the shrimps which were collected from tank 10 was down-regulated at week 5, after that, it was increased and became up-regulated in week 6. Meanwhile, the relative expression of Na^+-K^+ ATPase α-subunit of the shrimps which were collected from tank 13 was still down-regulated at week 5 and week 6. Overall, Na^+-K^+ ATPase α-subunit was most expressed in the shrimps which were cultured in tank 4 at week 5.

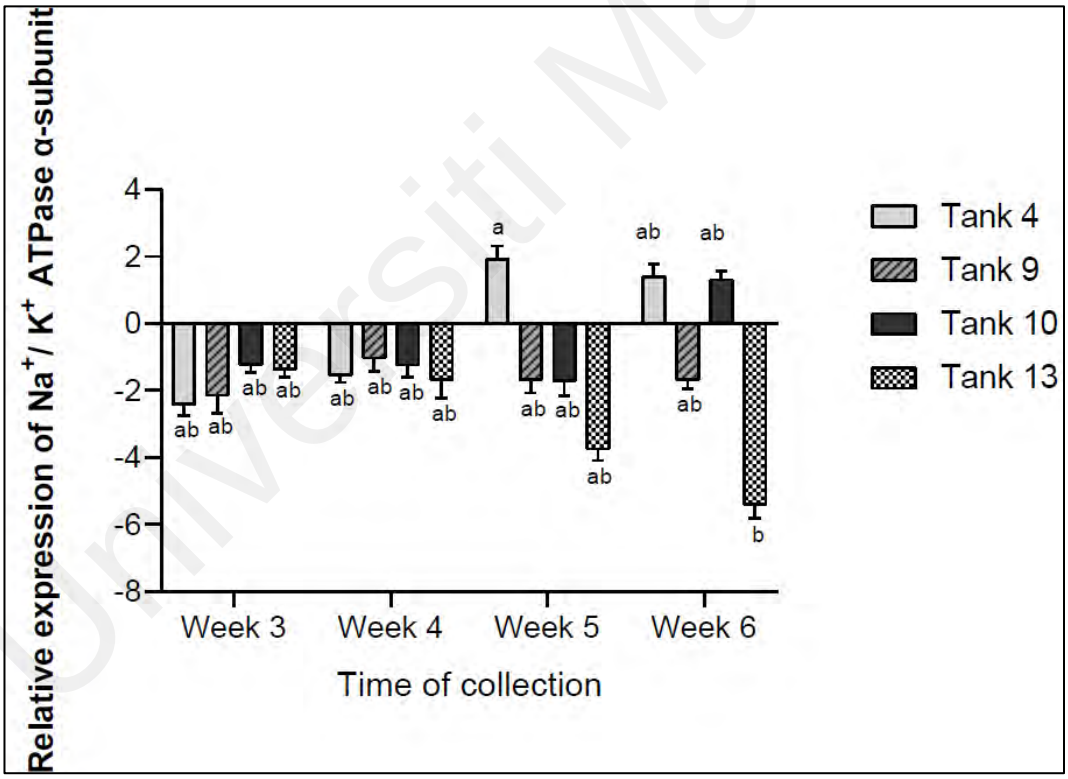


Figure 4.6: Histogram of relative expression of Na^+-K^+ ATPase α-subunit at different times. Shrimp samples were collected at week 2, 3, 4, 5 and 6 of the culture. The bars in the graph show the up and down-regulation of Na^+-K^+ ATPase α-subunit. Data are presented as means ± SE (standard error) from three independent biological samples (three shrimps) with three technical replicates each (n=3). Different lowercase letters indicate statistically significant differences ($p < 0.05$) in the relative expression of Na^+-K^+ ATPase α-subunit among different sampling time points.

4.4.3 Relative Expression of Hsp70

The levels of expression of the hsp70 gene of *P. vannamei* shrimps in the biofloc system are shown in Figure 4.7. Overall, the expression profiles of hsp70 in most of the samples varied, no significant differences were found among these relative expressions ($P > 0.05$). The shrimps that were collected from tank 4, tank 10, and tank 13 had a similar relative expression of hsp70 profiles since they were all up-regulated. Meanwhile, the relative expression of hps70 of the shrimps which were collected from tank 9 was down-regulated at week 3 and week 4, after that the relative expression was increased and became up-regulated at week 5 and week 6.

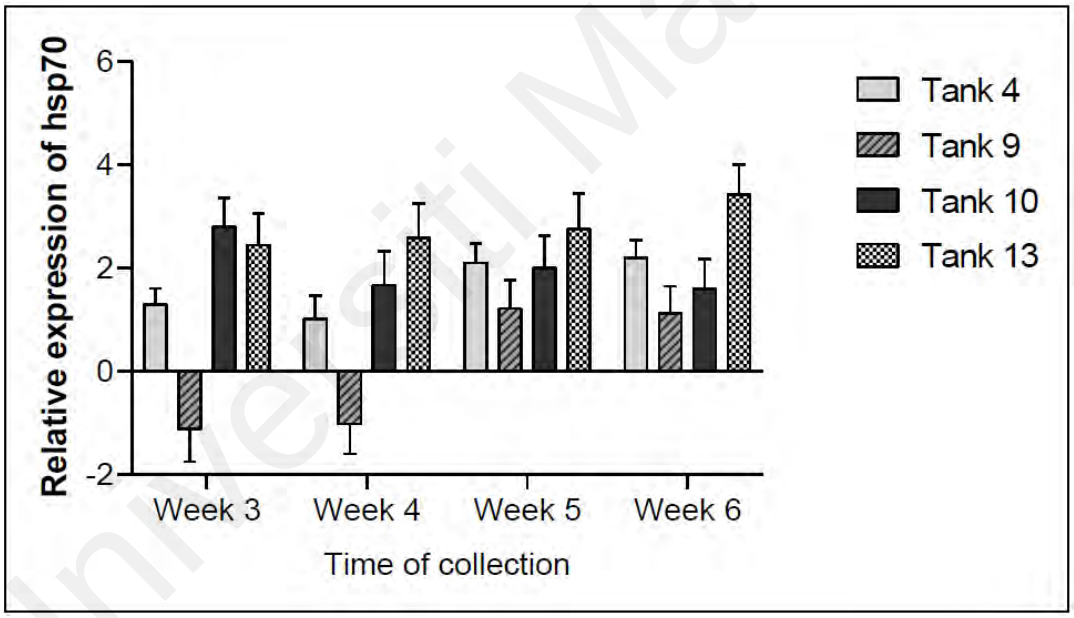


Figure 4.7: Histogram of relative expression of hsp70 at different times. Shrimp samples were collected at week 2, 3, 4, 5 and 6 of the culture. The bars in the graph show the up and down-regulation of hsp70. Data are presented as means ± SE (standard error) from three independent biological samples (three shrimps) with three technical replicates each (n=3). ($p > 0.05$).

4.5 Relationship Between Water Quality Parameters and Relative Expression of Stress-Related Genes

Figure 4.8 shows a negative linear regulatory relationship (R^2 = 0.064) between dissolved oxygen levels and relative expression of catalase. This regression was not significant since the p-value was greater than 0.05. Meanwhile, a positive linear regulatory relationship (R^2 = 0.117) between salinity and relative expression of Na^+-K^+ ATPase α-subunit is shown in Figure 4.9. This regression was not significant since p > 0.05. Lastly, Figure 4.10 presents a negative linear regulatory relationship (R^2 = 0.454) between temperature and relative expression of hsp70. This regression was significant since p < 0.05.

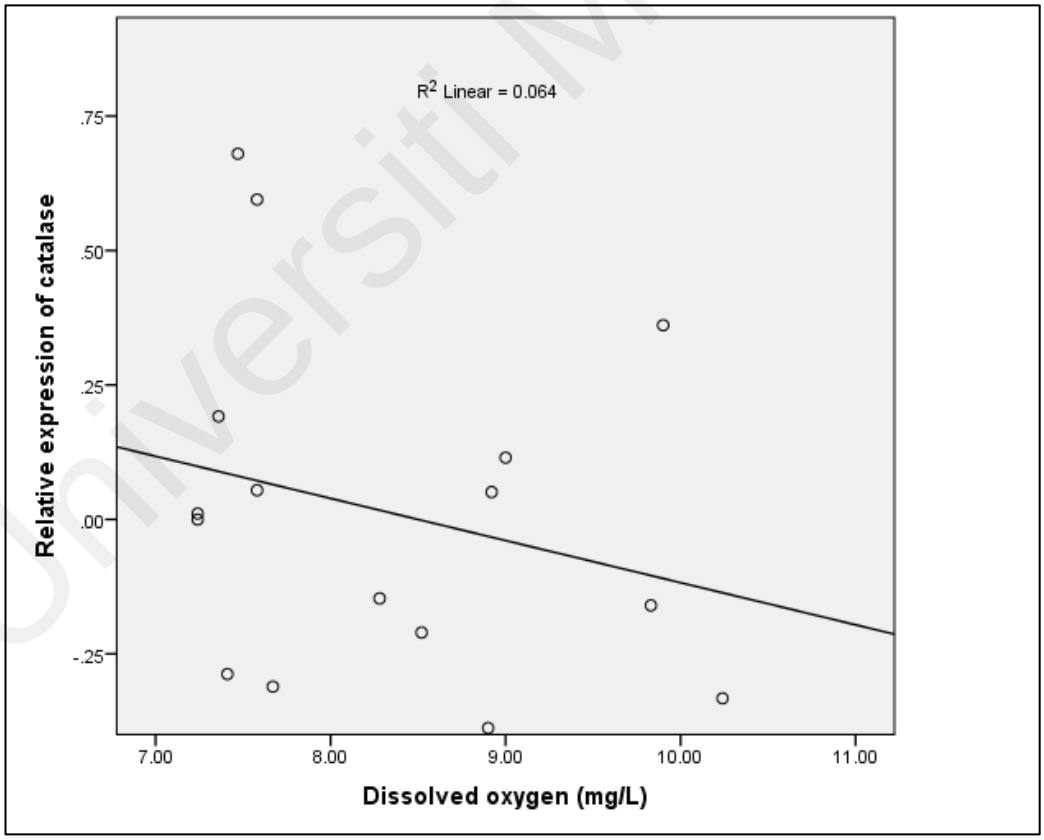


Figure 4.8: Scatter plot showing the relationship between dissolved oxygen level and relative expression of catalase (R^2 = 0.064; p > 0.05).

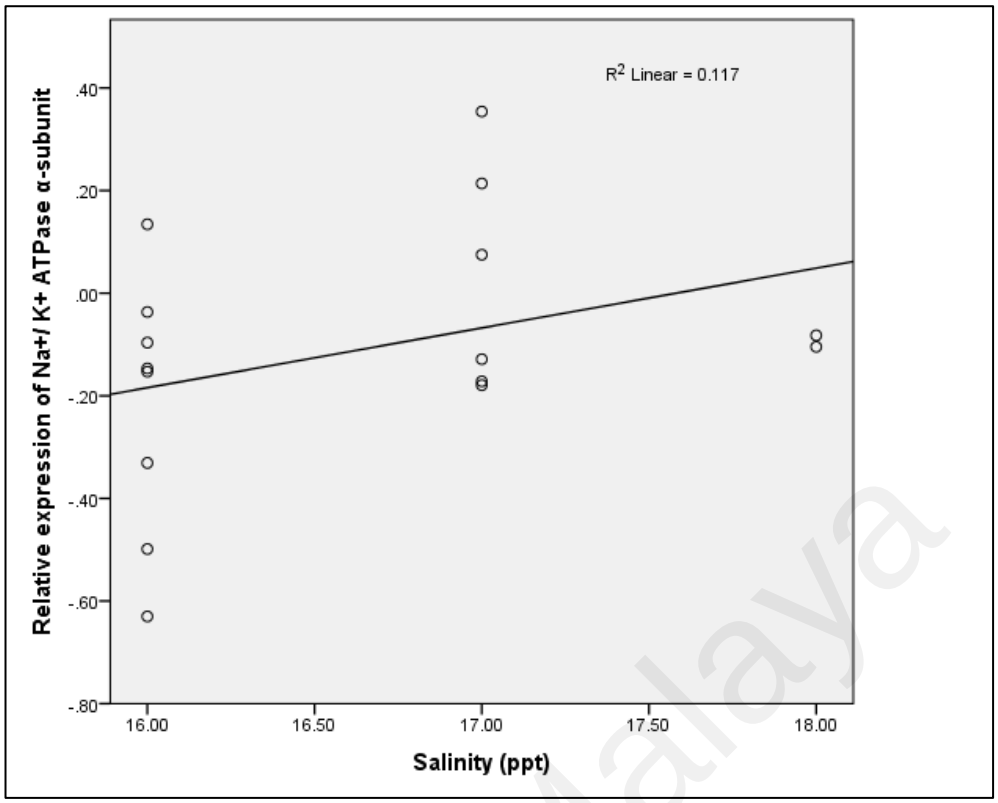


Figure 4.9: Scatter plot showing the relationship between salinity level and relative expression of Na^+-K^+ ATPase α-subunit ($R^2 = 0.117$; $p > 0.05$).

