Growth, Vibriosis, and Streptococcosis Management in Shrimp-Tilapia Polyculture Systems, and the Role of Quorum Sensing Gene cqsS in Vibrio harveyi Virulence

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By

Sidrotun Naim

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Sidrotun Naim

entitled "Growth, Vibriosis, and Streptococcosis Management in Shrimp-Tilapia Polyculture Systems, and the Role of Quorum Sensing Gene CqsS in *Vibrio harveyi* Virulence"

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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Signed: Sidrotun Naim

DEDICATION

In the loving memories of:
Father Abidullah, it was his dream to see his children achieve the highest education.
Maternal grandmother (Eyang Putri Kusniyah) and grandfather (Eyang Kakung M. Komar), who looked after me during high school, and taught me to be the best person in serving others, the same mystical teaching I received from my paternal grandparents.
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ABSTRACT

Tilapia culture in Indonesia was started with the Mozambique Tilapia (*Oreochromis mossambicus*) in the 1930's, and the Nile Tilapia (*Oreochromis niloticus*) the 1960's. The genetic improvement program of the Nile Tilapia, has led Indonesia to be one of the main tilapia producers in the world. On the other hand, shrimp aquaculture in the country was not started until the 1960's, it became more popular after the eye ablation technology for broodstock maturation was developed in the early 1980's.

The first experimental study was conducted to investigate the feasibility of low salinity shrimp farming in a polyculture system with tilapia. Polyculture increased the survival for shrimp (77% compared to 62%), but at the same time decreased the survival of tilapia (87% compared to 97%). Together, the data on survival, specific growth rates, and feed conversion ratios showed that the shrimp performed well at low salinity.

The second experimental study investigated the feasibility of brackishwater shrimp farming in a polyculture system with tilapia. Polyculture increased the survival for shrimp (82% compared to 65%), and had higher survival for the tilapia (60% compared to 43%). The Red hybrid Tilapia strain used in the study experienced mortalities after one month, suggesting the need for a salt tolerant strain. The presence of tilapia stimulated the growth of microalgae (*Chlorella* dominance), promoted higher numbers of heterotrophic bacteria in the water, and had lower presumptive vibrios on TCBS agar.

A challenge study was conducted by mixing pathogenic luminescent *Vibrio harveyi* UAZ-651 into shrimp and tilapia feed. The survival of shrimp in monoculture were significantly lower (20%) compared to in polyculture systems (75 - 95%). Mortality was not found in tilapia. Based on *16S rRNA* gene sequence, shrimp monoculture water was dominated by marine *Vibrio* spp., while the polyculture system had *Bacillus* spp. and *Vibrio* spp. with high homology to *V. cholerae*. The presence of *Bacillus* spp. which produce a lactonase enzyme AiiA, seems to inhibit vibrio growth. While providing advantages, shrimp-tilapia polyculture might also contribute to streptococcosis transmission. Injecting shrimp with *Streptococcus iniae* and *S. agalactiae* resulted in mortalities. *S. iniae* caused higher mortality in the shrimp cultured in 20 ppt (40%) compared to 10 ppt (20%), and no mortality in 5 ppt. *S. agalactiae* caused higher mortality in 5 ppt (40%) compared to 10 ppt (20%) and 20 ppt (20%).

Quorum sensing (QS) is a density dependent cell to cell communication process in bacteria. Based on challenge studies in shrimp, the luminescent *Vibrio harveyi* BB120 wild-type strain caused 75-90 % mortality through injection of 10^6 CFU/shrimp. The mortality patterns in the QS mutants suggest that QS defined, when specific virulence genes were expressed or repressed. As QS in *V. harveyi* consists of three different circuits, further experiments deployed six mutants lacking either a synthase or a receptor for each circuit. The highest survival in the CqsS (a receptor for CAI-1 circuit) mutant group indicates that the CAI-1 circuit is the most crucial for virulence, followed by the AI-2 and HAI-1 cascades. Chitin acquisition and oxygen scavenging may be two reasons for luminescence in *V. harveyi* evolution and why they infect shrimp.

CHAPTER 1

GENERAL INTRODUCTION

1.1. Shrimp-Tilapia Polyculture: Technical Feasibilities in Low and High Salinities

Shrimp aquaculture has been the main livelihood for coastal residents in many developing countries, including Indonesia, which has one of the longest coastal lines in the world, and it is one of the main shrimp producers in the world. Rural residents in the coastal areas do not have many alternative livelihoods so they rely highly on shrimp farming. Unfortunately, because of problems from diseases, poor management, or other causes, many shrimp ponds in developing countries have been idled. An alternative to better utilize these abandoned facilities is by co-culturing shrimp and tilapia, known as polyculture. The wide salinity, water quality tolerance, a relatively lower nutrition requirement for growth, and low disease risk compared to shrimp, make tilapia a good candidate for an alternative aquaculture species (Fitzsimmons et al., 2011).

However, for the farmers, shrimp culture is still preferable because of its high value compared to tilapia. With this regard, shifting completely from shrimp culture to tilapia culture is not an ideal solution from socio-economic point of view. Shrimp is, and might forever be, the most preferred seafood in the world. Shrimp-tilapia polyculture may provide an opportunity to re-establish a profitable and more sustainable aquaculture system at these dormant facilities with shrimp as the main species (Yi and Fitzsimmons, 2004). Farmers have reported that culturing shrimp and tilapia together can minimize the disease risk for shrimp (Fitzsimmons, 2001).

The idea to culture shrimp and tilapia in the same water column might seem an oddity at the beginning. In nature, shrimp and tilapia rarely live together because shrimp are raised in higher salinity and tilapia are freshwater species. In aquaculture settings, they can be reared together to maximize the space and available nutrients in the water (Fitzsimmons, 2001). From disease risk perspective, having tilapia in shrimp pond seem to provide advantages in several ways: improving water quality by disturbing/cleaning bottom sediment, disrupting shrimp cannibalism and minimizing disease transmission, and reducing bacterial infections by using their antimicrobial properties. On the other hand, shrimp lack of those capabilities because they have a less developed immune system (Cruz et al., 2008; Tendencia et al., 2010). The simplistic immune system put shrimp at a higher risk when a variety of disease agents are present in the water.

Theoretically feasible, there are technical questions to be answered regarding optimal salinities, stocking densities, feeding rates, disease and parasite infestations, cost benefits, and environmental impacts. In low salinity, the growth of shrimp will be affected and at the same time shrimp disease risk is minimal as the shrimp disease agents are optimum at high salinity. However, there will be a risk of new diseases being transmitted from tilapia to shrimp in low salinity as tilapia diseases (for example streptococcocal disease) are found at low salinity in different freshwater fish (Shoemaker et al., 2001). In contrast, in high salinity, tilapia may be compromised survival and growth. Development of salt tolerant tilapia and supermale tilapia (which grows faster compared to female tilapia) is needed. The risk of disease transmission from shrimp to tilapia (for example vibriosis) is considered low in salinity that is close to freshwater.

1.2. Disease Management and Quorum Sensing of Luminescent Vibriosis

While growth is the main economic issue in any aquaculture production, disease is considered as the most limiting factor in any operation. Addressing how a polyculture system would provide benefit in terms of disease management is as critical as the technical feasibilities. From the disease transmission point of view, culturing two species together might provide advantages (when one species compensate the lack immune system of another species), and disadvantages (when disease is transmitted from shrimp to tilapia, and vice versa). Chapter 5 discusses luminescent vibriosis in a polyculture setting and to less extent the risk of streptococcocal disease transmission in the system. Even though bacterial diseases are considered less important compared to viral diseases, in fact, the emergence of bacterial disease in shrimp aquaculture is as old as shrimp aquaculture itself (Lightner, 1993). With the tendency of high density aquaculture in a recirculating system and low-salinity (inland) aquaculture to minimize viral disease, the approach might provide favorable environment for pathogenic bacteria as side effects. With this regard, addressing bacterial disease and its pathogenicity mechanism will be critical for the future of aquaculture.

In the last two decades, the pathogenicity mechanisms of bacterial diseases have been studied extensively. Bacteria need to be in a high number to cause disease (Bassler et al., 1993). Quorum Sensing (QS), the process of cell to cell communication enable bacteria to do many tasks they cannot accomplish as an individual (Fuqua et al, 1994). The roles of QS in pathogenicity of *Vibrio harveyi* has been one of the most well studied areas in *in vitro* scale (reviewed in Bassler et al., 1997; Waters and Bassler, 2005; Ng and Bassler,

2009). Unfortunately, in terms of *in vivo* studies, the manifestation of bacteria in its host (shrimp) is still limited. The current state-of-the-art in QS study has been toward screening and formulating anti-QS molecules to disrupt the communication in harmful bacteria, and discovering pro-QS molecules for beneficial bacteria for humans (Ng et al., 2012; Wei et al., 2012). At the same time, shrimp disease studies should benefit from the cutting edge technology widely used in the human disease studies, and challenge study in shrimp may be simpler compared to clinical trials for new drugs for humans. Some of these anti-QS and pro-QS molecules would come from the natural environment, perhaps from tilapia skin-mucus or the microalgae and bacteria associated with the polyculture system.

1.3. Taxonomy

Experimental studies presented in this dissertation covered three main species: shrimp (*Penaeus vannamei*), tilapia (*Oreochromis niloticus*), and *Vibrio harveyi*. The taxonomy for each species presented in Table 1.1.

Table 1.1. Taxonomy of shrimp, tilapia, and *Vibrio harveyi* used in the studies

	Shrimp	**Tilapia**	***Vibrio harveyi***
Phylum	Arthropoda	Chordata	Proteobacteria
Class	Malacostraca	Actinopterygii	Gammaproteobacteria
Order	Decapoda	Perciformes	Vibrionales
Family	Penaeidae	Cichlidae	Vibrionaceae
Genus	*Penaeus*	*Oreochromis*	*Vibrio*
Species	*P. vannamei*	*O. niloticus*	*V. harveyi*
References	FAO website*	Trewavas, 1982	Baumann et al., 1980

*http://www.fao.org/fishery/species/search/31001/8502/en

1.4. Brief Descriptions of Dissertation Chapters

Chapter 2, Tilapia Culture and Development in Indonesia (1936 – 2012), covers an historical perspective that even though tilapia are not native to the country, the fish have been established for more than seven decades. In this regard, considering tilapia as invasive species which will endanger native fish is not relevant. Tilapia culture in Indonesia was started with the Mozambique Tilapia (*Oreochromis mossambicus*) in the 1930's and the Nile Tilapia (*Oreochromis niloticus*) in the 1960's. In the last decade, tilapia is considered as one of the main aquaculture species in Indonesia. Direct interviews with the remaining family members of the first person who cultured tilapia in the country provides a documentation how the original brackishwater fish were successfully cultured in freshwater environment in the 1930's. Starting in the late 1960's, an effort to improve the genetic quality through importation of improved strains, especially Nile Tilapia, was conducted. Since then, several strains from other geographic locations have been introduced. Genetically Supermale Indonesian Tilapia (GESIT) strains are discussed in more detail in this chapter. The genetic improvement program has led Indonesia to be one of the main tilapia producers in the world together with China, Thailand, Egypt, and the Philippines. By 2010, tilapia production in Indonesia had surpassed carp production (MMAF, 2010). This trend might be followed by other countries with the increasing demand for tilapia worldwide. Indonesia is not only a tilapia producing country, but it is also a major producer of shrimp, together with China, Thailand, and Vietnam (FAO, 2010). This reality makes a shrimp-tilapia polyculture study one that would benefit the country as both species are widely distributed.