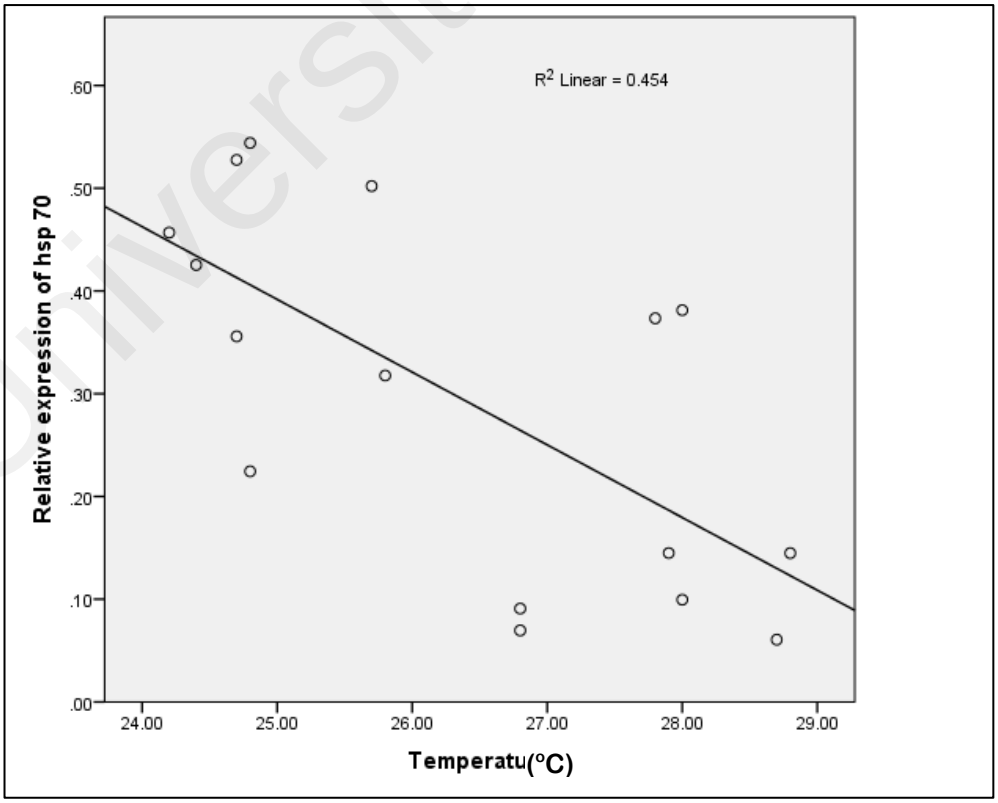


Figure 4.10: Scatter plot showing the relationship between temperature and relative expression of hsp70 ($R^2 = 0.454$; $p < 0.05$).

4.6 Relationship Between Dissolved Oxygen and Relative Expression of Catalase in Different Categories

Figure 4.11 shows a negative linear regulatory relationship (R^2 = 0.186) between dissolved oxygen and relative expression of catalase in tank 4. This regression was not significant since the p-value was greater than 0.05. A similar result was observed in tank 10 as there was a negative linear regulatory relationship (R^2 = 0.125) between dissolved oxygen level and relative expression of catalase, as shown in Figure 4.13. This regression was not significant since $p > 0.05$. The result obtained in tank 13 was in contrast with the above results, since there was a positive linear regulatory relationship (R^2 = 0.822) between dissolved oxygen level and relative expression of catalase, as shown in Figure 4.14. This regression was not significant since $p > 0.05$. Meanwhile, Figure 4.12 shows a very weak positive linear regulatory relationship (R^2 = 0.0001326) between dissolved oxygen level and relative expression of catalase in tank 9**.** This regression was not significant since $p > 0.05$.

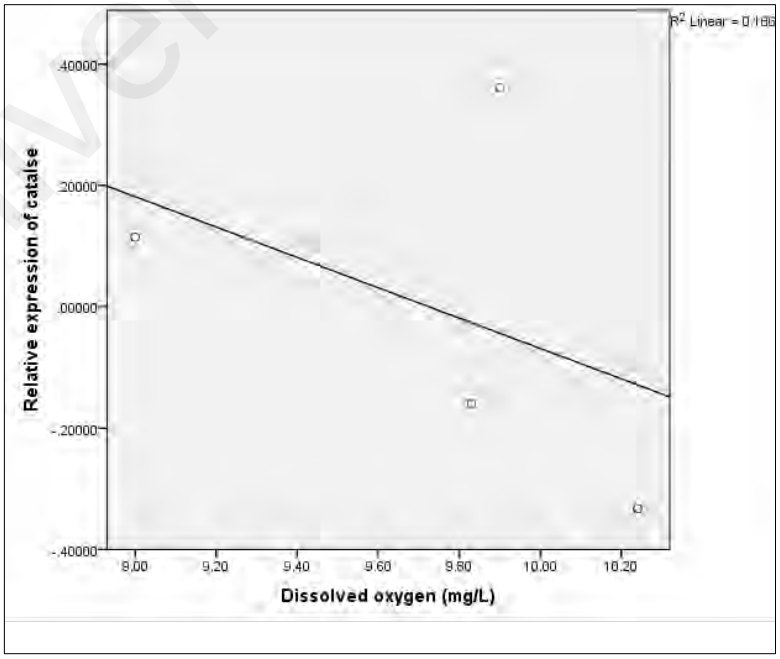


Figure 4.11: Scatter plot showing the relationship between dissolved oxygen level and relative expression of catalase in tank 4 (microbubble aeration) (R^2 = 0.186; $p > 0.05$).

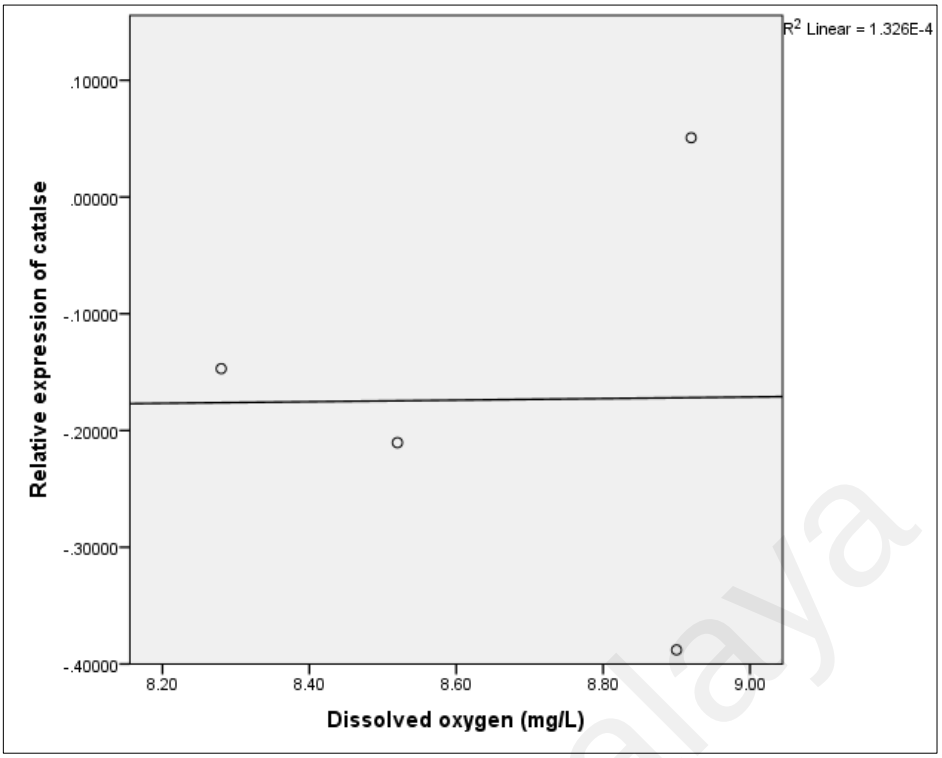


Figure 4.12: Scatter plot showing the relationship between dissolved oxygen level and relative expression of catalase in tank 9 (a mixture of microbubble and macro bubble) (R^2 = 0.000; p > 0.05).

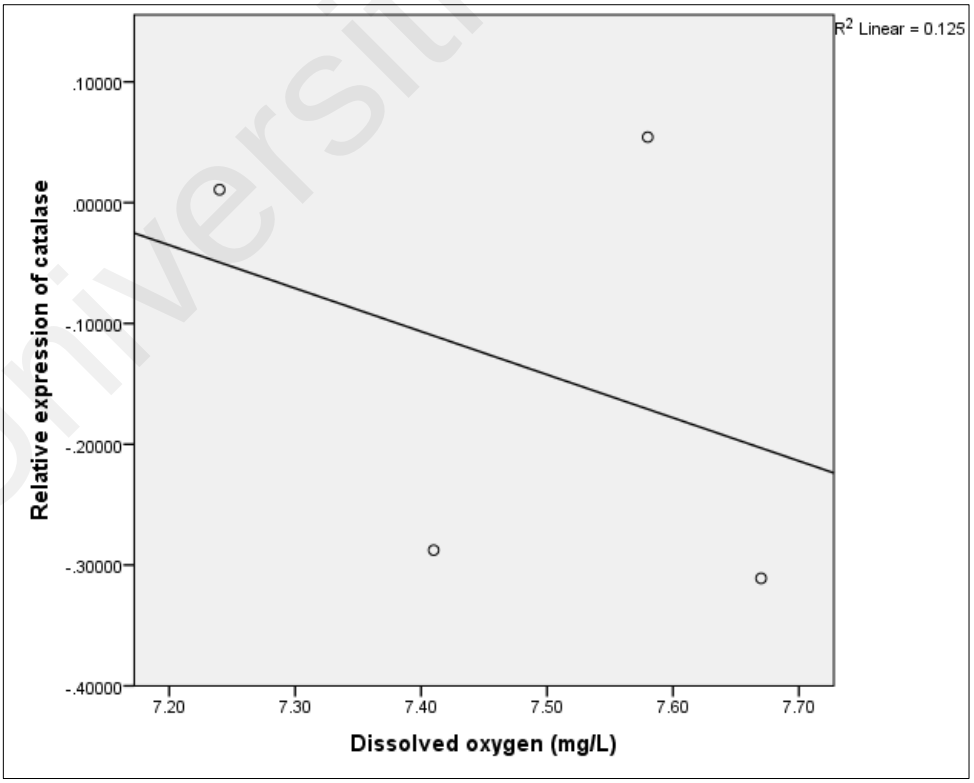


Figure 4.13: Scatter plot showing the relationship between dissolved oxygen level and relative expression of catalase in tank 10 (macro bubble aeration with bioballs) (R^2 = 0.125; p > 0.05).