Chapter 3, Feasibility of Shrimp-Tilapia Polyculture in Low Salinity, investigates the technical considerations of the system in an experimental study with emphasis on the survival and growth of shrimp. Inland and low salinity shrimp aquaculture have been implemented by farmers in different regions in the world in the effort to minimize shrimp disease risk (Fitzsimmons, 2001; Flaherty et al. 2000). The green water in tilapia culture and shrimp-tilapia polyculture is a nutrient-rich environment compared to the clearer water in shrimp monoculture. The presence of a higher number and diversity of bacteria, a relatively lower pH, and the presence of microalgae (*Chlorella* dominance), all together might play synergestic roles in the polyculture system to improve water quality and fitness of the animals. Polyculture increased the survival for shrimp compared to monoculture, but at the same time decreased the survival of tilapia, which was not expected as low salinity is the common habitat for tilapia. Together, the data on survival, specific growth rates, and feed conversion ratios showed that the shrimp performed well at low salinity. Water quality parameters (dissolved oxygen, pH, ammonia, nitrite, and nitrate) were all within acceptable ranges.

Chapter 4, Review on Shrimp Aquaculture in Indonesia, and Feasibility of Shrimp-Tilapia Polyculture in Brackishwater, describes the history of shrimp aquaculture in Indonesia and major diseases associated with the culture. Polyculture systems, adding tilapia to shrimp ponds with high salinity is believed will minimize to disease risk (Yi and Fitzsimmons, 2004). Compared to the results from a low-salinity experiment, survival of tilapia were lower (which was expected), but the growth rates of tilapia survivors were higher as a result of fewer survivors. An interesting point, that the survival

of tilapia in the polyculture system was higher compared to tilapia monoculture. This result suggested that in higher salinity, the presence of shrimp might help tilapia fitness. For shrimp, as expected the animals had higher survival, growth rates, and more efficient Feed Conversion Ratio (FCR) compared to the performance at low salinity. The advantages of brackishwater polyculture for both shrimp and tilapia will lead to the need to develop salt-tolerant tilapia strains to optimize the system. Compared to shrimp monoculture, total heterotrophic bacterial counts were higher in tilapia monoculture and polyculture and, at the same time, lower presumptive vibrio counts, suggesting that the presence of tilapia, or tilapia and algae, stimulates higher diversity of bacteria which in turn competes and inhibits vibrio growth.

Chapter 5, Reduced Luminescent Vibriosis in Shrimp-Tilapia Polyculture and Low Susceptibility of Shrimp to Streptococcosis Infections, further investigates the beneficial effects of polyculture in terms of minimizing disease risk. The experimental study was conducted in brackishwater for two reasons. First, the growth study experiments (Chapter 3 and Chapter 4) suggest that higher salinity is more feasible for both shrimp and tilapia compared to low salinity, not to mention the socioeconomic and environmental issues which might rise from low-salinity polyculture in the field. Second, the most important bacterial disease in shrimp, luminescent vibriosis, is optimum at higher salinity (Lightner, 1993) and, therefore, it is necessary to run the experiment at the appropriate salinity. By mixing luminescent *Vibrio harveyi* UAZ-651 (originally isolated from diseased shrimp in the Philippines in 1990) into shrimp and tilapia feed, the survival of shrimp in monoculture was significantly lower compared to in polyculture systems.

Mortality was not found in tilapia suggesting that the risk of vibriosis transmission from shrimp to tilapia was low.

Sequence data from the *16S rRNA* gene of different bacteria community revealed that water from shrimp monoculture was dominated by marine *Vibrio* spp., while the polyculture system had *Bacillus* spp. and *Vibrio* spp. with high homology to *V. cholerae*. The sequence confirmed previous finding that *Bacillus* sp produces AiiA lactonase enzyme which inhibits acyl homoserine lactone/AHL (Bassler and Losick, 2006). AHL is the structure of the HAI-1, one of the three autoinducers for *V. harveyi* virulence.

While providing advantages, on the other hand, shrimp-tilapia polyculture might also contribute to disease transmission. *Streptococcus iniae* and *S. agalactiae* which are pathogenic for tilapia and other freshwater fish (Shoemaker et al., 2001), might be transmitted to shrimp (Hasson et al., 2009; Lightner, 2009). In places where polyculture is being implemented, tilapia is considered as the secondary species, and, therefore, the density is low. When both species are raised in high densities, tilapia might be infected with *Streptococcus*, and it could be transmitted to shrimp. Injecting shrimp with *S. iniae* and *S. agalactiae* resulted in mortality. *S. iniae* caused higher mortality in 20 ppt (40%) compared to 10 ppt (20%), and no mortality in 5 ppt. *S. agalactiae* caused higher mortality in 5 ppt (40%) compared to 10 ppt (20%) and 20 ppt (20%). This result provides information that the density of tilapia in polyculture system both in low and high salinities is critical, not only in terms of growth but also to minimize streptococcus transmission.

Chapter 6, *CqsS* Gene in CAI-1 Circuit Determines *Vibrio harveyi* Virulence in Shrimp, highlights a series of experimental challenge study to confirm the *in vitro* findings in the last two decades that quorum sensing (QS) controls the pathogenicity of luminescent *Vibrio harveyi* (reviewed in Bassler et al., 1997; Waters and Bassler, 2005; Ng and Bassler, 2009). All experiments used *V. harveyi* BB120 strain (also known as ATCC BAA-1116) and its derivative mutants which are widely used in the QS study. BB120 strain was isolated from marine environment in 1993 and not associated with shrimp disease. The strain is one of the only two *V. harveyi* which has its full genome sequenced, so the genetics are known very well. Recent genomic studies (Lin et al., 2010) proposed that the BB120 strain, and *V. harveyi* HY01 strain which have full sequence, are *V. campbellii,* and, therefore, no *V. harveyi* full genome sequence is available. Efforts to sequence the first full genome for *V. harveyi* CAIM 1792 (Espinoza-Valles et al., 2012), and *V. campbellii* DS40M4 strain (Dias, 2012) are currently undergoing, and the data is not yet available. For consistency, this dissertation refers the BB120 as *V. harveyi* because sequence comparison for BB120, HY01, CAIM 1792, and DS40M4 strains could not be established at this moment.

The first QS experiment suggested the nature of vibrio infection which is a systemic, secondary, and opportunistic pathogen (Lightner, 1996). These facts lead to the difficulties in establishing experimental infection. During the experiment, mortalities were only achieved by injection of 10^6 CFU/shrimp and not by reverse gavage, feeding or immersion. As one of the autoinducers for virulence, the AI-2, needs boron as co-factor to be functional (Chen et al., 2002), an additional challenge study was done by adding

boric acid into water in the feeding and immersion groups, which resulted no mortality. A disadvantage of injection is, it bypasses the shrimp primary defense mechanisms such as the cuticle, cuticular epithelium and midgut mucosa (Smith, 1991). The second experiment employed two different mutants locked at low cell density/LCD conformation (QS is repressed) and high cell density/HCD conformation (QS is constitutively expressed). Compared to the wild-type, the LCD mutants caused an immediate infection and the HCD ones experienced delayed virulence, and led to delayed mortalities in shrimp. As the course of infections was skewed, these findings suggest that QS plays roles in the pathogenicity. QS controls the expression of specific genes at specific time (Waters and Bassler, 2006), and consists of three different circuits (Henke and Bassler, 2004b). The third experiment further investigated if one circuit is the most important determinant for the pathogenicity. The experiment used six different mutants with knock-out in one gene either the synthase or the receptor mutant for each circuit. Defining the most important cascade(s) would provide useful information to block that specific pathway as anti-QS molecules for two cascades (HAI-1 and CAI-1) are already available (Ng et al., 2012; Wei et al., 2012). Scientists are currently screening the anti-QS molecule for the third circuit (AI-2).

Mortality data showed that the wild-type and CqsA- mutant (lack of CAI-1 system) caused the highest final mortality rate (80%) in shrimp, followed by LuxM- and LuxN-mutants (lack of HAI-1 system) with 70% mortality, LuxS- and LuxPQ- (lack of AI-2 system) with 40-50% mortality, and CqsS- (lack of CAI-1 system) with 20% mortality. The findings indicated that CAI-1 circuit is the most crucial for virulence followed by AI-

2 and HAI-1. The CqsA- mutant showed similar behavior to the wild-type because it had the other two systems to compensate, and it possibly used CAI-1 or CAI-1 like molecules from other bacteria in the water which were recognized by CqsS receptor. On the other hand, the CqsS- mutant resulted in lowest mortality in shrimp because even though CAI-1 molecules were available in the water, the cascade had no function in the absence of receptor. Microbiology, histopathology and PCR analysis confirmed the presence of *V. harveyi* in the hemolymph of dead shrimp.

Histopathology analysis revealed that the bacteria colonized the hindgut lumen. Vibrio have unique ability to survive on chitin and their association with chitin may be a key to their evolution and why they colonize shrimp, which have chitin in different areas such as the cuticle and cuticle epithelium in the stomach and hindgut. Huq et al. (1986) demonstrated that *V. cholerae* attach exclusively to crab hindguts, which are lined with chitin, as opposed to the crab midguts which are endodermal and not lined with chitin.

To the best of authors' knowledge, this is the first report that *cqsS* gene (or CqsS receptor) in the CAI-1 system is the most important determinant for the virulence of *V. harveyi* in shrimp. The finding is different from *in vitro* result that HAI-1 is the most crucial pathway. The difference is not unexpected, as *in vitro* study does not account the host immune response. This result has a significant relevance in the study of vibriosis in shrimp, as closely related pathogenic species (*V. campbellii* and *V. parahaemolyticus*) share the same pathogenicity pathways (HAI-1, CAI-1, and AI-2) (Henke and Bassler, 2004a; Defoirdt et al., 2008).

CHAPTER 2

TILAPIA CULTURE AND DEVELOPMENT IN INDONESIA (1936 – 2012)

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2.1. Overview of Tilapia Worldwide

Tilapia have become a shining star of aquaculture with farms starting and expanding across the globe. In 2010, farmed tilapia exceed 3.2 million metric tons per annum, surging further ahead of the salmon and catfish industries (Fitzsimmons et al., 2011). The global adoption of tilapia as a substitute for all kinds of wild-caught fish has driven demand higher every year, even though there has been a global recession in recent years. The description of tilapia as an "aquatic chicken" becomes more accurate every day. The wide acceptance of this fish across all cultural, religious, and economic groups is similar to chicken. A variety of breeds and strains have been developed and by most measures, tilapia are now the most highly domesticated of farmed fish species. Unique among the major farmed fishes, tilapia maintain a key role in rural aquaculture improving the welfare of the poorest farmers, while at the same time, they are reared in the most high tech production systems and are sold into up-scale international markets. Tilapia are still the darling of the environmental community and the industry continues to polish and promote its "green" credentials.