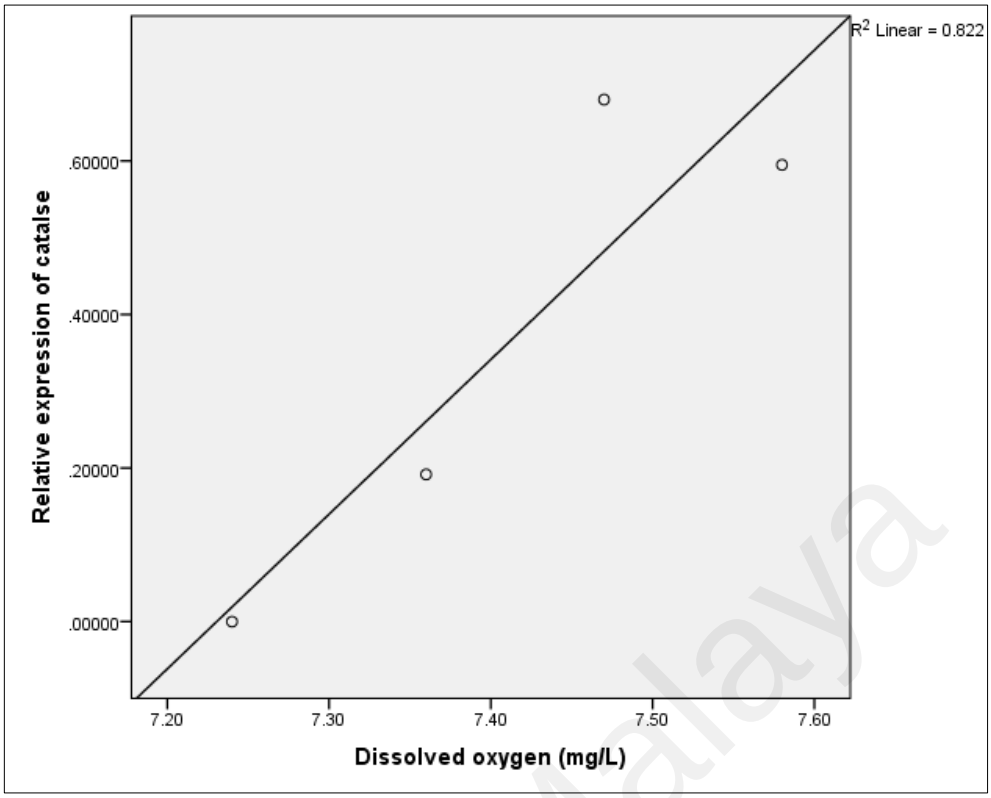


Figure 4.14: Scatter plot showing the relationship between dissolved oxygen level and relative expression of catalase in tank 13 (macro bubble without bioballs) (R^2 = 0.822; p>0.05).

4.7 Relationship Between Relative Expression of Catalase and Time of Cultivation in Different Categories

Figure 4.15 shows a positive linear regulatory relationship (R^2 = 0.621) between the time of cultivation and the relative expression of catalase in tank 4. This regression was not significant since the p-value was greater than 0.05. Similarly, tank 9 and tank 10 obtained a positive linear regulatory relationship (R^2 = 0.057 and R^2 = 0.714, respectively) between the time of cultivation and relative expression of catalase, as shown in Figure 4.16 and Figure 4.17, respectively. These regressions were not significant since p > 0.05. The result obtained in tank 13 was in contrast with the above results, since there was a negative linear regulatory relationship (R^2 = 0.069) between the time of cultivation and relative expression of catalase, as shown in Figure 4.18. This regression was not significant since p > 0.05.

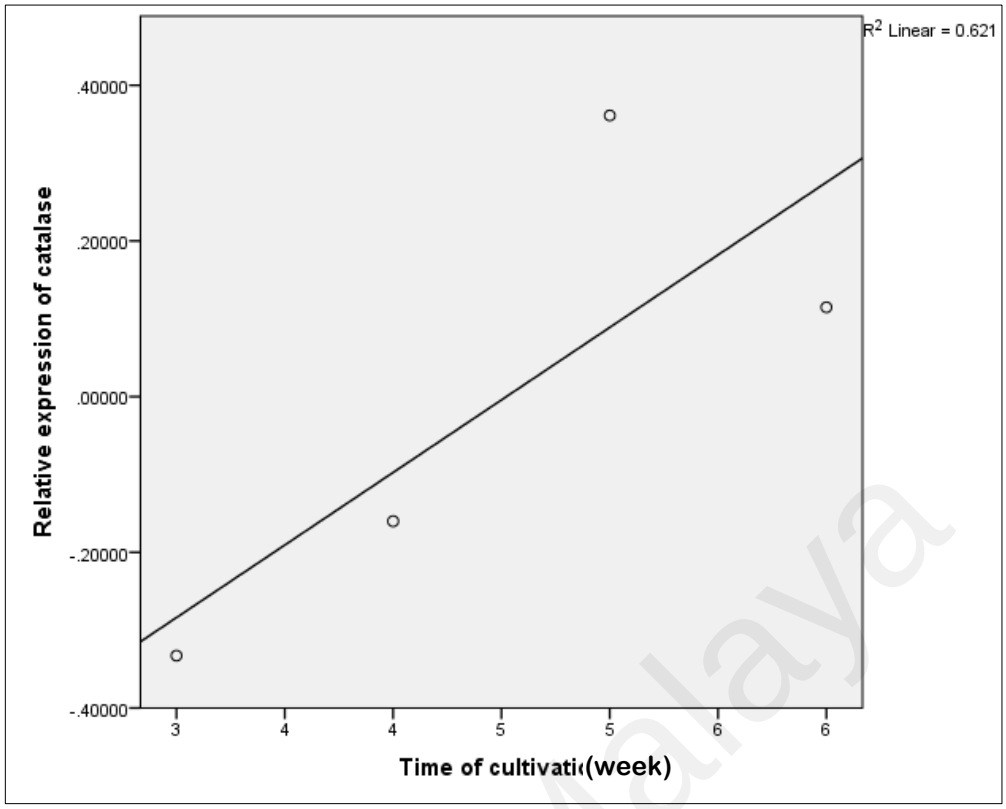


Figure 4.15: Scatter plot showing the relationship between time of cultivation and relative expression of catalase in tank 4 (microbubble aeration) ($R^2 = 0.621$; $p > 0.05$).

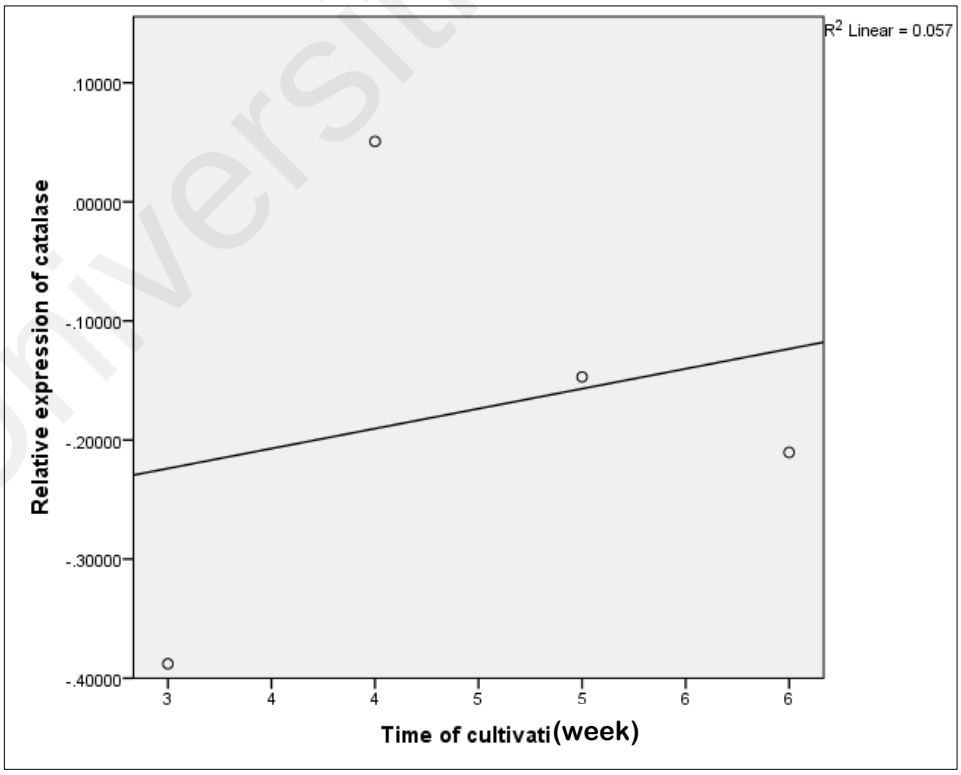


Figure 4.16: Scatter plot showing the relationship between time of cultivation and relative expression of catalase in tank 9 (a mixture of microbubble and macro bubble) ($R^2 = 0.057$; $p > 0.05$).

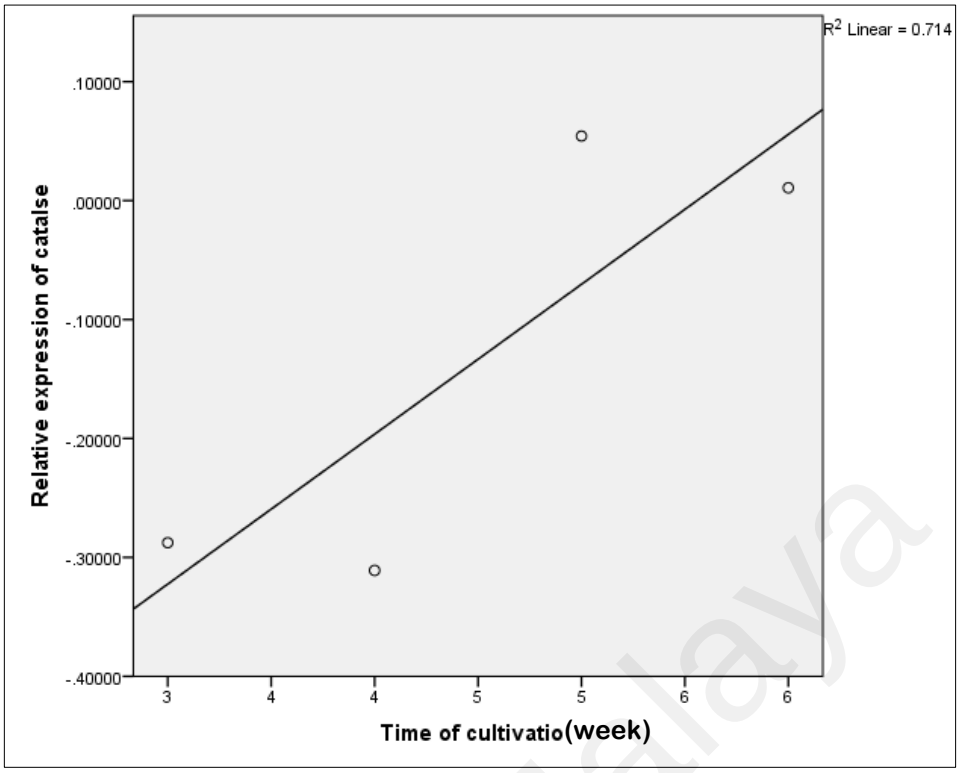


Figure 4.17: Scatter plot showing the relationship between time of cultivation and relative expression of catalase in tank 10 (macro bubble with bioballs) ($R^2 = 0.714$; $p > 0.05$).

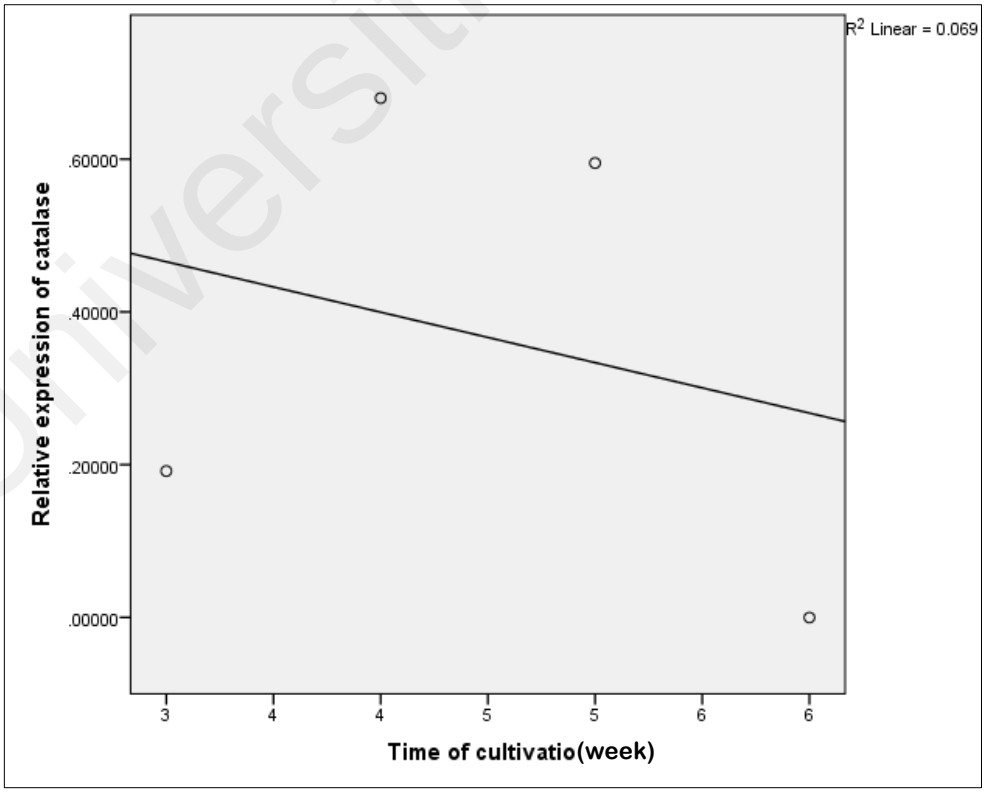


Figure 4.18: Scatter plot showing the relationship between time of cultivation and relative expression of catalase in tank 13 (macro bubble without bioballs) ($R^2 = 0.069$; $p > 0.05$).

CHAPTER 5: DISCUSSION

5.1 Expression of catalase

Reactive oxygen species (ROS) damage organisms' DNA, as well as other essential elements such as lipids and protein. Studies have shown that the ROS homeostasis could be broken by dissolved oxygen, and therefore will lead to steady oxidative damage due to the toxicity of ROS (Trasviña-Arenas et al., 2013; Chen et al., 2019a).

It has been investigated that some crustaceans including the whiteleg shrimp *P. vannamei* can well cope with hypoxia. It is suggested that these animals are able to survive due to an adjustment of the obligated metabolic and physiological changes. However, the details of this are still not clear.

Unfortunately, hypoxia still negatively affects the aquatic culture by reducing approximately 25% of the energy canalized to biomass production. Consequently, this will lower the shrimp farming profits. Therefore, in order to preserve the health of cultured animals, the concentration of ROS must be controlled during hypoxia (Trasviña-Arenas et al., 2013). It has been investigated that the balance between generation and elimination of ROS is maintained by the antioxidant defense systems which include non-enzymatic and enzymatic components (Chen et al., 2019a).

Catalase is a vital antioxidant enzyme that involves in the antioxidant defense systems of the shrimps. Catalase has the ability to catalyze the dismutation of $2H_2O_2$ to O_2 and $2H_2O$. There have been studies regarding the shrimp *P. vannamei* catalase gene, the expression of it, and enzyme activity during hypoxia. The results of these studies showed that catalase activity has a response to hypoxia (Trasviña-Arenas et al., 2013).

Results of a study of Tavares-Sa´nchez et al. (2004) indicated that the transcript of catalase is most abundant in hepatopancreas, it is also high in gills, however, it is not detected in the muscle of the shrimp. In addition, results of a study of Trasviña-Arenas et al. (2013) showed that expression in gills of the shrimp *P. vannamei* increased 3.2 – fold in response to hypoxia. It has been suggested that the expression of catalase and its activity might be related to oxygen sensing and physiological functions. Gills play a vital role in the gases' exchange between the aquatic environment and the organism, consequently becoming the first tissue that physiologically and biochemically responds to oxygen, and therefore, being more sensitive to the variation of oxygen (Trasviña-Arenas et al., 2013). Meanwhile, hepatopancreas has been investigated to be high in metabolic activity, consequently, is able to generate a great amount of ROS. Thus, catalase is required for the detoxification (Trasviña-Arenas et al., 2013). Therefore, in this study, for the use of catalase expression measurement, gills and hepatopancreas were used to extract the RNA of the shrimp samples.

It has been investigated that up or down changes do occur in catalase expression upon different stress (Trasviña-Arenas et al., 2013). In general, the expression level of catalase mRNA is up-regulated when the shrimps face environmental challenges, including heat stress, nitrite stress, and pH stress (Chen et al., 2019a). According to the results of the study of Trasviña-Arenas et al. (2013), catalase expression is up-regulated during hypoxia, and data showed that catalase activity had a significant increase of 3.2 and 2.4-fold at 6 h and 24 h of hypoxia. On the other hand, higher catalase activity occurred also in the gills of the crab *Chasmagnathus granulata* after 8 h of hypoxia. However, in rats, the activity of catalase in their liver was decreased when they were exposed to low levels of oxygen. This indicates that responses of antioxidant systems to oxygen variability in vertebrates or terrestrial animals and crustaceans are different (Trasviña-Arenas et al., 2013).

Chen et al. (2019a) carried out a study to examine the effects of dietary *myo*-inositol (MI) supplementation on survival, antioxidant abilities, and immune response in the whiteleg shrimps when they are being exposed to acute hypoxia stress. The results showed that under the experimental conditions, a certain level of MI down-regulated the expression of catalase. Both glutathione peroxidase and catalase can disintegrate H_2O_2 into O_2 and H_2O, however, glutathione peroxidase removes the H_2O_2 more effective than catalase. In addition, glutathione peroxidase activities in the hepatopancreas of shrimp showed the inverse variation trend with catalase expression. When the shrimps are exposed to hypoxia stress, glutathione peroxidase activities in hepatopancreas increased gradually with increasing dietary MI levels. It can be concluded that glutathione peroxidase may play a more important role than catalase under hypoxia due to supplementation of MI, and therefore, it possibly contributes to resulting in the down-regulation in the expression of catalase.

In the current study, the DO was inconstant and likely to vary among different time points and among different types of culture. Particularly, DO levels were statistical differences among the four conditions with the highest level was archived in tank 4, the second-highest level was found in tank 9, and the lowest level was obtained in tank 10 and tank 13. This indicates that different types of aerations used in the system give different effects on the DO in the culture, resulting in variation of this parameter. Tank 4 was applied with microbubble generator. Microbubbles are extremely small bubbles with a diameter of ≤ 50 μm and have several special physicochemical properties (Khuntia et al., 2012) such as high dissolubility in the surrounding water (Sadatomi et al., 2012). It has been investigated that microbubbles rise slowly to the liquid surface due to their low buoyancies, so dwell longer times in the liquid, resulting in very high mass transfer rates during these longer residence times (Kaushik et al., 2014). Therefore, the higher level of DO in tank 4 was due to the long exist of microbubbles under the water surface. However,

a negative correlation between different time points of the culture and DO levels in tank 4 was found, suggesting that the DO decreased over culture time. A possible explanation for this is the dissolution of the interior into the water around (Shangguan et al., 2018). One of the special characteristics of microbubble is its high interior gas pressure since a decrease in the diameter of the bubble results in the high internal pressure of the gas inside it. As time goes by, the bubble becomes smaller, the interior gas pressure increases higher, results in shrinkage of the bubble. Although the microbubbles can exist for a long time underwater, they tend to gradually decrease in size and eventually disappear (Shangguan et al., 2018). The DO level obtained in tank 4 was high at the beginning of the culture, but it then decreased in the end as the microbubbles had been shrunk. Meanwhile, tank 10 and tank 13 were applied with airstone diffuser that generates macro bubbles. Macro bubbles are ordinary bubbles that have a diameter in a range of 2-5 mm (Khuntia et al., 2012). In contrast to microbubble, macro bubble has higher buoyancies, therefore it couldn't exist under the liquid for a long period of time. In fact, macro bubbles will rapidly rise in the liquid then burst at the liquid surface. Therefore, DO levels in tank 10 and tank 13 were lower than the DO levels in tank 4. In addition, a negative correlation between different time points of the culture and DO levels in tank 10 as well as in tank 13 was found, however, it was not statistically significant. This indicates that macro bubbles are more stable in the water than microbubbles. Besides, tank 9 obtained the second-highest DO levels among the four tanks, and no statistically significant correlation was found, this suggests that the mixture of microbubbles and macro bubbles is the most suitable candidate for the maintenance of stable DO levels in biofloc culture.

Regarding the growth performance of the cultured shrimps, according to the results provided by the co-workers (Ezzah et., unpublished), the cultured shrimps in tank 4 were higher in weight and length than the shrimps which were cultured in the other tanks. In particular, the average height of the shrimps cultured in tank 4 was 7.594 (cm), and the

average weight was at 3.88 (g); while in tank 9 the average height and average weight was at 7.22 and 3.21, respectively; the average height in tank 10 was 7.117 and the average weight was 2.29; meanwhile, those shrimps in tank 13 obtained the average height at 7.185 and the average weight at 2.63. The higher growth performance obtained in tank 4 was related to the use of microbubbles in the system. It has been reported that the enrichment of oxygen by microbubbles into water culture promotes the consumption of oxygen by the cultured animals as well as their blood circulation and metabolism, resulting in the speed-up of their growth. However, the survival rate obtained in these tanks is in contrast to their DO levels as well as growth performance. Indeed, the lowest survival rate was obtained in tank 4 (38%), while tank 10 archived 100% of survival rate, tank 13 was 97.5%, and 96.67% in tank 9. This indicates that although the aeration condition could provide high levels of DO and could well maintain the stable of this parameter, there are some other factors such as microbial communities can negatively affect the quality of the culture as well as the cultured animals, resulting in low growth performance, and high mortality.