Tilapia continue to march towards eventually overtaking carp as the most important farmed fish crop. With a much wider distribution of production and consumption and a huge base of value added product forms, this fish will someday, most likely, eclipse carp production. As production and consumption grow globally, tilapia are likely to become the foundation product for all farmed fishes, just as chicken is the base for the poultry industry. So, someday soon, instead of referring to tilapia as the aquatic chicken we may be referring to chicken as the "terrestrial tilapia".

In terms of geographic distribution, FAO reports tilapia production from over 100 nations (FAO, 2010). This vast base of production and interest in the fish vastly exceeds any other farmed fish. The consumer demand is equally widespread. There are no reports of cultural or religious restrictions on consuming tilapia, contrasted to other terrestrial animal products such as beef and pork, and bottom dweller aquatic species.

The major countries in tilapia production harvested just over 3,200,000 metric tons of in 2010 (FAO, 2010) (Figure 2. 1). China continues to be the main tilapia producer in the world, with Egypt, Indonesia, the Philippines and Thailand increasing production every year. As one of the main tilapia producers in the world, unfortunately, literature on tilapia culture in Indonesia is limited. Therefore, this chapter reviews the history of tilapia culture and development in Indonesia, which was started in the 1930's.

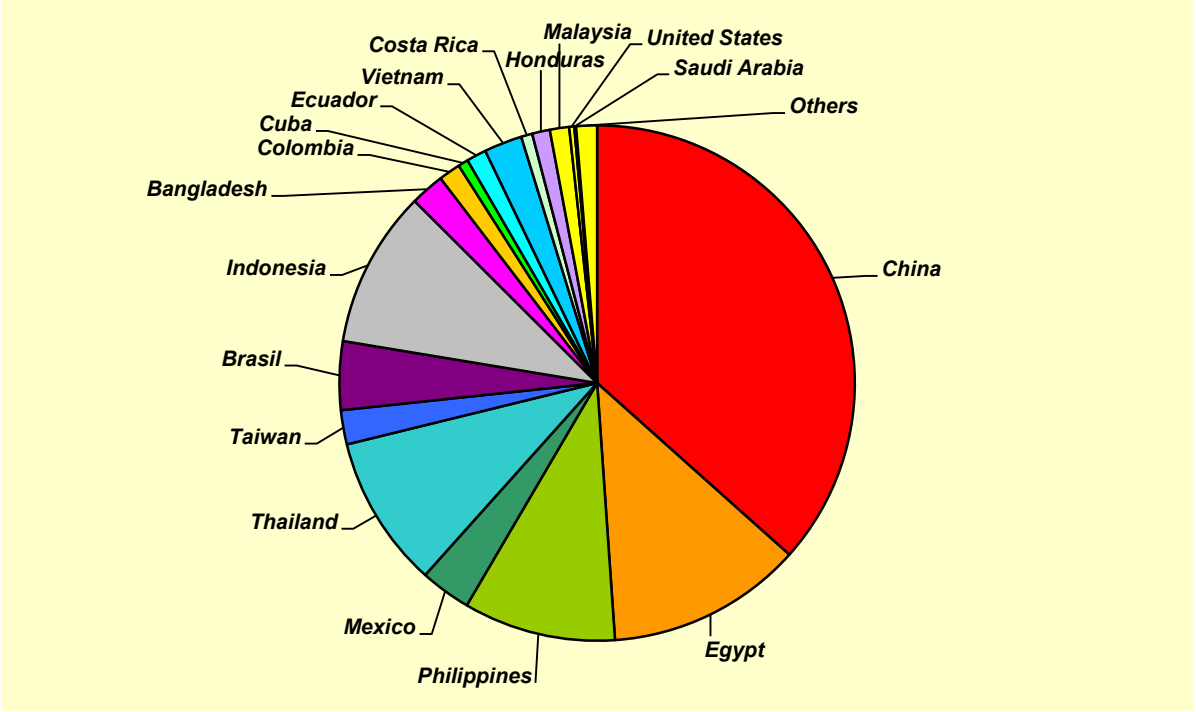

Figure 2.1. World tilapia production in 2010

2.2. Tilapia in Indonesia

In Indonesia, tilapia are known as 'ikan nila' (for the Nile Tilapia, *Oreochromis niloticus*) and 'ikan mujair' (for the Mozambique Tilapia, *O. mossambicus*). Not native to Indonesia, the local name 'mujair' for the Mozalmbique Tilapia came from the person who found this fish in 1936 in the Serang River, Blitar, East Java. The most likely explanation for the appearance of this non-native fish was that the Dutch, during the colonial era, shipped live fish from its native habitat in Africa to Indonesia. Both South Africa and Indonesia were colonized by the Dutch during the same period. Shelton and Popma (2006) speculated the introduction from aquarium trade. Nevertheless, this history

also explains how the common name for Mozambique Tilapia at some point became Java Tilapia, as it was already found in Java, one of the islands in Indonesia in 1930's (Bardach et al., 1972). During the Japanese occupation in Indonesia (1942 – 1945), the Japanese spread the fish to other occupied territories, mostly in Asia, and people called the fish as Java Tilapia. Based on the identification by taxonomists, later it was determined that what had been famous as Java Tilapia, was in fact the Mozambique Tilapia (Shelton and Popma, 2006).

The fish was first regarded as a nuisance, but when facilities for milkfish culture deteriorated under the Japanese occupation, the ease of tilapia culture became apparent. By the end of the Second World War, tilapia were already found in many locations in Indonesia (Bardach et al., 1972).

The development of tilapia culture in Indonesia was quite rapid. In the beginning, tilapia farming concentrated on optimizing the technique/method for cultivation, including the fingerling supply to increase production. However, the genetic improvement program lagged behind production. Starting in the late 1960's, an effort to improve the genetic quality, through importation of improved strains, especially the Nile Tilapia, was conducted. Since then, several strains from other geographic locations have been introduced into Indonesia.

2.3. Moedjair, the Mozambique Tilapia Aquaculture Pioneer from Indonesia

The history of tilapia in Indonesia was started by a single person, Moedjair (old Indonesian spelling), or Mujair (modern spelling). For consistency, this chapter will use the old spelling when referring to his name, and the new spelling for the name of the fish which was derived from his name.

Based on personal communications with the remaining family members, his real name was Iwan Dalauk, but he was better known as Moedjair. He was born in 1890, in a village 3 miles east of Kuningan in Blitar, one of the regions in East Java. He was the promoter for *Oreochromis mossambicus* (the Mozambique Tilapia) in Indonesia. Born into a family with nine children, his father was Bayan Isman and his mother was Rubiyah. Later in life, he married Partimah, a daughter of an Islamic cleric in the village. Together, they had seven children. Out of these seven, by 2012, only two children survived, Ismoenir who continues to live in Kanigoro, Blitar, and Djaenuri who lives in Kencong, Jember. The interview was conducted with the family in Blitar.

During his life, Moedjair was selling goat satay (an Indonesian traditional food, a kind of grilled goat). His restaurant was quite famous in those days in Kanigoro. Customers came from different ethnic groups, including the Dutch, the Arabs, and the Chinese. As a result, soon he became a wealthy man. Unfortunately, he had one bad habit. Since he had a lot of money, he started gambling. Moedjair did not gamble with Indonesian natives, only amongst the rich Chinese people, and he did not allow his children to gamble. Moedjair was not good at gambling, and soon he went into bankruptcy.

During this difficult time in his life, Moedjair travelled and contemplated on a special day, at a special place close to Serang River in Blitar. He did traditional ritual bathing to cleanse himself from the bad things he had done in the past. At one point during the ritual bath, he saw a number of fish. He thought the fish were so unique. The fish carried the babies in their mouths when they were in danger, and then released them in safer situations.

Because of this unusual mouth breeding behavior, which he had never seen before, together with his two friends (Abdulah Iskak and Umar), Moedjair brought the fish back to his home in Papungan village in Kanigoro, Blitar. Due to different habitats, the fish died when he put them into freshwater. This drove his curiosity to culture the fish in freshwater environment. In fact, the distance from his village to the river was quite long (about 35 kilometer), and passed the jungle and hills. Difficult road access made the trip take two days and two nights in total.

When he saw similar fish again, he mixed seawater and freshwater. He continued mixing the water by decreasing the amount of seawater and increasing the freshwater overtime. After eleven generations, he was able to successfully keep four fish without seawater. Starting from that day, the fish were known as freshwater fish in the area. Moedjair recorded this achievement on March 25, 1936.

Moedjair was very satisfied with his experiment. He started to culture the fish in one pond and later three ponds. Moedjair found that the fish grew very rapidly. He shared his fish for free with residents in his village, and sold them in other places in Blitar.

The discovery of this new species of fish came to the attention of a Dutch officer who was based in in Kediri, East Java. The officer was a scientist (his name could not be verified), and he tried to identify the fish based on the literature. He interviewed Moedjair and he was impressed by this traditional farmer's accomplishment. As the Dutch authority for the area, he announced that he named the fish as mujair, to honor the founder. Later, by taxonomy, it was identified that the fish was the Mozambique Tilapia (Shelton and Popma, 2006).

Again, Moedjair became popular, not as a goat satay restaurant owner, but as a fisherman. After the Indonesian independence in 1945, with the help of his eldest son, Wahanan, he marketed the fish to almost the entire mainland of East Java. By the local government, he was appointed as an officer in Papungan Village and received a monthly salary. The new Indonesian government appointed him as a fisheries extension specialist.

Several recognitions were awarded to Moedjair. The first one was during the sixth Indonesian Independence Day, on August 17, 1951. The Minister of Agriculture (Soewarto), on behalf of the Government of Indonesia, gave the citizenship award to him. The second award was from the Executive Committee of the Indonesian Fisheries Council. This award was given in Bogor, West Java, on June 30, 1954.

Soon, Moedjair was recognized as a tilapia expert. He constructed different ponds in East Java. Many people visited him to gain knowledge on fisheries. During his later years and as his health declined, Moedjair started three ponds in Krajan Village. He passed away September 1, 1957 because of asthma, and he was buried in the cemetery in Papungan village. In 1960, upon the initiative of the Department of Fisheries of Indonesia, his tomb was moved to the area south of the village which also serves as the family tomb. In recognition of his service, his tombstone reads: "Moedjair, the founder of Moedjair Fish". The road to the cemetery was also named Moedjair Street to honor him.

On April 6, 1965, the Indonesian Government, through the Department of Fisheries and Marine Affairs, recognized him as the aquaculture pioneer in Indonesia.