As explained above, the DO level was inconsistent as it varied in different time of cultivation or in different culture conditions. However, its level was still within the suggested optimal range of DO level for maintaining the biofloc system, which is between 7 and 8 mg-L (Kasan et al., 2018). In particular, the average DO level in tank 4 was at 10.09 ± 0.57, 8.85 ± 0.35 in tank 9, in tank 10 was at 7.48 ± 0.14, and 7.44 ± 0.11 in tank 13. Since hypoxia is caused when DO level in the culture water goes below 2 mg/L (Chen et al., 2019a), it can be concluded that DO levels in this study did not cause hypoxia stress. However, there were presences of up-regulated expression of catalase in some points of time during the cultivation. In particular, the up-regulated in catalase expression was found in tank 4 and tank 10 during the last two weeks of the culture. While in tank 13, catalase expression was high up-regulated from week 3 to week 5, the highest levels of

expression were obtained in week 4 and week 5. The presence of these up-regulated catalase expressions suggests a possibility of exposure to hypoxia of the cultured shrimps. In fact, a general definition of hypoxia is: “Hypoxia is a condition in which there is an inadequate supply of oxygen to the tissues of the body. If the oxygen entering inside a cell is not matching the oxygen demand of the same cell, a hypoxic condition is created” (Sridharan et al., 2017). Besides, many authors have assumed that hypoxia is not only caused by the low level of DO in the water, but it can also occur when the amount of consumed oxygen by the organisms, which are living in the environment, is greater than the amount of supplied oxygen that has been provided into the environment, consequently being limited (Trasviña-Arenas et al., 2013). Other resources also suggested that when there are too many microorganisms or cultured animals in the culture, they may overpopulate and consume DO in great amounts, leading to the decline of oxygen since more oxygen is consumed than is produced, consequently causing negative effects on the cultured animals (Lenntech, n.d; EPA, n.d.). Mahasri et al. (2018) also suggested that dissolved oxygen in water is not only used for the respiration of cultured animals but also used by other aquatic microbial communities that live in the water, consequently, decrease DO levels in the culture. In addition, an increase of organic waste in the culture water will lead to a decrease of DO level, since oxygen is consumed by decomposing bacteria to decompose organic substances into inorganic substances (Mahasri et al., 2018). Meanwhile, Hargreaves et al. (2002) suggested three factors that affect the DO in a culture. The first factor is that oxygen is not very soluble in water, therefore water has only a limited capacity to hold oxygen. Secondly, the rate of oxygen consumed by the cultured animals and other organisms living in the culture can be high. Lastly, the diffusion of oxygen from the atmosphere into undisturbed water is very slow. The combination of the three factors will lead to rapid changes in the concentration of DO, consequently. Mahasri et al. (2018) carried out a study to investigate the enrichment

process of DO in fish culture using nanobubble technology. Two treatments were conducted, the first treatment contained tilapia fishes, while the second treatment was conducted without any fishes, only culture media was added. The results showed that the rate of decrease in DO in the treatment without fish was 3.08 ppm/day, while the rate of decrease in DO in the treatment containing fishes was 0.23 ppm/minute. Therefore, it can be concluded that the decrease in DO concentration will decline with the increase of stock densities since the cultured animals have to compete for space and oxygen in the culture. Similarly, Kim et al. (2014) carried out a study to investigate the effect of biofloc on the Pacific white shrimp, the results showed significant differences in DO levels between control (containing clear water) and biofloc, the average DO concentration in control was at 5.8 (mg/L), while its value in biofloc treatment was at 4.9 (mg/L). The lower levels of DO in biofloc treatment was caused by the greater demand of oxygen by the bacteria and other microorganisms since the total number of bacteria in biofloc group was approximately 10 times higher than in control. Last but not least, the level of oxygen consumption is also related to the ability of the individuals to absorb oxygen to support their life processes. Some factors that affect the oxygen consumption of an individual are temperature, body size, and activity (Galang et al., 2019). According to Zarain-Herzberg et al. (2006), the grow-out period of *L. vannamei* starts when they are at 30 days old, during this period, the shrimps grow rapidly, resulting in rapid increase of their weight and length. In this study, up-regulated in the expression of catalase was mostly found in the last few weeks of the culture, because the cultured shrimps were in their grow-out period, they had larger size, and performed more activities, therefore consumed more oxygen than before.

Unfortunately, this study couldn't manage to determine the oxygen consumption rate in order to investigate if the total amount of oxygen consumed by the cultured shrimps and other organisms was greater than the total amount of oxygen that had been applied to

the culture. This limits the finding of this study since there was a lack of evidence to support the argument that hypoxia stress might occur due to the great demand for oxygen by the organisms living in the culture.

5.2 Expression of Na^+-K^+ ATPase α-subunit

It has been investigated that the variation of salinity is directly related to osmoregulation. By osmoregulation, the shrimp can regulate hemolymph osmolytes to control hemolymph pressure (Hu et al., 2015). However, the mechanism of osmoregulation and molecular response to salinity stress in marine animals is not clear. Hu et al. (2015) carried out a study to detect the transcriptomic responses of the gills in the whiteleg shrimps that had been exposed to low salinity stress using Illumina's digital gene expression system. The results found out many different genes and pathways that have a relation to the salinity stress and osmoregulation in the whiteleg shimps *L. vannamei*. The results showed that there were some significantly differentially expressed genes related to osmotic regulation. These genes were categorized into three types based on their function, including ion transporters, ion transport enzymes, and genes that participate in intracellular signaling pathways.

The gene which was focused in this present study is sodium-potassium-transporting ATPase subunit alpha. It has been investigated that Na^+-K^+ ATPase is a vital participant in regulating Na^+ uptake. And the catalytic site, Na^+-K^+ ATPase α-subunit, is associated with vectorial and electrogenic ion transport processes. In some crustacean including shrimp, after they were exposed to dilute media, transcriptions of the α-subunit gene or expression of the α-subunit will be increased. In addition, the stimulation of Na^+-K^+ ATPase activity has been investigated to be attached with the increase of transcription of the α-subunit gene or expression of the α-subunit. This is an explanation for the function

of Na^+-K^+ ATPase α-subunit when crustacean, as well as shrimps, suffer from ambient salinity challenge. Salinity change will affect the Na^+-K^+ ATPase α-subunit expression as well as regulate the activity of Na^+-K^+ ATPase. Consequently, Na^+-K^+ ATPase will lead to the flux of cell ions to maintain the osmotic pressure balance (Hu et al., 2015).

Previous studies have investigated that the expression of Na^+-K^+ ATPase α-subunit is strongly up-regulated after acute salinity stress (Gao et al., 2016). According to the results of the study of Hu et al. (2015), the expression of Na^+-K^+ ATPase α-subunit in gills of the Pacific white shrimp *L. vannamei* was up-regulated in response to ambient salinity challenge. A study which was conducted by Gao et al. (2016) also showed similar results. In order to examine how long-term salinity stress affects the growth performance and expression of osmolarity and metabolism-related genes in whiteleg shrimp *L. vannamei*, including Na^+-K^+ ATPase α-subunit in gills and trypsin and chymotrypsin in hepatopancreas, an experiment was conducted in eight weeks, with four different categories at four salinity levels. The shrimps that were reared at high salinity level (above 20 ppt) were significantly higher in the final weight, weight gain and specific growth ratio in comparison with those were reared at low salinity (at 2 ppt and 10 ppt). More importantly, the shrimps which were reared at low salinity 2 ppt were not only low in growth performance, but they were also low in survival. In addition, results of quantitative Real-Time PCR (qRT-PCR) showed that relative expression levels of Na^+-K^+ ATPase α-subunit at salinity 2 ppt and 10 ppt were significantly increased in comparison with the expression of this gene at salinity 20 ppt and 30 ppt.

There have been studies to investigate the level of salinity that is optimal for the growth performance of the shrimp *P. vannamei*, and lots of results showed that shrimps had optimal growth performance near isotonic point (Gao et al., 2016). According to the biology dictionary, a cell in an isotonic solution is in equilibrium with its surroundings,

this means the solute concentrations inside and outside are the same. When the cell is in this state, water molecules can freely move in and out of the cell, but the rate of movement is the same in both directions, thus there is no large movement of water in or out of the cell (Isotonic vs. Hypotonic vs. Hypertonic Solution, n.d.). This conclusion was supported by the results of the study of Gao et al. (2016). Salinity 20 and 30 ppt used in the study were closed isotonic point, and the survival rate, as well as the growth performance of the reared shrimps, were significantly higher than those shrimps were reared at salinity 2 ppt and 10 ppt. Moreover, the transcript levels of Na^+-K^+ ATPase α-subunit at salinity 20 and 30 ppt near isotonic point was significantly lower than salinity 2 and 10 ppt, and the levels of this gene were increased higher with the decreasing salinity. This indicates that the stress stage of *L. vannamei* could be reflected by the transcript levels height of Na^+-K^+ ATPase α-subunit, the closer the isotonic point, the lower of salinity stress pressure, and the lower of Na^+-K^+ ATPase α-subunit (Gao et al., 2016).

In addition, it has been investigated that different development stages of *P. vannamei* will have different adaptive ability to low salinity. Studies have shown that there was no difference in survival or growth between *P. vannamei* juveniles which had been cultured at salinity 2 ppt in comparison with those had been cultured at salinity 8 ppt. Similarly, no difference found in the growth of *L. vannamei* postlarvae which had been grown at 2 ppt and those had been grown at 16 ppt. Moreover, it has been investigated that both *L. vannamei* postlarvae and juveniles can be grown successfully under low levels of salinity such as at 4 ppt, however, survival and growth of postlarvae were affected negatively (Gao et al., 2016).

In this current study, tank 4 and tank 9 had similar salinity levels, while salinity levels in tank 10 were similar to salinity levels in tank 13. In particular, the average salinity level in tank 4 and tank 9 was at 17 ppt, while it was at 18 ppt in tank 10 and tank 13, however,

these levels of salinity were not significant difference. Regarding the changes of salinity throughout the culture, tank 4 and tank 9 had a different variation of salinity than tank 10 and tank 13. In particular, salinity in tank 4 and tank 9 decreased in week 2 and week 3 of the culture, then increased in the last three weeks. No statistically significant correlation found between different time points and salinity levels. However, the negative correlation between salinity and time of cultivation in tank 10 and tank 13, indicates that salinity tends to decrease over the culture. Indeed, at the beginning of the culture, salinity in tank 10 and tank 13 was at 20 ppt, then it decreased throughout the time and was at 16 ppt at the end of the culture. Two factors that could contribute to this change of salinity including evaporation and additional culture. Evaporation is defined as a process by which water changes from a liquid to a gas or vapor (USGS, n. d). When the temperature increases, the evaporation rate will be increased since there is a higher amount of energy available to convert the liquid water to water vapor. (NCSU, n. d). Evaporation can significantly affect water salinity, when evaporation rate increases, the amount of water loss is increased, resulting in the increase of the salinity levels (Suantika et al., 2018a). In addition, it is necessary to balance the water level in the culture by adding water to replace water loss caused by evaporation. Subsequently, the quality of current water in the culture tank is affected by the quality of added water (Junda, 2018). In particular, the salinity of the culture is usually decreased when new culture water is added. According to the study co-workers (Ezzah et al., unpublished), tank 4 and tank 9 had a higher evaporation rate than tank 10 and tank 13 due to the recirculation process, therefore additional of water in tank 4 and tank 9 was usually done twice per week, while in tank 10 and tank 13 was refilled with new culture water only once a week. The salinity in tank 4 and tank 9 was increased by evaporation and was decreased by the addition of water. That is the reason why the salinity in tank 4 and tank 9 decreased at this time but increased at the other time. Meanwhile tank 10 and tank 13 had lower temperature and lower evaporation rate, the

concentration of salinity was mostly affected by the salinity of the added water, therefore the salinity levels in these two tanks were dropped throughout the culture. However, this current study could not manage to measure the total amount of water that had been added to the culture tanks as well as its salinity level, therefore it could not be able to provide enough evidence to support the argument above. This is also one of the limitations of the study.

In the present study, mRNA expression levels of Na^+-K^+ ATPase α-subunit were rarely different. In particular, the expression levels of Na^+-K^+ ATPase α-subunit of the samples were down-regulated during the 3rd and 4th week of the culture, indicating that the shrimps might not be under salinity at these points of time. However, there was a difference in the expression level of Na^+-K^+ ATPase α-subunit in different culture conditions during the last two weeks. Particularly, relative expression of Na^+-K^+ ATPase α-subunit was still down-regulated in those samples which were reared in tank 9 and tank 13. Meanwhile, the expression of this gene became up-regulated in those shrimps reared in tank 4 during the last two weeks. Similarly, the Na^+-K^+ ATPase α-subunit expression also became up-regulated in those shrimps reared in tank 10 in the last week of the culture. The high up-regulated of the expression of Na^+-K^+ ATPase α-subunit may be related to the changes of salinity as discussed above. However, since the two tanks 4 and 9 had same range of salinity, and tank 10 and tank 13 also obtained same salinity levels, but the changes of Na^+-K^+ ATPase α-subunit expression occurred in tank 4 and tank 10 only, this suggests that the expression of this gene was also be affected by other factors such as pH.