All pictures presented in this chapter regarding Moedjair were obtained from his family.

Figure 2.2. Portrait sketch of Moedjair (Photo by Dodit Ari G.)

Figure 2.3. The tombstones of Moedjair (right) with the fish relief and his wife (left). Photo by Ating Yuniarti

Figure 2.4. The Mujair Street in Blitar, East Java. Photo by A. Yuniarti


REPUBLIK INDONESIA
DEPARTEMEN PERIKANAN
DARAT/LAUT

MENTERI PERIKANAN

PIAGAM NELAJAN PELOPOR

HAMZAH ATMOHANDOJO

Figure 2.5. Aquaculture Pioneer recognition certificate, given by the Indonesian Minister of Fisheries in 1965. Photo by Dodit Ari G.

2.4. The Nile Tilapia

The Nile Tilapia was introduced from Taiwan in 1969, followed by a special black colored tilapia, the Chitralada, from Thailand in 1989, and GIFT (Genetic Improvement of Farmed Tilapia) from the Philippines in 1994. Another strain, NIFI (Thai Red Tilapia Strain), was imported from Thailand in 1989 (MMAF, 2000).

After six generations, in 1997, GIFT tilapia, which was the main tilapia strain in Indonesia at that time, experienced a decrease in the genetic quality because of reproduction management which was not accurate and therefore influenced growth. As a result, the decrease in production was unavoidable. This encouraged the establishment of the National Tilapia Broodstock Development Center in Sukabumi, West Java. By gathering fish genetic experts, the center was expected to develop several new strains of tilapia from the genetic resources existing in Indonesia (MMAF, 2000).

In 2002-2004, a Freshwater Research Centre in Jambi, Sumatra, under the Indonesian Ministry of Marine Affairs and Fisheries (MMAF) developed JICA (Japan for International Cooperation Agency) Nile Tilapia, which was originally from Kagoshima Fisheries Research Station in Japan. JICA Tilapia development was fully funded by the JICA, therefore the strain's name indicates the donor. Based on MMAF report, the JICA strain grows faster, produces more eggs (better fecundity), and reduce the feed cost up to 25% compared to the GIFT strain (MMAF, 2005).

In 2006, the Agency for the Assessment and Application of Technology, an Indonesian government research body, introduced a new strain named "Genetically Supermale Indonesian Tilapia" (GESIT). GESIT are genetically manipulated to hatch eggs that will produce 98% - 100% male tilapia. This will benefit fish farmers to culture all male tilapia which grow faster compared to mixed sexes as females spends energy for reproduction (Mair et al., 1997; Phelps, 2006).

There have been many other improved strains produced, such as Nirwana from Wanayasa (West Java) in 2006, Umbuwan from East Java (2008), and BEST (Bogor Enhanced Strain Tilapia) from Bogor in 2009. The development of GESIT strain will be discussed in more detail in the next section following the review of production data (MMAF, 2010).

2.5. Tilapia Production and Market

Tilapia have become popular with local fish farmers because they are easy to farm and grow fast. Major tilapia production areas are in West Java and North Sumatra, where Regal Springs has one of its operations. Regal Springs Tilapia, the world's largest aquafarmer of tilapia, discovered two decades ago that Indonesia was favorable for farming the affordable whitefish so popular with the Americans. In a rural landscape of volcanoes, rice fields and fresh water springs in Central Java, Regal Springs began farming tilapia in 1988. The company's founder had previously worked with the United Nations Food and Agriculture Organization in West Java (Regal Springs' website).

In 2004, the national production of tilapia in Indonesia was 97.116 MT, and increased by 500% in six years to 464,191 MT in 2010 for the Nile Tilapia only, as statistic for other tilapia species is not available (MMAF, 2011). This figure means that the production increased significantly, despite the global economic crisis in 2008. Most of this production is dedicated to domestic demand, and not the export market. Based on FAO Fish Report (2008), Indonesia is among the five top cultured fish producers, but its export growth is not even in the top ten. Based on MMAF predictions (2011), there are about 14 million hectares of river streams and lakes that can be used for freshwater aquaculture development in Indonesia. Currently, only 10.1% are being used for tilapia and other fish culture.

Table 2.1 outlined the top eight aquaculture major commodities in Indonesia including tilapia and shrimp. Compared to other species, both tilapia and catfish show promising trends in terms of production. With the emergence of KHV (Koi Herpes Virus) disease in carp, tilapia became an alternative freshwater fish, and one of the major commodities in the Indonesian Aquaculture Revitalization program for the following reasons: (1) high economic value, (2) culture technology is available and widely known to public, (3) high demand in export and domestic markets, and (4) can be massively cultured and developed.

Table 2.1. Major aquaculture production in Indonesia from 2003 – 2010 (in metric tons)

Species	2003	2004	2005	2006	2007	2008	2009	2010	% increase (2003-2010)
Shrimp	192,912	238,854	280,629	327,610	358,925	409,590	338,060	380,972	97
Nile Tilapia	**71,947**	**97,116**	**148,249**	**169,390**	**206,904**	**291,037**	**323,389**	**464,191**	**545**
Milkfish	227,854	241,438	254,067	212,883	263,139	277,471	328,288	421,757	85
Common Carp	219,385	192,462	216,920	247,633	264,349	242,322	249,279	282,695	29
Clarias (catfish)	58,614	51,271	69,386	77,272	91,735	114,371	144,755	242,811	314
Pangasius (catfish)	12,904	23,962	32,575	31,490	36,755	102,021	109,685	147,888	1046
Giant gouramy	22,666	23,758	25,442	28,710	35,708	36,636	46,254	56,889	151
Tilapia rank	4	4	4	5	6	2	3	2	

Note : excluding seaweed production. Source: MMAF statistics (2011)

In Subang, West Java, farmers culture the Nile Tilapia in running water ponds. After 6-7 months, the weight reached 700 grams, a minimum for fillet fish production. Tilapia are also cultured in floating cages in Indonesia, particularly when the location is an open lake, or open brackish water with fish density usually around 10 fish per square meter. During the harvest time, the farmers pull out the floating cage or floating net to collect the fish. Typical harvest per 1,000 square meter and 1.5 meter depth is 7 metric tons of whole fish or equal to 2.8 metric tons of fillet fish (personal observation and interview with farmers).

Tilapia culture in Indonesia shows promising trends, with West Java being the top producer for many years. Table 2.2 describes the top ten producers for Nile Tilapia in Indonesia from 2005-2009. The significant increase for North Sumatera in 2008 came from Regal Springs Tilapia operation in Toba Lake. Similarly, West Sumatera also shows increased production as they utilize Maninjau Lake. Different from other locations,

tilapia are cultured in brackish water in Central Java. The production in Banjar and Tabalong in Indonesia suggests that tilapia culture could be a promising alternative livelihood for coastal communities. All other locations in Indonesia show increasing production trend.

Table 2.2. Top ten producers for Nile Tilapia in Indonesia (in Metric Tons)

No	Province	Year				
		2005	2006	2007	2008	2009
1	West Java	48,069	76,163	85,954	100,454	87,397
2	South Sumatera	18,617	24,980	28,783	40,154	48,991
3	**North Sumatera**	**1,465**	**1,210**	**3,435**	**36,290**	**39,614**
4	West Sumatera	20,661	14,498	18,791	31,963	30,847
5	Central Java	9,860	11,686	12,362	14,095	20,073
6	**South Kalimantan**	**2,936**	**5,130**	**3,750**	**4,815**	**19,637**
7	North Sulawesi	9,557	4,445	10,476	10,831	11,123
8	Jambi	4,891	4,857	6,695	7,874	9,848
9	East Java	6,027	6,182	6,981	7,660	8,521
10	Bengkulu	2,668	2,732	4,191	5,738	7,134

Source: Ministry of Marine Affairs and Fisheries/MMAF (2010)

Based on MMAF database (2010), the total production for both species (Nile Tilapia and Mozambique Tilapia) was 328,831 MT in 2008. Table 2.2 shows the detail production for each province. For Nile Tilapia only, in 2009, the total production increased by 11.12% in one year (from 291,037 MT to 323,389 MT).

Table 2.3. Total production of tilapia in Indonesia for the year 2008 (in metric tons)

Province	*O. niloticus*	*O. mossambicus*
Nanggroe Aceh Darusalam	3,650	1,860
North Sumatera	36,290	1,120
West Sumatera	31,963	2,407
R i a u	5,290	-
Riau Islands	23	1
J a m b i	7,874	21
South Sumatera	40,154	1 659
Bangka Belitung	325	1
Bengkulu	5,738	773
Lampung	4,471	130
DKI Jakarta	578	228
Banten	1,521	2,986
West Java	100,454	12,492
Central Java	14,095	2,672
D.I. Yogyakarta	2,915	21
East Jawa	7,660	8,153
B a l i	364	4
West Nusa Tenggara	2,122	224
East Nusa Tenggara	222	24
West Kalimantan	844	-
Central Kalimantan	1,601	-
South Kalimantan	4,815	-
East Kalimantan	3,298	35
North Sulawesi	10,831	590
Gorontalo	1,422	-
Central Sulawesi	557	3
West Sulawesi	40	22
South Sulawesi	405	2 136
Southeast Sulawesi	137	125
Maluku	47	10
North Maluku	145	50
Papua	837	45
West Papua	349	-
Total for Indonesia	291,037	37,794

Tilapia production in Indonesia is expected to continue to grow, as global demand increases. Demand for tilapia continues to grow particularly in the United States, where shrimp, with 1.86 kg annual per-capita consumption, was the most popular seafood in 2009 (Fitzsimmons et al., 2011). Per-capita consumption of tilapia was 0.54 kg, making tilapia the fifth most popular fish for the Americans, just behind shrimp, tuna, salmon, and pollock. This consumption is the equivalent of 453,264 MT of live-weight fish. In U.S. retail stores, tilapia is the second best-selling fish behind salmon. Total import and farm gate sales for 2008 were $784.5 million (USD). Domestic growers received about $50 million of that total, while the balance went for tilapia grown outside the United States (Fitzsimmons et al., 2009).

2.6. Aquaculture Stewardship Council

In 2009, The Aquaculture Certification Council, the World Wildlife Fund, and Whole Foods unveiled plans for the next step in tilapia regulation. The creation of the Aquaculture Stewardship Council (ASC) which is a third-party group that audits and certifies fish farms that meet stringent requirements. These new standards grew out of open discussions between the stakeholders in the tilapia industry and their desire to see uniform standards applied to an industry that struggles with uneven product quality from its farmers across the globe. The decision depends on multi-stakeholders, and not merely buyer based as in the past. In fact, there are about 150 stakeholders, including a group of tilapia producers, seafood buyers and non-profit organizations (Fitzsimmons et al., 2009). In August 2012, ASC announced that tilapia from Indonesia have become the first fish to meet its standards. This should allow the products to be sold at a higher price to

environmentally conscious consumers (Cressey, 2012). The idea behind the establishment of the certification was because aquaculture has been attacked by conservation groups for the pollution issues and the use of wild-caught fish as part of the feed ingredient. In fact, tilapia are significantly different from aquaculture species such as salmon and tuna. Tilapia are easily cultured in places where other species would not grow, and the feed is grain based (Cressey, 2012).