Similar to salinity, pH obtained in tank was the same as pH obtained in tank 9, while tank 10 and tank 13 also had a similar value of pH. The average pH value obtained in tank 4 and tank 9 was 7.69, while in tank 10 it was 7.84, and 7.86 in tank 13. The Pearson's correlation coefficient results showed no statistically significant correlation found

between time of cultivation and pH levels in tank 4, tank 9 and tank 10, while a negative correlation was found in tank 13 indicating that pH decreased over the culture times. In addition, there were significant differences in pH levels among these tanks, the highest level was obtained in tank 10 and tank 13, while the lowest level was found in tank 9 and tank 10. It has been investigated that factors that might cause a rapid change of pH including sudden change or disappearance of dominant species of plankton, and pollution of water. Crustacean like shrimp has been investigated to be able to adapt to the variety of pH (Pan et al., 2007) due to the role of ion transport and osmoregulation (Han et al., 2018). And Na^+-K^+-ATPase has been proven to be central molecular components of the systemic process of ion transport and osmoregulation (Han et al., 2018). Previous studies have reported the relationship between pH and Na^+-K^+-ATPase. For instance, Han et al. (2018) studied the adaption of whiteleg shrimp *P. vannamei* to the variation of pH. The experimental shrimps were cultured in three different levels of pH, including low (6.65-8.20), normal (8.14-8.31) and high (8.20-9.81) levels. Results showed that there was an increase in Na^+-K^+-ATPase expression in both low and high levels of pH. In detail, in the treatment of high pH, Na^+-K^+-ATPase expression increased at first, then decreased in the end. This result indicated that those shrimps that were reared in a high level of pH, at an early stage, enhanced the expenditure of energy to keep the balance of acid and base, however, at the final stage, the osmoregulation was failed. Meanwhile, in the treatment that had low levels of pH, the expression of Na^+-K^+-ATPase increased continuously throughout the experiment. This indicated that high osmoregulatory ability of the whiteleg shrimp *P. vannamei* is the major adaption mechanism of this species under low levels of pH. However, since the shrimp can tolerate to a wide range of pH, it is important to identify the optimal range for their growth. Pan et al. (2007) carried out a study to investigate the effect of salinity and pH on the growth of *P. vannamei* as well as the ion-transport enzymes of this species. The study was conducted with a wide range of pH, at

7.1, 7.6, 8.1 (control), 8.6 and 9.1. Results showed that the activities of Na^+-K^+-ATPase in experimental treatment group (pH=7.1, 7.6, 8.6, 9.1) showed peak change within 72 h of pH variation, after that dropped to the level of the control group. In addition, the results also indicated that variation of pH did not give significant effects on the survival rate of the shrimps. However, the weight gain was distinctly affected in experimental groups of pH = 7.1, 7.6 and 9.1. In conclusion, the study also suggested that the variation of salinity and pH during the cultivation of the shrimp *P. vannamei* should not more than 3 and 0.5, respectively. Since the activity of Na^+-K^+ ATPase can reflect the osmoregulation of the shrimp (Pan et al., 2007), the up-regulated in the expression of Na^+-K^+ ATPase obtained in the current study suggests that the shrimp might experience enhanced energy expenditure to maintain the acid-base balance (Han et al., 2018) at that time. This may be because the variation of pH obtained in the culture tanks is not optimal for the growth of the shrimps. According to the results of the above studies (Pan et al., 2007; Han et al., 2018), it is assumed that the optimal pH for the growth of the shrimp *P. vannamei* is close to 8.1, however, the pH obtained in this current study was below that point, in addition, it tended to decrease during the last few weeks of the culture, consequently affected the cultured shrimps. Unfortunately, this current study could not manage to set up a culture tank at pH of 8.0-8.1 to investigate the expression of Na^+-K^+ ATPase at these pH values, resulting in a lack of evidence to support the above argument.

5.3 Expression of Hsp 70

Qian et al. (2012) carried an experiment to investigate the expression profiles of four HSP genes of the whiteleg shrimp *P. vannamei*, including hsc70, hsp60, hsp70 and hsp90, under different environmental stresses such as pH challenge, acute thermal stress, and exposure of heavy metal. And the results indicated that the four genes exhibited quite different expression profiles when the experimental shrimps exposing to each of these

stressors. In particular, when the shrimps were exposed to acute thermal stress, the expression levels of all the four genes were significantly induced, and the transcription level of hsp70 was the most sensitive to temperature fluctuations, indicating that hsp70 may be most suitable for the use as a biomarker indicating thermal stress. Particularly, the study's results indicated that the transcript level of hsp70 was induced immediately by heat stress, it was up-regulated and reached the maximum level at 1 h post-treatment. The level of expression was then slowly decreased; however, it was still higher than that of the control at 2.5, 4, and 6 h post-treatment.

In this present study, the temperature obtained in tank 4 and tank 9 higher than the temperature in tank 10 and tank 13. In particular, the average temperature in tank 4 and tank 9 was at 27.4 (ºC), while in tank 10 and tank 13 it was at 24.6 (ºC), the difference between these values was statistically significant. Besides, no statistically significant correlation found between the time of cultivation and temperature in these tanks, indicating that the temperature was stable and did not vary much during the culture. A possible factor that caused this difference in temperature among the four tanks is the use of the recirculation system. As described in the previous chapter, tank 4 and tank 9 were applied with a recirculation system, which was operated during the daytime and unactivated at night. According to Bregnballe (2015), recirculation is referred to as a technique for aquatic organisms farming by reusing the water in the production. The principle of this technique is based on the use of mechanical and biological filters to treat the water continuously to remove the wastes produced by the cultured animals. Basically, the culture water flows from the outlet of the culture tanks into a mechanical filter, then keeps flowing on to a biological filter – where it is aerated and stripped of carbon dioxide, after that it returns to the culture tanks. Depending on the exact requirements, the system can be added with some other facilities including oxygenation with pure oxygen, ultraviolet disinfection, automatic pH regulation, denitrification, and heat exchanging.

However, one of the issues that commonly find while using recirculation is the heat in the water culture (Bregnballe, 2015). It has been investigated that high temperature in the system is a common problem in the indoor recirculation system. The heat will slowly build up in the water since energy in the form of heat is released from the metabolism of the culture animals, and the activity of bacteria in the biofilter. In addition, heat from friction in the pumps and from the use of other installations will also accumulate to the rise of heat (Bregnballe, 2015). Therefore, the higher temperature obtained in tank 4 and tank 9 can be explained. Previous studies also obtained a higher temperature while using recirculation (Chen et al., 2019c) carried out a study to examine the effect of different recirculation rate on the microorganisms and water quality and their relation to the survival and the growth performance of the shrimp *P. vannamei*. Results showed that all three treatments obtained high temperatures. In particular, the temperature in treatment with low recirculation rate was at 28.19 (ºC), it was at 28.27 (ºC) in treatment with medium recirculation rate, and 28.31 (ºC) in treatment with a high rate of recirculation. The study of Suantika et al. (2018b) also showed a similar result. The study conducted an indoor recirculation system for the culture of *P. vannamei* at the condition of low salinity. The recirculation system was operated continuously for 24 hours without water replacement. According to the study results, the temperature obtained in all the treatments was between 28.20 to 28.40 (ºC). In this current study, the temperature obtained in the tanks that applying recirculation system was similar to the above results, in particular, the temperature in tank 4 varied from 26.2 to 28.8, and from 26.5 to 28.7 in tank 9. Meanwhile, tank 10 and tank 13 were conducted as same as other conventional intensive systems, therefore also obtained lower temperature like these systems. Maicá et al. (2014) studied the effect of salinity on the Pacific white shrimp reared in a super-intensive system without any exchange of water. The temperature obtained in treatment with low salinity

levels (4 ppt) was at 23.29 (ºC), while it was at 22.92 (ºC) in treatment with medium salinity levels (16 ppt), and 22.93 (ºC) in treatment of high salinity levels (32 ppt).

Since the use of recirculation results in high temperature in the culture, consequently, give negative effects on the cultured shrimps, it is necessary to regulate the temperature of the water culture. According to the co-workers (Ezzah et al., unpublished), the culture area was applied with air conditioner, when the recirculation system was operated at daytime, the air conditioner was also operated to reduce the heat in the tanks through the movement of water molecules. It has been investigated that the atmosphere has an influence on the water temperature since atmospheric heat transfer occurs at the water's surface (Fondriest Environmental, 2014). As heat always flows from a higher temperature to a low a temperature (Fondriest Environmental, 2014) by the circulation of the body of the water which is caused by the movement of water molecules ("About water temperatures", n.d.), this transfer can go both ways (Fondriest Environmental, 2014). If the air is cold, warm water will transfer energy to the air and cool off. When the air is hot, cold water will receive the energy and warm up. The extent of this transfer is based on the thermal inertia and specific heat of the water. Fluctuations of water are more gradual than fluctuations of air temperature (Fondriest Environmental, 2014).

However, the use of air-conditioning to reduce high temperatures produced during the recirculation led to continuous changes of temperature in the recirculated tanks as well as in the non-recirculated tanks, consequently caused thermal shock to the cultured shrimps. This current study obtained high-regulated expression of hsp70 in all of the four tanks at most of the culture times. In particular, high up-regulated in the expression of hsp70 was found in tank 4, 10 and 13 at all the time points throughout the cultivation, meanwhile, in tank 9 the expression of hsp70 become up-regulated in week 5 and week 6. The high-up

regulated in hsp70 expressions indicated that the shrimps were exposed to thermal stress almost of the culture times.

In addition to the inefficiency of maintaining temperature, another factor that could contribute to the occurrence of up-regulated in hsp70 expression is that the temperature obtained in the system is not optimal for the growth of the shrimps. According to Wyban et al. (1995), the growth of *P. vannamei* is extremely sensitive to small changes in temperature. In addition to the optimal temperature for the growth of this species is size-specific, meaning that it decreases as the shrimp size increases. For small shrimps that weight below 5 g, the optimal temperature may be greater than 30 ^{o}C, while it is at about 27 ^{o}C for large shrimps. According to this study's co-workers (Ezzah et al., unpublished), the average weight of shrimps cultured in tank 4 was about 3.88 (g) at the end of the culture, while shrimps reared in tank 9 obtained average weight of 3.21 (g), in tank 10 was 2.29 (g) and 2.63 (g) in tank 13. It can be seen that the weight of all the reared shrimps was below 5 (g), therefore they should be cultured at temperature of 30 ^{o}C or above. However, all of the four tanks in the study used lower temperature than 30 ^{o}C, in particular, the highest temperature obtained in tank 4 and tank 9 was 28.8 and 28.7, respectively, while it was at 25.8 and 25.7 in tank 10 and tank 13, respectively. None of these temperatures are optimal for the growth of the shrimp *P. vannamei,* consequently limited the growth or caused stress on the cultured shrimps.

Besides, the limitation of this current study is that the temperature measurement was done at daytime only, while the temperature of the cultured tanks during the night time was not measured. At night, the recirculation system, as well as the air-conditioning, were not operated, water temperature in the tank was affected by room temperature at this time, resulting in increase or decrease in temperature. However, since the temperature at this

time was not measured, it is unknown if the changes of temperature between daytime and night were significant and consequently caused heat shock on the reared shrimps.

5.4 Relationship Between Water Quality Parameters and Relative Expression of Stress-Related Genes

5.4.1 Relationship Between Dissolved Oxygen and Relative Expression of Catalase

As described previously, the biofloc system used in this current study had been differently conducted. In particular, tank 4 was applied with microbubble aeration and recirculation system, while tank 9 was applied with a combination of microbubble and macro bubble, and recirculation system, while macro bubble was applied into tank 10 with additional of bioballs, lastly tank 13 was applied with microbubble only. These types of aerations have been proved to generate different values of water parameters, especially DO, therefore the differences of DO among the culture tanks were predicted. In addition, the DO level is suggested to be inverse to catalase expression.

Overall, the results of this current study showed a negative linear regulatory relationship between DO level and relative expression of catalase obtained in all four tanks, indicating that as the DO increases, the relative expression of catalase tends to decrease. However, the correlation coefficient was not significant ($p>0.05$).

In particular, a negative linear regulatory relationship between DO level and catalase expression was found in tank 4 and tank 10, however the correlation coefficient obtained in both tanks was not significant ($p>0.05$). Meanwhile, a positive linear regulatory relationship between DO and expression of catalase in tank 9, indicating that when the DO increases, the relative expression of catalase tends to increase. Nonetheless, the

correlation coefficient was not significant ($p>0.05$). Lastly, tank 13 obtained a strong positive linear regulatory relationship between the level of DO and expression of catalase. However, the correlation coefficient was also not significant ($p>0.05$).

The insignificant of the correlation coefficient as described above indicates that change in DO level is not associated with the change in the relative expression of catalase. However, it could not be concluded that the DO level and catalase expression have no relationship. In other words, it could not be concluded that there is no relationship between the DO levels and types of aeration since this is in contrast to the results obtained. As discussed in the previous chapter, the four tanks differently affected the DO levels in the culture, in addition, the levels of DO also gave influences in the expression of catalase. The reason for this argument will be given afterward.

5.4.2 Relationship Between Relative Expression of Catalase and Time of Cultivation in Different Categories

Time of cultivation has also been investigated to be one of the key factors that have an effect on the expression of the stress-related gene, since the animals have different exposure to short term and long term stress, resulting in different levels of expression. Therefore, it is important to investigate if the time of cultivation gives any effects on the expression of stress-related genes. A simple linear regression analysis was performed to develop the relationship between the time of cultivation and the relative expression of catalase in different types of categories. Positive linear regulatory relationship between time of cultivation and relative expression of catalase was obtained in tank 4, tank 9 and tank 10, indicating that as the time of cultivation increases, the relative expression of catalase tends to increase. In other words, the long term culture is associated with a high level of catalase expression, while the short term of culture results in a low catalase level.

A possible explanation matter is the formation of microbial communities in the culture and the growth of the shrimps. The principal of the biofloc system is based on encouraging the growth of microorganisms in the culture through the addition of carbohydrates to the water (Liu et al., 2019). As explained in the previous chapter, these microorganisms also require oxygen for their growth and activities, when the time of culture increases, the number of them also increases, consequently the amount of oxygen consumed by them also increases. Besides, over the culture times, the shrimps will grow and become larger, shrimps with larger size require more oxygen for their growth and activities. As a result, the amount of oxygen consumed by the reared shrimps and the microbial communities will be increased over the time and will exceed the oxygen supplied, thus the DO being limited, and hypoxia being produced.

Meanwhile, the result obtained in tank 13 (macro bubble without bioballs) was in contrast with the above results, since there was a negative linear regulatory relationship between time of cultivation and relative expression of catalase, indicating that as the time of cultivation increases, the relative expression of catalase tends to decrease. This statement is likely related to the lack of bio balls in the system. Bio balls play a vital role in biofloc systems as they provide a large surface for the colonization of microorganisms in biofloc. Especially, bioballs carry nitrifying bacteria, which require oxygen for the nitrification process, since this species live in the surface of objects in the culture (Sterling, n.d.). If there is a lack of bioballs in the culture, the number of microbial communities especially nitrifying bacteria will be decreased, since there is a limited object for them to climb on. As the microorganism densities reduce, the amount of oxygen consumed by them also reduces, DO levels not being limited, and hypoxia is not being occurred, as a result. However, this current study could not manage to investigate the densities of microorganisms in the culture tanks, especially the densities of nitrifying bacteria. This limits the finding of the study since there is no evidence support for the

argument. In addition, the correlation coefficient between DO level and catalase expression obtained in all four tanks was not significant ($p>0.05$).

5.5 Relationship Between Salinity and Relative Expression of Na^+-K^+ ATPase α-subunit

Previous studies have investigated that the expression of Na^+-K^+ ATPase α-subunit is strongly up-regulated after acute salinity stress. According to the results of these studies, a decrease in salinity level was associated with an increase in expression of Na^+-K^+ ATPase α-subunit (Hu et al., 2015; Gao et al., 2016). However, a positive linear regulatory relationship between salinity and relative expression of Na^+-K^+ ATPase α-subunit was investigated in this present study. This positive coefficient contracts with other published results as this suggests that as the level of salinity decreases, the relative expression of Na^+-K^+ ATPase α-subunit tends to decrease. The high tolerance of the cultured animal to salinity could possibly explain this positive relationship. It has been investigated that the whiteleg shrimp *L. vannamei* has a high tolerance to a wide range of salinity since they can survive in both low and high salinity environment, as low as 1 ppt to as high as 50 ppt (Martínez-Antonio et al., 2019). Results of various studies showed support for this statement. In these studies, the *L. vannamei* shrimps were reared in a various range of salinity, at salinity 2, 8, 10, 16, 20 and 30 ppt, results showed that the shrimps survived even at low salinity as 2, 8 and 10 ppt. In addition, there was no difference in the growth of those grown at salinity 2 ppt and those grown at salinity 16 ppt (Gao et al., 2016). Therefore, it can be concluded that the whiteleg shrimps that were cultured in the biofloc system in this study grew much better at low salinity than high salinity. As those shrimps can grow well at low salinity, they do not have to regulate a great number of stress-related genes such as Na^+-K^+ ATPase α-subunit to mitigate the

stressor. As a result, in this case, a low level of salinity is associated with a low level of relative expression of Na^+-K^+ ATPase α-subunit.

However, the p-value here was not statistically significant since it was greater than the usual significance level of 0.05. This indicates that, overall, the change in the salinity level was not associated with the change in the relative expression of Na^+-K^+ ATPase α-subunit.

The insignificant correlation coefficient indicates that there is no relationship between DO, salinity and their related genes. However, this is in contrast to the results obtained in this current study. In fact, it has been suggested that a p-value which is greater than the significant level of 0.05 indicates that there is insufficient evidence in the samples to conclude that a zero correlation exists. A possible explanation for this insignificance result is that the sample size is not large enough to provide enough evidence to reject the null hypothesis for the entire population (Frost, n.d.). This is referred to as type II error that commonly occurs in the statistical tests due to lack of statistical power to detect sufficient evidence for the alternative hypothesis (CFI, n. d). Consequently, it causes the user to incorrectly accept the null hypothesis resulting in a false-negative (Columb et al., 2016). In order to avoid type II error, the power of the test must be increased to minimize the probability of the occurrence of the error by increasing the sample size used in a test, since bigger sample size increases the chances to capture the differences in the statistical tests, as well as the power of a test (CFI, n. d).

5.6 Relationship Between Temperature and Relative Expression of Hsp70

It has been investigated that the expression level of hsp70 was significantly induced and was up-regulated when the shrimps were exposed to acute thermal stress (Qiagen, 2004). This indicates that the high temperature of the culture environment will be

associated with a high level of expression of hsp70 as a stress response. However, in this study, a negative linear regulatory relationship between temperature and relative expression of hsp70 was investigated. This negative coefficient contracts with other published results as this suggests that as the temperature increases, the relative expression of hsp70 tends to decrease. The high tolerance of the cultured animal to a wide range of temperatures could possibly explain this negative relationship. There have been studies to investigate an optimal temperature for the growth of *P. vannamei* shrimps. According to the study of Ponce-Palafox et al. (1997), the range of temperature for the best survival of the *P. vannamei* shrimps was between 20 ºC and 30 ºC. In addition, the range of temperature for the best growth of this species was between 25 ºC and 35 ºC. Meanwhile, the results of the study of (Wyban et al., 1995) indicated that this species can grow at between 23 ºC to 27 ºC. Especially, this study also investigated that temperature optima, which is defined as the temperature of fastest growth, is size-specific. When the size of the shrimp increases, this temperature optima will decrease. According to this study, those shrimps that weighted at smaller than 10 grams (small and medium-size shrimps) could grow faster in higher water culture temperature (such temperature was up to 30 ºC). Meanwhile, the temperature optimum for larger shrimps (those weighted at greater than 15 grams) was at about 27 ºC. In the present study, the size of most of the *P. vannamei* shrimps that were cultured in biofloc systems was at small (below 5 g), therefore, they grew much better at a higher temperature than lower temperature. As those shrimps can grow well at high temperatures, they do not have to regulate a large amount of hsp70 as a stress-related gene to mitigate the stressor. As a result, in this case, a high degree of temperature is associated with a low level of relative expression of hsp70.

In addition, the correlation of 0.674 and the R^2 value of 45.4 % suggests a moderate linear relationship between temperature and hsp70 expression. In addition, the p-value here was statistically significant since it was smaller than the usual significance level of

0.05. This indicates that, overall, the changes in the degree of temperature were associated with the changes in the relative expression of hsp70.

5.7 Microorganism Community Present in The Biofloc System

Microorganisms have been investigated to have an important role in enhancing the quality of water in the biofloc system by taking in the compounds of nitrogen and producing microbial protein which can be consumed back by the cultured animals, this help to decrease the cost of feed, subsequently increase the feasibility of culture (Emerenciano et al., 2013). In biofloc systems, there are three pathways for the remediation of ammonia, including algal assimilation, chemoautotrophic bacterial oxidation, and heterotrophic bacterial assimilation (Ray et al., 2014). Among these three pathways, the nitrification process by chemoautotrophic bacteria is the most efficient process in long term (Emerenciano et al., 2017), since the amount of oxygen consumed by this process is less than the heterotrophic process (Ray et al., 2014). In addition, the autotrophic process involves two bacterial groups, the first group is the ammonia-oxidizing bacteria that obtain their energy by catabolizing unionized ammonia to nitrite, they have higher efficiency in nitrogen transformation than heterotrophic bacteria, however, they take longer to oxidize the ammonia in nitrite, some genera belong to this group such as *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*; meanwhile, the second group is the nitrite-oxidizing bacteria that can metabolize a significant amount of nitrite to nitrate, involving *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitrospina*, they have a slower rate than the first group (Emerenciano et al., 2017; dos Santos et al., 2019). Meanwhile, heterotrophic bacteria also play an important role in the biofloc system. Firstly, they utilize the feces, dead organisms, and unconsumed food to produce bacterial biomass (Emerenciano et al., 2017). Secondly, they can assimilate nitrogen. It has been investigated that by adding carbohydrates to the

water, heterotrophic assimilation can rapidly remove ammonia. Moreover, the assimilation process of heterotrophic bacteria consumes a greater amount of oxygen than nitrification (Ray et al., 2014). More importantly, the immobilization of ammonium by heterotrophic bacteria occurs much more rapidly higher than nitrifying bacteria since heterotrophic bacteria have a high growth rate and high microbial biomass yield (Crab et al., 2012). Therefore, the identification of the type of microorganisms present in the biofloc is important. The isolation and identification of microbial communities had been done by the co-workers of this study (Ezzah et al., unpublished).

According to Ezzah et al. (unpushlised), the microbial communities obtained in tank 4, tank 10 and tank 13 at the 30th day and the 50th day of cultivation (the cultured was stopped at the 37th day, however, the culture tanks were kept for few more days for the formation of microbial flocs) were isolated and were characterized. Results indicated that *Proteobacteria* and *Bacteroidetes* were the most dominant bacteria presenting in biofloc. In particular, in tank 4, on the 30th day of cultivation, the density of *Bacteroidetes* was at about 42%, higher than the density of *Proteobacteria* which was approximately 32%. However, on the 50th day of cultivation, the density of *Proteobacteria* was higher than the density of *Bacteroidetes*, at 53% and 12%, respectively. In addition, the *Planctomycetes* was also found with densities of about 10% and 22% at the 30th and 50th day of the cultivation. While in tank 10, the density of *Proteobacteria* was higher than the density of *Bacteroidetes* throughout the culture. In particular, the density of *Proteobacteria* and density of *Bacteroidetes* was about 82% and 5% (on the 30th day), respectively, on the 50th day their densities were about 52% and 11%, respectively. Meanwhile, the *Planctomycetes* had a density of 10% and 27% at the 30th and 50th day, respectively (Ezzah et al., unpublished). Lastly, in tank 13, on the 30th day of cultivation, the density of *Proteobacteria* was higher than the density of *Bacteroidetes*, about 50%, and 16%, respectively. However, on the 50th day of cultivation, *Proteobacteria* kept

presenting in the biofloc at about 75%, while there was no presence of *Bacteroidetes*. In addition, the density *Planctomycetes* at the 30th and 50th day was about 31% and 10%, respectively (Ezzah et al., unpublished).