2.7. Development of GESIT Strain

Tilapia are omnivores in nature, and they can be maintained in an extensive cultivation system which depends on the natural productivity from the water, or in an intensive cultivation system which can be operated at a lower cost. In terms of reproduction, tilapia are a paradox. The relative fecundity of the *Oreochromis* genus is low, at 6,000-13,000 eggs/kg/spawn. But this is compensated for by the high survival of fry due to their large size at hatching, their large yolk reserves, the mouth-brooding maternal care given until the fry are 10 mm or larger, and frequent spawning (Phelps, 2006).

Tilapia also present some challenges to fish culturists. Most *Oreochromis* species can reach sexual maturity within six to eight months of hatching at sizes often less than 100 gram. Under some conditions, they mature in less than five months at 20 to 30 g. Unless controlled, the fish continue to reproduce, and off-spring compete with the initial stock for food, often resulting in stunted growth and unmarketable fish. Therefore, all-male monosex culture of tilapia is preferred because of males' fast growth and larger average size (Rothbard et al., 1987). Therefore, the culture of monosex male tilapia is done to

overcome the problem of uncontrollable mating (Mair et al., 1997), although the supply of the male monosex tilapia is still very limited.

Several techniques have been adopted to produce all-male tilapia, including manual sexing, hybridization, genetic manipulation and sex reversal through sex hormone administration (Adel et al., 2006). Human error in manual sexing can be high, and the method also wastes the females. The problems associated with hybridization are the difficulties in maintaining the pure parental stocks that consistently produce a high percentage of male offspring, and reduction in egg fertilization. The use of hormones to produce monosex fish has been limited or prohibited in some countries because of market and/or environmental concerns. Therefore, the production of genetically "supermale" YY Tilapia has been suggested as the safest, most efficient and effective technology. When crossed with normal female (XX) fish, YY tilapia produce 98 to 100% male tilapia (XY) or genetically male tilapia (GMT). All-male tilapia result in more uniform culture populations and faster growth compared to mixed sex populations.

Considering the significance of tilapia, research on developing genetically male tilapia has been conducted on Nile tilapia in Indonesia since 2001. BPPT (Badan Pengkajian dan Penerapan Teknologi/ the Indonesian Agency for the Assessment and Application of Technology) and IPB (Institut Pertanian Bogor/ Bogor Agricultural Institute) initiated the YY male tilapia development program, production and began a cooperative effort with the "Research Centre for Freshwater Aquaculture" in Sukabumi, West Java, in 2002. The collaboration of researchers in three government institutions suggested that the YY male

tilapia be named ”Nila GESIT” (**Ge**netically **S**upermale **I**ndonesian **T**ilapia). The production of the super YY male tilapia and GESIT was conducted at the Doc Experiment Pond – IPB, from 2001 to 2005, and the Research Centre for Freshwater Aquaculture, Sukabumi, from 2002 to 2006. Even though the research was done six years ago, the results have never been published for international readers. This paper is the first report of the development of the Super-YY Tilapia from Indonesia, an extended version from Aliah et al. (2010).

As outlined in Figure 2.6 and Table 2.3, hormonal treatment resulted in XY females, which were then crossed with XY males to produce YY males. Further progeny testing and hormone teatment generated YY females, and mass production of all-male YY fish.

Genetically Supermale Indonesian Tilapia (GESIT) were officially released on December 2006, by the Indonesian Ministry of Marine Affairs and Fisheries, with the biological performance outlined in Table 2.5. Over 100,000 GESIT fish were distributed to 22 provinces by 2008. Based on reports from Cianjur, West Java, GESIT reach a size of 6 to 8 cm size in 15 days, faster than previous tilapia strains. Others reported that 100 kg of GESIT fingerlings resulted in 1,300 kg at harvest, double the weight when compared to the regular harvest.

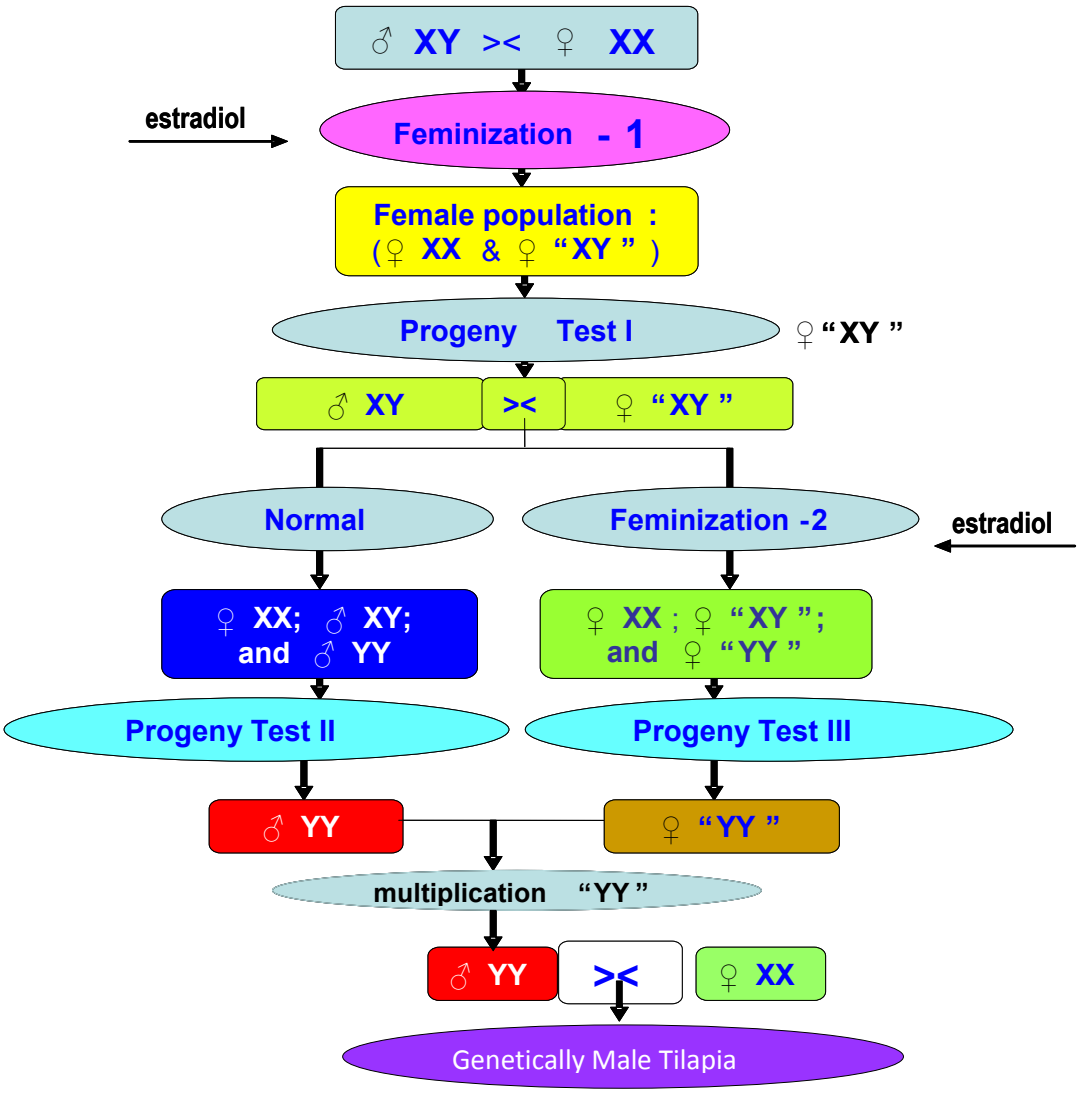

Figure 2.6. Steps in developing GESIT strain

Table 2.4. Development of YY male tilapia in Indonesia

Time	Activity	Result
July - Dec 2001	Feminization through feeding which contains estradiol 17β 50 mg/kg (10 day fries) for 30 days.	120 female
Jan – Jun 2002	Feminization	59 female
	Progeny test to produce ”XY” female	47 female
Jul - Nov 2002	Progeny test I, ”XY” female crossed with XY male.	3 ”XY” female
Dec 2002 – Jul 2003	”XY” female crossed with XY male to produce YY male. Only YY dan XY males were selected for further steps.	421 YY + 28 XY (all male)
Jun 2003 – Nov 2004	”XY” female crossed with XY male, the result then fed with feeding contains estradiol 17β to produce ”YY” female.	129 XX, ”XY”, and ”YY” Female
Dec 2003 - Jul 2005	Progeny test II, XY crossed with YY male from ”XY” to produce YY male.	19 YY male 9 → 100% ♂; 10 → ≥96% ♂
Aug 2004 – Oct 2005	Progeni test III, (”YY” , ”XY” dan XX) females crossed with XY (normal male) to produce ”YY” female.	2 fish, (♀: 98 – 100%)
Dec 2004 – Oct 2005	Progeny test III for female (”YY”, ”XY”, dan XX).	2 YY female
Jul 2005 - Oct 2006	Multiplication YY males through crossing YY male and ”YY” female from progeny test.	Parental = 663 fish > 8 cm = 797 fish 5 – 8 cm = 454 fish 3 – 5 cm = 2401 fish 2-3 cm = 125 fish
Jul 2006 - Oct. 2006	Mass production of YY male, where YY male crossed with YY female	98-100% male tilapia

Table 2.5. Description of GESIT Tilapia

I	**Source**	GIFT Sukabumi
II	**Morphology dan morphometrics**	
2.1	Age	10 month
2.2	Culture system	Hapa net in pond
2.3	Maximum Length	30 – 31.5 cm
2.4	Average Length	24 – 25 cm
2.5	Weight	500 – 680 g
2.6	Colour	Black
2.7	Ll	38
2.8	Vertebra	28
2.9	Dorsal Ventral Pectoral Anal Caudal	D. XVI – XVII. 12 – 13 V. I.5 P. 13 – 14 A. III. 9 – 10 C. 2.16
2.10	% fillet	36,20 – 44 %
III	**Reproduction**	
3.1	Maturity	6 months
3.2	Weight at maturity	300 – 350 g
3.3	Fertility	Normal
IV	**Genetics**	
4.1.	Male progeny	98 – 100%

In Situbondo, East Java, the monosex fish have been cultured in abandoned shrimp ponds in 12 ppt salinity. At a density of 10 fish/m^2 starting with 1-2 cm fish, GESIT reach 300 g after 120 days with 60% survival, and a feed conversion ratio (FCR) of 0.8. In Subang, West Java, GESIT fry demand 30% higher prices than local fry. It took 60 days to reach 10-g size with FCR of 1.1 to 1.2, compared to 75 days for local fry with 1.4 FCR.

Further experiments have been conducted to measure the survival and growth performance of fingerlings crossed with female tilapia (JICA strain) in hapa nets in 300-m^2 concrete ponds with aeration. The densities were 250 fish/hapa net with three replicates.

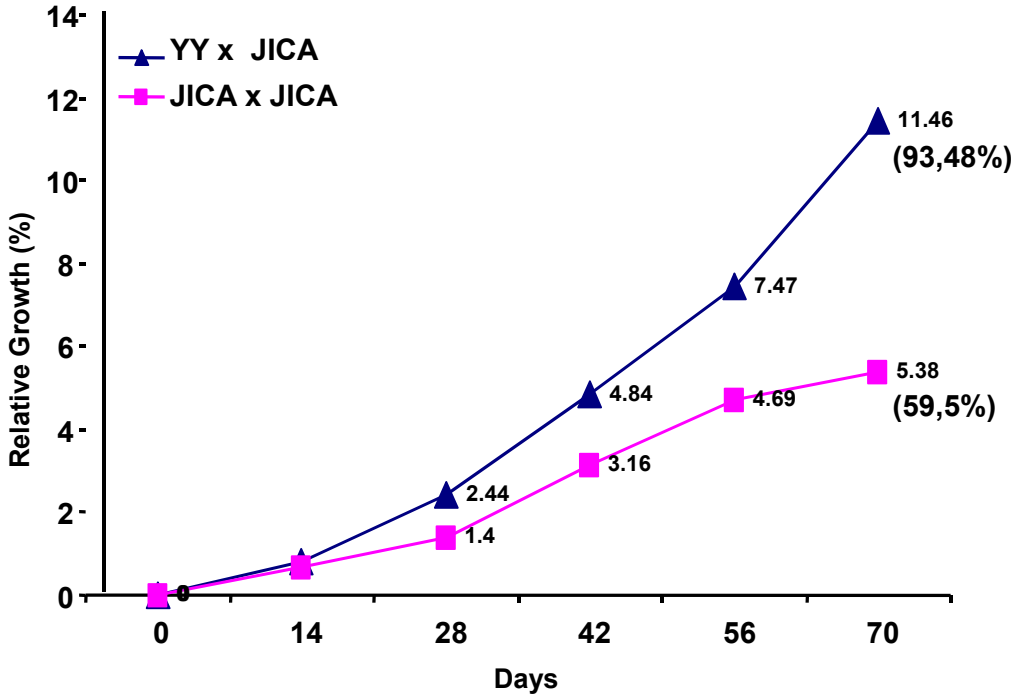

Figure 2.7. The growth of the YY ♂ x JICA ♀ and the JICA ♂ x JICA ♀

After 70 days, GESIT x JICA reached 11.46 g ± 1.20% compared to JICA x JICA at 5.38 g ± 1.51%. Figure 2.7 shows the growth curve. The FCR for GESIT x JICA was 2.11, lower than JICA x JICA's 3.04 after 70 days. Survival for GESIT x JICA was 82.8 ± 1.1%, compared to JICA x JICA at 89.1 ± 5.7%. The GESIT x JICA cross resulted in 93.8% males, compared to 59.5% males for JICA x JICA.

In recent years, efforts have been made to develop salt tolerant tilapia strains to utilize many abandoned shrimp ponds in brackishwater area throughout the country. This will also give opportunities to do shrimp-tilapia polyculture in high salinities. Even though

tilapia can tolerate wide range of salinities, reproduction is limited in salinity higher than 15 ppt (Popma and Masser, 1999).

The technical feasibility of shrimp-tilapia polyculture in low and high salinities is presented in Chapter 3 and Chapter 4 of this dissertation.

2.8. Acknowledgement

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CHAPTER 3

FEASIBILITY OF SHRIMP-TILAPIA POLYCULTURE IN LOW SALINITY

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3.1. Abstract

Historically, most shrimp farming has been done in coastal areas to meet the demand for large volumes of salt water during the grow-out period. Unfortunately, shrimp diseases, caused by different agents, are optimum in the salinity and temperature where shrimp are normally being cultured in brackish water. In an effort to reduce the disease risk, low salinity culture has been practiced in different parts of the world. This experimental study investigated the technical feasibility of low salinity shrimp farming in a polyculture system with tilapia. Polyculture increased the survival for shrimp (77% compared to 62%) but at the same time decreased the survival of tilapia (87% compared to 97%). Together, the data on survival, specific growth rates, and feed conversion ratios showed that the shrimp (*Penaeus vannamei*) performed well at low salinity. The presence of tilapia stimulated the growth of microalgae (*Chlorella* dominance) and promoted higher numbers of heterotrophic bacteria in the water. Water quality parameters (dissolved oxygen, pH, ammonia, nitrite, and nitrate) were all within acceptable ranges.

Keywords: low salinity, shrimp-tilapia polyculture, *Chlorella* dominance, water quality

3.2. Introduction

3.2.1. Free water, not free of disease

Shrimp farming is one of the fastest growing components of the global aquaculture industry. Most shrimp farming is done in coastal areas which accommodates the demand for large volumes of salt water during the grow-out period. The salinity levels of coastal (brackish water) shrimp farms are normally in the range of 20 – 35 ppt. Water exchange in intensive farming systems can reach 30 to 40% each day, to replace water loss due to seepage and evaporation and to maintain environmental conditions (Flaherty et al. 2000).

The abundant availability of seawater has been the major factor that has contributed to the fast growing aquaculture industry around the world. Unfortunately, this free water is not free of disease agents. Disease is considered as one of the most important limiting factors in many aquaculture practices, particularly in shrimp farming. Shrimp hatchery and farm production losses due to diseases have been increasing overtime. Compared to fish, shrimp have a less developed and more simplistic immune system, which put them at a higher risk when a variety of disease agents are present in the water. Both biological and non-biological agents can cause or contribute to disease in shrimp. Viruses, rickettsia/chlamydia, bacteria, fungi, protozoa, metazoa, feed factors (limiting nutrients or toxins), environmental factors (soil and water both physical and chemical, biotoxins and pesticides), and other factors have been found to be contribute to shrimp disease (Lightner, 1996).

Shrimp pathogens thrive in the salinity and temperature where shrimp are being cultured, normally in brackish water. One approach to reduce the disease risk that is being practiced in different parts of the world is low salinity culture (Fast and Menasveta, 2000).

3.2.2. Shrimp performance and disease risks in low salinity

When considering the environmental factors, theoretically, both shrimp and its pathogens will favor the natural parameters where shrimp are growing in the wild (temperatures around 27-28°C and salinity of 25 – 30 ppt). Three of the most important parameters would be temperature, salinity, and pH (Gao et al., 2011). Salinity and water temperature affect aquatic host health by directly influencing their metabolism, oxygen consumption, molt cycle, and growth.

Several studies have demonstrated the effects that environmental factors have on the pathogenicity of biological agents that cause shrimp disease. Gao et al (2011) mentioned that for Fleshy prawn (*Penaeus chinensis*), the optimal environmental conditions for white spot syndrome virus (WSSV) proliferation would be 30°C, 35 ppt, and 8.0 for pH. Water temperature was considered as the most determinant factor. The experiment examined temperature ranges of 15 - 35°C, salinities of 15 – 35 ppt, and pH values from 6.5 to 9.0. WSSV is the most important shrimp pathogen which causes the most economic loss since the first outbreak in China in 1992. Hsieh et al. (2007) confirmed the strong relationship between vibrio abundance and environmental paramaters such as

salinity, temperature, and attachment to planktonic organisms, and vibriosis is the most important bacterial disease in shrimp.

Even though temperature is likely one of the most determinant environmental factors for pathogenicity in aquaculture operations, the control of water temperature during the grow-out period is normally not feasible. One approach to increase the water temperature, with the hope of minimizing a disease outbreak, is by covering the pond with plastic. In many regions, this is not practical due to the large size of the ponds during the grow-out periods, and also the investment. Shrimp industries might be able to do this, but not traditional farmers. Adjusting for low temperature is possible during hatchery periods in a closed and controlled space.

Another approach to prevent or minimize disease risk, which has been practiced in several places in the world is by culturing shrimp at low salinity (Xingqiang et al., 2010; Prapaiwong and Boyd, 2012). The interest in low-salinity shrimp culture has increased recently through the opportunity of farming inland areas, as an attempt to minimize the risk of disease outbreaks that normally occur in coastal marine or brackish water farms. However, this idea has two important consequences that should be considered. First, low salinity, while preventing shrimp disease agents would likely decrease the growth performance of shrimp. Second, fresh water is not as abundant as seawater. The use of freshwater for shrimp culture could cause competition between aquaculture and agriculture for this limited resource (Flaherty et al., 2000).

Nevertheless, Samocha et al. (2004) indicated that the *Penaeus vannamei* can be raised at very high densities with good survival using low-salinity groundwater. To improve shrimp performance, metabolic and haematological responses in low salinity, Flores et al. (2007) recommended dietary astaxanthin supplementation of 80 mg/kg. Liang et al. (2008) compared shrimp performance at high and low salinities. Shrimp cultured in high salinity had a higher content of crude protein, lower moisture, and a higher flesh pH. No significant differences were found in crude lipid and ash and amino acid composition. Overall, seawater shrimp had tasted sweeter with better flavor, and less of an earth-musty taste compared to low salinity shrimp.

In the Yucatán Peninsula, Mexico, Gullian et al. (2010) reported that the Northern pink shrimp (*Penaeus duorarum*) can be successfully acclimated to low-salinity (5 ppt) conditions with a high percentage of survival and an acceptable growth rate in a nursery phase experiment run for 45 days. Esparza-Leal et al. (2010) also suggested that the Pacific white shrimp can be successfully grown in low salinity well water. The growth, production output and survival are significantly higher when shrimp are acclimated to the lower salinity for longer periods. Maica et al. (2012) concluded the viability of rearing *P. vannamei* at low salinity under zero-water-exchange conditions based on microbial floc composition and growth performance.

Roy (2009) noted that variable survival of shrimp in low salinity can be attributed to a combination of several factors, including environmental, but also is largely due to poor handling of postlarvae and stocking errors. Further studies are needed to correlate the

ionic composition of water, as well as the duration of acclimation time, rate of salinity reduction and post-larvae age to obtain optimum survival.

Other than low salinity shrimp culture, another aquaculture practice with a long history has become popular in the last decade to minimize shrimp disease risk. The approach is called polyculture, where two or more species are cultured together in the same water body (Fitzsimmons, 2001; Yuan et al., 2010). Co-culturing shrimp (a marine species in nature) and tilapia (a freshwater species) is one of the most popular polyculture practices as both species have high demands in international markets (Fitzsimmons et al., 2011).