The most dominant species presenting in the biofloc system used in this current study were *Proteobacteria* and *Bacteroidetes*. The obtained results are similar to the result of a study by Cardona et al. (2016). The study investigated the bacterial community that presents in the cultural environment of the biofloc system as well as in the intestine of the *L. stylirostris* shrimps that were reared in the biofloc system. The results showed that *Proteobacteria* was the most abundant phylum in the culture water, while the second most abundant phylum was *Bacteroidetes.* Meanwhile, Kasan et al. (2017) isolated and identified the bacteria that forming biofloc in the culture of Pacific Whiteleg Shrimp, *L. vannamei.* The results indicated that most of the isolated bacteria belong to Proteobacteria and Firmicutes families. According to Cardona et al. (2016), *Proteobacteria* is widely dispersed in the aquatic environment. In addition, it plays a vital role in a nutrient cycling process, as well as the mineralization of organic compounds. Meanwhile, *Bacteroidetes* is referred to as a dominant member of the aquatic heterotrophic bacterioplankton.

However, nitrifying bacteria only found at the end of the culture with a small amount (Ezzah et el., unpublished). This is because the nitrifying bacteria are naturally promoted by the presence of ammonia and nitrite (Emerenciano et al., 2017) as well as other factors such as the carbon/nitrogen ratio, pH, temperature, DO, alkalinity and organic and inorganic carbon (dos Santos et al., 2019). Since there was a lack of nitrifying bacteria in the culture, the nitrification process mainly lay on the *Bacteroidetes*. In the present study, the average of ammonia in tank 4 was 0.21 ppm resulting in high amount of *Bacteroidetes* (about 42% at the 30th day); while the average ammonia observed in tank 10 was 0.18 ppm, leading to increase of *Bacteroidetes* from 5% (day 30th) to 11% (day 50th); lastly,

tank 13 obtained the average of ammonia at 0.11 ppm at the beginning of the culture, and the amount of *Bacteroidetes* was 16% during these times, lately, ammonia in this tank decreased and was not found in the last few weeks, consequently presence of *Bacteroidetes* was also not found. Moreover, no nitrite found in tank 4, 9 and 10 over the cultivation, while in tank 13 obtained nitrite of 0.18 ppm, this is within ideal range of ammonia, since ideal range of ammonia in a biofloc system is less than 1 mg/L (≈ 1 ppm) (Emerenciano et al., 2017).

It has been investigated that *Proteobacteria* comprises many important groups of Gram-negative bacteria, including several pathogens to humans and animals, especially Vibrionaceae, which is the most affected bacterial family by the presence of the virus. The genus *Vibrio* - which belongs to the mentioned bacterial family – is able to colonize the digestive tract of penaeids. Many *Vibrio* species are considered opportunistic pathogens for shrimp under stressful conditions, for instance, poor nutrition, low water quality, and immune depression. In farmed shrimp, this bacterial group has been repeatedly implicated in gastro-intestinal diseases, causing high mortality in shrimp culture worldwide (Pilotto et al., 2018). Since *Proteobacteria* is dominant and present in the culture tanks at a high amount, it is necessary to identify whether *Vibrio,* as well as other pathogens, present in the shrimp culture. In the current study, tank 4 obtained a low survival rate of 38%, in other words, mortality was about 62%. Two possible factors could contribute to the death of the reared shrimps in the tank. Firstly, the condition and quality of the tank itself were not suitable for the growth of the shrimp. As discussed in the previous chapter, hypoxic conditions might be created in the shrimps' cells due to inadequate supply of oxygen to the body although the DO levels in the tank were high. In addition, the level of dietary protein supplied is considered as a possible factor, since both high and low levels of dietary protein supplement negatively affect the culture environment and the cultured animals. Secondly, high mortality may be caused by the

presence of pathogens. This suggests that the low survival rate in tank 4 might be caused by pathogens although this tank obtained the highest DO level; while there might be no pathogens present in tank 13, resulting in a high survival rate although this tank obtained lower DO levels than tank 4. However, the current study could not manage to perform an identification test to verify the presence of pathogens such as *Vibrio* in the culture. This limits the finding of the study due to a lack of evidence to support the argument.

Besides, the biofloc system used in this study had been installed in a closed building with no exposure to natural light. Such systems like this are referred to as "brown-water" biofloc system, in which water quality is controlled by bacterial processes only (Hargreaves, 2013). Therefore, no presence of algae was found in the culture tanks.

CHAPTER 6: CONCLUSION

In this study, the shrimp *P. vannamei* were cultured in four different types of categories, including tank 4 (microbubble aeration), tank 9 (a mixture of microbubble and macro bubble), tank 10 (macro bubble with bioballs) and tank 13 (macro bubble without bioballs). In addition, tank 4 and tank 9 were applied with a recirculation system which was operated during the daytime. The expression profiles of catalase, Na^+-K^+-ATPase α-subunit, and hsp70 in response to hypoxia, salinity and heat shock, respectively, in each category, were well developed.

In tank 4 (microbubble aeration), up-regulated of catalase expression during the last two weeks indicated that hypoxic condition might occur inside the body of the shrimp. Meanwhile, the expression of Na^+-K^+-ATPase α-subunit was down-regulated during most of the time the cultivation, indicating that no salinity stresses had occurred. However, the results of up-regulated in the expression of Na^+-K^+-ATPase α-subunit in the last two weeks of the culture might be related to the variation of pH. Lastly, the expression of hsp70 was high up-regulated throughout the culture, indicating that the heat shock stress might occur in this system. In addition, the survival rate obtained in this tank was at 38%, this low rate indicates that this type of system is not suitable for the growth of the shrimp.

In tank 9 (a mixture of microbubble and macro bubble), the expression of catalase was down-regulated throughout the culture, indicating that the condition of this system did not cause hypoxia stress for the shrimps. Meanwhile, the expression levels of Na^+-K^+-ATPase α-subunit were down-regulated throughout the cultivation, indicating that salinity did not occur in this system. Lastly, the expression of hsp70 was down-regulated in the 3rd and 4th weeks of the culture after that become up-regulated at the end of the culture. Such up-regulated in hps70 expression indicates a presence of heat shock stress

at these points of time. Moreover, the survival rate obtained in this tank was at 96.67 %, this high rate indicates that the condition of this system is good for shrimp growth.

In tank 10 (macro bubble with bioballs), up-regulated of catalase expression during the last two weeks indicated that hypoxic condition might be created inside the cells of the shrimps. Meanwhile, the expression of Na^+-K^+-ATPase α-subunit was down-regulated during most of the time the cultivation, indicating that no salinity stresses had occurred. However, there was up-regulated in Na^+-K^+-ATPase α-subunit expression at the last week of the culture, this might be related to pH changes in the culture. Lastly, the expression of hsp70 was high up-regulated throughout the culture, indicating that the heat shock stress might occur in this system. Furthermore, the survival rate obtained in this tank was at 100%, this absolute percentage indicates that the condition of this system is extremely suitable for shrimp growth.

In tank 13 (macro bubble without bioballs), the expression of catalase was up-regulated at the beginning of the culture, after that became down-regulated in the last two weeks. The results indicate a possibility of hypoxic conditions that occur inside the shrimp tissues. Meanwhile, the expression levels of Na^+-K^+-ATPase α-subunit were down-regulated throughout the cultivation, indicating that salinity did not occur in this system. Lastly, the expression of hsp70 was high up-regulated throughout the culture, indicating that the heat shock stress might occur in this system. In addition, the survival rate obtained in this tank was at 97.50 %, this high rate indicates that the condition in this system is suitable for the growth of the shrimp.

In conclusion, all of the four categories above might cause a different type of stress in the culture environment, especially heat shock stress, since this stress was likely occurred in all of the categories. Among these four types of categories, the microbubble aeration

is not suitable for the shrimp culture, as this system might cause the hypoxic condition in the shrimp tissues, and heat shock stress. More importantly, the shrimp could not grow well in this system and could be died easily. Meanwhile, the two systems, macro bubble with bioballs and macro bubble without bioballs, providing a better environment for the shrimp growth and therefore they can survive and grow well, however, both systems can cause hypoxic condition and heat shock stress. In addition, the tank 13 had lower efficiency in nitrogen assimilation. Therefore, they could not be considered as the most appropriate for the shrimp culture. Lastly, the mixture of microbubble and macro bubble, could be suggested as the most appropriate technique and could be applied widely in the culture of whiteleg shrimp *P. vannamei* as well as other shrimp species. This system promises an efficient and stable culture since it did not produce hypoxia and salinity stress, while the heat shock stress occurred only at the end of the cultivation. In addition, the cultured shrimp in this system could grow well and have a high survival rate.

In addition, in this study, the relationship between water quality parameters and relative expression of stress relates genes were also developed. Particularly, the relationship between the DO level and catalase showed a negative linear regulatory. While a positive linear regulatory relationship was obtained between salinity and relative expression of Na^+-K^+-ATPase α-subunit. Lastly, the relationship between temperature and hsp70 showed a negative linear regulatory. However, only this regression was significant as p-value < 0.05; the other two regressions were not significant since p-value > 0.05.

Furthermore, since DO was the most unstable parameter and was varied among different types of systems, and it was also changed throughout the experiment, this study also developed the relationship between DO and relative expression of catalase in different categories, as well as the relationship between relative expression of catalase

and time of cultivation in different categories. However, most of the regressions showed a very weak relationship, as the R^2 values obtained at very low values. More importantly, all of these regressions were not significant, since their p-value > 0.05.

This study has several limitations, including the inability to investigate significant differences in the water quality parameters in the tank during the cultivation due to insufficient total number size. In addition, another limitation of this study is the non-significant results. As explained above, this insignificance might be caused by type II error due to the low power of a statistical test. Therefore, in order to prevent this issue for future research, a suggestion is that the cultivation of the shrimps in biofloc systems must be prolonged to several months, at least for more than two months. In addition, the number times of the sampling should be increased, and time between two samplings should be shortened. For instance, sampling should be done every three days or every five days. In addition, more biological replicates should be collected at each sampling time.

Besides, the presence of microorganisms in the system is suggested to be an important factor and is needed to be considered. This is because the microbial communities presenting in the environment can compete with the animals living in the environment for oxygen consumption. Consequently, this will lower or even limit the amount of oxygen in the culture, and causing hypoxia, as a result. Therefore, it is important to investigate whether the presence of microorganisms in the bioflocs system influences the levels of dissolved oxygen. However, the results of this study only can investigate types of microorganisms present in the system, but could not measure the density of them, consequently, could not able to develop the relationship between microorganisms' densities and levels of dissolved oxygen. Therefore, further work is required to measure the number of microorganisms that present in the culture of the shrimp *P. vannamei* bioflocs system in order to develop the relationship between microbial communities and

the variation of DO levels, as well as investigate the rate of oxygen consumption by the organisms present in the culture. This will help to investigate if the amount of oxygen consumed by the organisms is greater than the amount of oxygen supplied, leading to limitation of oxygen, consequently resulting in a hypoxic condition in the tissue of the body of the cultured shrimps. Besides, it is important to identify if the pathogens including *Vibrio* present in the culture.

Moreover, one of the limitations of this current study is the lack of control. All the comparisons were only made among the four tanks. Therefore it could not be concluded if the results obtained herein were different than the normal system. A suggestion for future work is the establishment of a control tank using a traditional technique. This will help to compare the gene expression levels in biofloc and non-biofloc system consequently will help to prove if the biofloc technology is beneficial than the traditional technique.

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