3.2.3. Shrimp-tilapia polyculture

Despite the fact that shrimp aquaculture is one of the main livelihoods in coastal areas of tropical countries, disease outbreaks in shrimp farming have caused major economic losses in many parts of the world where shrimp are being cultured. As a consequence, some farmers have abandoned their shrimp ponds, while other farmers have shifted to tilapia culture, due to the belief that this species is more resistant to diseases. Yi and Fitzsimmons (2004) demonstrated that shrimp-tilapia polyculture is technically feasible under an appropriate feeding strategy. The use of cost effective diets and optimization of feeding inputs can make the shrimp-tilapia polyculture more economically attractive.

The addition of tilapia into shrimp ponds is also environmentally friendly. As a filter feeder, tilapia reduce excessive phytoplankton biomass and recycle nutrients effectively (Stickney et al. 1979). Akiyama and Anggawati (1999) suggested that stocking tilapia (50

– 100 g in size) at a rate of 20 – 25 gram fish/ m^2 or one fish in every 2 - 3 m^2 will improve shrimp performance by stabilizing the water quality, foraging and cleaning the pond bottom, and having a probiotic type effect in the pond.

In terms of reducing shrimp disease, Cruz et al. (2008) suggested that tilapia reduce luminous vibriosis by feeding on organic wastes, increasing beneficial phytoplankton (*Chlorella* spp.) dominance, bioperturbating the pond sediments, and releasing antimicrobials or stimulating beneficial bacteria from the skin and gut mucus of tilapia. Tendencia et al. (2010) summarized which densities and feeding strategies reduced vibriosis in shrimp-tilapia polyculture.

Most of the previous research on penaeid shrimp-tilapia polyculture was done in brackishwater and a relatively low density for either shrimp or tilapia or both (Yuan et al., 2010). For low salinity, giant freshwater shrimp (*Macrobrachium* spp.) and tilapia polyculture may be preferable (Tidwell et al, 2010). As penaeid/marine shrimp are more favorable in the market, the experimental studies in this chapter will highlight the survival and growth performance of high densities of penaeid shrimp-tilapia polycultured in low salinity, along with the water quality dynamics, as well as the bacteria and microalgae composition. All these factors together provide opportunities and constraints in low salinity penaeid shrimp-tilapia polyculture.

3.3. Materials and Methods

3.3.1. Experimental tanks

The experimental study was conducted in a greenhouse with transparent roof at the Research Center for Brackishwater Aquaculture, Bangil, East Java, Indonesia for 90 days (three months), to investigate the growth performance of shrimp (*Penaeus vannamei*) and Red Hybrid Tilapia (*Oreochromis niloticus x O. mossambicus*) in low salinity (3 ppt). The salinity was achieved by pumping the seawater adjacent to the research center and mixed with well water. There were three treatments (shrimp monoculture, tilapia monoculture and shrimp-tilapia polyculture). Each treatment was conducted with three replicates in a one cubic meter indoor concrete tank covered with plastic on each side. The depth of the water was adjusted to 80 cm. In shrimp monoculture, the stocking density was sixty (60) shrimp. The tilapia monoculture tanks had ten (10) fish each. In the shrimp-tilapia polyculture group, sixty (60) shrimp and ten (10) fish were combined in one tank. Each tank was supplied with a biofilter to maintain the aeration. Shrimp were placed inside a sinking hapa net in the tank while tilapia freely moved in the water columns outside the hapa nets.

3.3.2. Growth parameters

Shrimp post-larvae (average weight of 0.0168 gram) and Red Tilapia (average weight of 97.645 gram) were obtained from local hatcheries. Feeding was given at 8 am in the morning and 4 pm in the afternoon. Shrimp were fed by hand spreading, 6% of body weight for the first two months, and 3% of body weight for the last month. Tilapia received floating feed (18% protein) throughout the duration of study by hand spreading.

Shrimp and fish were weighed biweekly to determine the survival percentage and the specific growth rate (SGR) for each species, as well as the feed conversion ratio (FCR). Formulas used to calculate the survival and growth parameters

$$Survival = [\frac{(A-B)}{A}]X\ 100 \quad \text{(unit in \%)}$$

A = number of animals at the beginning of study

B = number of animals at the termination of study

$$Specific\ Growth\ Rate = \frac{Ln(WT)-Ln\ (W0)}{T}\ X\ 100 \quad \text{(unit in \% per day)}$$

WT = Weight at the termination of the study

W0 = weight at the beginning of study

T = number of days

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{Total\ weight\ of\ feed\ fed\ for\ each\ species}{Total\ weight\ gain\ by\ each\ species}$$

FCR is unitless

3.3.3. Water quality monitoring

During the trial, Dissolved oxygen (DO), temperature, salinity, and pH were measured daily at 8 A.M. and at 4 P.M. Water samples were taken biweekly for analyses of total ammonia nitrogen (TAN), nitrate nitrogen (nitrate-N), nitrite nitrogen (nitrite-N), and total phosphorus (TP) taken at 12 P.M. using a kit and following the manufacturer's instructions.

3.3.4. Bacterial count and identification

Prior to the termination of study, the culturable bacteria in the water were counted by taking water samples and plating on Nutrient Agar (NA) with 2.5% salt for total bacteria and TCBS agar for presumptive *Vibrio* spp. Heterotrophic plate count (HPC) were done in three replicates. The BBL Crystal™ Identification System was used to determine the bacteria to species level according to the protocol by the manufacturer.

3.3.5. Microalgae identification

Microalgae composition was identified by taking water samples and observing the microalgae shape and morphology under a light microscope. The identification followed the Marine Plankton Identification Key published by Project Oceanography and GloBallast Monograph Series No. 7 Phytoplankton Identification Catalogue, a joint initiative from the Global Environment Facility, United Nations Development Programme and International Maritime Organization (Botes, 2001).

3.4. Results and Discussions

Penaeid shrimp and tilapia rarely meet naturally because of the different habitats they live in. Penaeid shrimp throughout most of their life cycle live at high salinity and tilapia in fresh water (low salinity). In an aquaculture setting, both species can be reared together due to their tolerance to wide range of salinities and their different ecological niches (Fitzsimmons, 2001).

Polyculture offers another opportunity to improve culture practices in the field. Because some aquaculture species grow optimally in different seasons, there is a growing demand to utilize the time and space efficiently, for example through crop rotation and sequential culture. For example, shrimp are normally cultured during summer seasons, while tilapia can tolerate temperature above 15°C. During Summer and Fall, shrimp-tilapia polyculture can be done in the same pond, and during the end of Winter and Spring, farmers can utilize the ponds to culture tilapia. This practice is not only better utilize space and time, but also preparing a cleaner pond bottom for the following shrimp culture cycle during Summer.

The survival and specific growth rate (SGR), as well as the feed conversion ratio (FCR) are the main parameters of growth performance in any aquaculture species. Table 3.1. summarizes the parameters for both shrimp and tilapia in monoculture and polyculture culture systems.

Table 3.1. Growth parameters of shrimp and tilapia after 90 day of culture

Parameters	Shrimp		Tilapia	
	monoculture	polyculture	monoculture	polyculture
Survival (in %)	62.33 ± 4.51*	77.22 ± 2.55*	97.67 ± 5.77*	86.67 ± 5.77*
Specific Growth Rate (SGR in % per day)	7.75 ± 0.15*	7.05 ± 0.24*	1.06 ± 0.03	0.92 ± 0.01
Feed Conversion Ratio (FCR)	1.25 ± 0.08*	1.45 ± 0.50*	1.26 ± 0.84*	1.47* ± 0.13

Data presented is the average of three replicates (mean ± standard deviation),
*indicated significant difference by *t*-test comparison at $p < 0.05$

Based on the survival data, polyculture increased the survival for the shrimp but at the same time decreased the survival of the tilapia (significantly different by *t*-test comparison) for each parameter. The lower survival for the tilapia in this experimental study might due to the space limit for the fish to move and to grow, as the middle of the tank was occupied with the hapa net. As a consequence of the higher survival, shrimp in the polyculture system gained less weight compared to monoculture. The lower growth rate may have been due to the nutrient availability in the water. The more animals that survived in the tank, the more nutrients that were needed. In the field, the case might be different because of the natural food availability in the earthen ponds. Another explanation for the lower growth rate for shrimp and tilapia in the polyculture system compared to monoculture can be explained by comparing the SGR and the FCR data. Shrimp and tilapia had higher FCR rate in the polyculture system in this experimental

study, partly because of the high density and the space limitation in the hapa net (for shrimp), and for tilapia in the rest of the water column which does not occur in nature. If the fish or shrimp had a relatively large space to move and to eat, growth rate would most likely not be affected.

Yi and Fitzsimmons (2004) described that in extensive systems at low density, the tilapia can filter feed on phytoplankton and zooplankton in the upper water column, while the shrimp spend most of the time at the pond bottom grazing on bacterial films and on the detritus settling from above. In an intensive culture where both species are receiving pelleted feeds, tilapia would monopolize floating feed and shrimp would need sinking feed. Because of the gravitation force, some particles from uneaten feed in the upper water column will sink to the bottom where the shrimp will consume it (Fitzsimmons 2001). Another important point, the fecal matter from the tilapia could be a detrital feed that support the shrimp.

On the other hand, some of the sinking feed might be caught by tilapia on the way to the bottom. Differing from tilapia, which mostly eat during the day, shrimp are physiologically active during day and night. Therefore, most shrimp farmers have practiced four feeding times a day: 6 A.M. in the morning, at noon, 6 P.M. in the afternoon and 10 P.M. at night. In a polyculture setting, the feeding at night for the shrimp will minimize the risk of sinking feed being eaten by the tilapia.

Together, the data on survival, specific growth rate, and feed conversion ratio show that marine shrimp (*P. vannamei*) can grow well at low salinity. The success of low-salinity shrimp-tilapia polyculture largely depends on effective feeding strategies and species densities. In practice, in a pond setting, it would be easier to harvest tilapia if the fish are caged. As the tilapia occupy the upper-part of the water, the fish need to be harvested or moved to other ponds prior to harvesting the shrimp. Culturing tilapia in cages would make the process more feasible.

In addition to achieving good growth performance, maintaining water quality is another critical issue in any aquaculture operation. Boyd & Tucker (1998) suggested the acceptable ranges of the most important water quality parameters. Dissolved oxygen (DO) needs to be in the range of 5 – 9 ppm, pH in between 7 – 8.3, unionized ammonia of less than 0.03 ppm, nitrite should be less than 1 ppm, nitrate is less than 60 ppm, and hydrogen sulfide of less than 2 ppm. Table 3.2. summarizes the water quality during the experimental study. In general, all the parameters were within the acceptable levels, and not significantly different, except for pH level which was higher in shrimp monoculture, compared to tilapia monoculture and polyculture.

Table 3.2. Water quality parameters in different system

	Shrimp monoculture	**Tilapia monoculture**	**Polyculture**
Salinity (ppt)	3	3	3
Temperature (°C)	28.7 ± 1.1^{a}	28.4 ± 0.7^{a}	28.9 ± 0.9^{a}
Dissolved Oxygen (ppm)	4.9 ± 0.2^{a}	4.9 ± 0.2^{a}	4.9 ± 0.3^{a}
pH	8.4 ± 0.5^{a}	7.8 ± 0.5^{b}	7.5 ± 0.6^{b}
Ammonia	0.10	0.10	0.01
Nitrite	0.07	0.10	0.10
Nitrate	0.10	0.15	0.20
Phosphate	2.01	1.53	1.82

pH in shrimp monoculture is higher and significantly different from other groups at $p < 0.05$

The main concern in water quality monitoring relates to uneaten feed and feces. This effluent from aquacultural activity contains ammonia, phosphorus, and organic matter. Toxic inorganic compounds, in the form of ammonia or ammonium, are very critical in aquaculture, and ammonia is more toxic compared to the latter (Boyd and Tucker, 1998). Other than measuring ammonia, monitoring the nitrite level in the water is equally important. Similar to terrestrial processes in the soil, the nitrification in the water is carried out by chemoautotrophic bacteria under aerobic conditions and nitrite is one of the compounds resultant from the nitrogen cycle process. Higher levels of nitrite are more common in intensive systems where the ammonia concentration is high, and the rate of nitrite oxidation to nitrate is lower compared to ammonia oxidation to nitrite. High accumulation of nitrite causes mortality in some aquaculture species (vertebrates) by transforming hemoglobin to methaemoglobin. In this form, binding with oxygen is not possible and results in anoxia (Almendras, 1987). Phosphate is also included in water quality monitoring because phosphorus will precipitate and bind with sediments in the pond bottom.

An interesting point from Table 3.2 is that the pH level in the shrimp monoculture is significantly higher compared to the tilapia monoculture or polyculture system. In shrimp monoculture, the growth of algae is less, compared to the other groups. The waste from tilapia, in the form of urea and uneaten feed which are rich of nitrogen, stimulates algae growth. Algal bloom during the day will absorb carbon dioxide and oxygen from the water for photosynthesis. The change in carbon dioxide level in the water will cause pH swings, as what seen in tilapia monoculture and polyculture groups.

In the water, bacteria and microalgae are important biological components which contribute to the pond dynamics (Burford, 1997; Moriarty, 1997). Table 3.3 lists the total heterotropic plate count (HPC) for total bacteria and presumptive *Vibrio* spp. In general, the total heterotrophic plate count in polyculture and tilapia monoculture is one log higher compared to shrimp monoculture. As expected, in lower salinity, the *Vibrio* spp. counts were very low.

Table 3.3. Total bacteria and presumptive *Vibrio* spp. in the water

	Total bacteria on Nutrient Agar (CFU/mL)	**Presumptive Vibrio on TCBS agar (CFU/mL)**
Shrimp monoculture	3.5×10^3	< 25
Tilapia monoculture	7.5×10^4	< 25
Polyculture	2.3×10^4	< 25

CFU = colony forming unit

The green water in tilapia culture and polyculture is a nutrient-rich environment compared to the clearer water in shrimp monoculture. The presence of a higher number and diversity of bacteria, a relatively lower pH at noon, and the presence of microalgae, all together might play synergestic roles in the polyculture system to improve water quality and fitness of the animals. Table 3.4 highlights the most dominant microalgae in each system. The presence of tilapia stimulates microalgae growth and more specifically promotes *Chlorella* sp. dominance which was not found in the shrimp monoculture group. In earthern ponds, the availability of microalgae is more abundant and there is an interaction between all components in the water with the pond bottom. The presence of

tilapia did not lower the diatom level, but added *Chlorella* sp., so both diatom and green microalgae became available in the water.

Table 3.4. Microalgae composition in different system

	Microalgae species	**Abundance (cells/mL)**
Shrimp monoculture	*Navicula* sp. (diatom) *Nitzschia* sp. (diatom)	1.0×10^4 5×10^4
Tilapia monoculture	*Chlorella* sp. (green) *Navicula* sp. (diatom) *Prorocentrum* sp. (dinoflagellate)	1×10^5 1.5×10^4 5×10^4
Polyculture	*Chlorella* sp. (green) *Navicula* sp. (diatom)	5×10^4 5×10^4

In earthern ponds, after harvesting and draining the water for the next cycle, it can be observed that tilapia ponds or polyculture ponds have very little black sediment in the bottom, most of the time the bottom is clear. This clear bottom is different from shrimp monoculture ponds, and therefore it necessary in shrimp ponds to do plowing of the pond bottom soil before starting another culture cycle.

3.5. Conclusions and Future Directions

The data presented in this chapter showed that shrimp-tilapia polyculture in low-salinity is technically feasible. Overall, polyculture resulted in better growth performance by increasing the survival of the shrimp when compared to monoculture. In low salinity and where space is not limited, survival and growth of tilapia might be improved. Polyculture, with the presence of microalgae, has the tendency to decrease the pH but remain in

acceptable levels for aquaculture practices. This low pH might have consequences in terms of disease risk as pH affects not only growth and virulence of aquatic pathogens.

While preventing viral and bacterial diseases in shrimp, low salinity shrimp-tilapia polyculture might lead to streptococcal disease transmission from tilapia to shrimp. Streptococcocis is more prevalent when tilapia are cultured at high densities (Shoemaker et al., 2001). To date, there have been no reports on streptococcal disease in polyculture settings, presumably because tilapia density is normally low and shrimp is the main species. Even though Gram-positive infection is less common in shrimp compared to Gram-negative bacteria, in recent years the streptococcal disease has been infected shrimp at low salinity in nature (Hasson et al., 2009), suggesting disease transmission from freshwater fish (Lightner et al., 2009). Therefore, in low salinity polyculture, maintaining low density of tilapia may be advisable to prevent streptococcal transmission.

Environmental deterioration risk might be another critical issue for low salinity polyculture in the longer term. Land use will always be a controversy as well as the salt accumulation in the soil. Different from freshwater fish, shrimp needs salt even at low level. While some growers prefer low salinity shrimp farming to minimize the disease risk, most of them might not realize that investment in wastewater treatment in the inland environment is urgently needed. But, as most shrimp farming are done in tropical countries, tropical rains would likely flush salt quickly from ponds, different from arid areas where salt accumulation is a bigger problem.

This experimental study is limited to a laboratory-scale, and therefore economic feasibility and cost benefit analysis are not calculated for a large scale operation in the field.

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CHAPTER 4

REVIEW OF SHRIMP AQUACULTURE IN INDONESIA, AND FEASIBILITY OF SHRIMP-TILAPIA POLYCULTURE IN BRACKISHWATER

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4.1. Abstract

Brackishwater polyculture provides advantages as water resources are more abundant from the ocean. Unfortunately, shrimp diseases, caused by different agents, are optimum in the salinity and temperature where shrimp are normally being cultured in brackish water. This experimental study investigated the technical feasibility of brackishwater shrimp farming in a polyculture system with tilapia. Compared to monoculture system, polyculture increased the survival for shrimp (82% compared to 65%) and tilapia (60% compared to 43%). Together, the data on survival, specific growth rates, and feed conversion ratios showed that the shrimp performed well in brackishwater. Mortalities were found in the Red Hybrid Tilapia strain after 40 day of culture. The presence of tilapia stimulated *Chlorella* dominance, promoted higher numbers of heterotrophic bacteria in the water, and had lower presumptive vibrios. Water quality parameters (dissolved oxygen, pH, ammonia, nitrite, and nitrate) were all within acceptable ranges.

Keywords: brackishwater, shrimp-tilapia polyculture, *Chlorella* dominance, water quality

4.2. Introduction

4.2.1. Aquaculture and polyculture in Indonesia

The practice of culturing two or more species in one water column, better known as polyculture, has a long tradition in Asian countries, including Indonesia. Milkfish (*Chanos chanos*) culture was already started in the country back in the 15th century, and by the 18th century, there were over 80,000 acres (32,389 hectares) of brackish water ponds (Pillay, 1990). Shrimp aquaculture was not initiated until the beginning of 1980's (Purnomo, 2001). Since then, shrimp-milkfish polyculture has been practiced in extensive and semi-intensive system. In most shrimp-milkfish polyculture systems, shrimp is cultured as the primary species for the first month while milkfish and later tilapia act as the secondary species stocked during the second month to reuse shrimp feed wastes and to improve water quality (Akiyama and Anggawati, 1999).

Having one of the longest coastlines (81,000 km) in tropical countries, most people in Indonesia are more familiar with marine fish compared to freshwater fish. Only in the last decade have tilapia become popular in the country as a seafood. What has happened in Aceh is a good illustration how the culture changed. Aceh province was the epicenter of the 2004 Indian Ocean tsunami. Prior to the tsunami, this north-tip province of Indonesia experienced civil and military war for almost two decades. The tsunami wiped-out coastal areas and a quarter million people in the area. People across the globe came in the rehabilitation and reconstruction efforts. People thrived together. The civil war ended. People from the Java mainland who work for the international organizations used to consume freshwater fish (carp, catfish, or tilapia). Soon, restaurants that offered

freshwater fish became available to accommodate different people around the world. The freshwater fish are also available in the market and groceries. Later, local Acehnese people started to eat catfish or tilapia or both.

4.2.2. Beneficial effects of brackishwater polyculture

As tilapia became popular, the shrimp-tilapia polyculture was already started in some places. The use of water from a tilapia culture pond reduced the prevalence of bacterial infections in shrimp ponds from luminous vibriosis (Yap, 2001). *Vibrio harveyi* and most other bacterial pathogens common in shrimp culture are Gram-negative, while water which have been used for fish culture tend to be predominated by Gram positive bacteria (Yi and Fitzsimmons, 2004).

Several research have been conducted to observe the potential of shrimp-tilapia polyculture in reducing Vibriosis (Tendencia, et al., 2010). The use of 'green water', a technique that involves the stocking of tilapia (*Oreochromis* sp.) in the reservoir for the production of microalgae *Chlorella* is believed to help in the reduction of the incidence of luminous vibriosis. Green water from brood stock tank of tilapia has the ability to inhibit vibriosis, and the brood stock are a better source of green water than juveniles in controlling the disease (Huervana et al., 2006). The effectiveness of the green water in preventing outbreaks of vibriosis can be attributed to the presence of anti-luminous vibrio factors in the bacterial, fungal, phytoplankton micro biota, and the skin mucus of tilapia. Polyculture also improves the nutrient conversion rate of the harvest organism, both shrimp and tilapia. Feeding enhances the antibacterial activity, the efficiency of tilapia at

a biomass of 500g/m^3 is reduced if the shrimp biomass is greater than 80g/m^3 (Tendencia et al., 2006).

4.2.3. Brackishwater, a hotspot for viral diseases: Indonesia cases

As the shrimp-tilapia polyculture is partly practiced to minimize shrimp disease risk, this chapter will describe the history of shrimp farming in Indonesia and the associated diseases that emerged in the country overtime.

Purnomo (2001) put a detail review on shrimp aquaculture history in Indonesia. Prior to 1964, milkfish farming was the most popular aquaculture practices in Indonesia and was mainly concentrated in Java, South Sulawesi and Aceh. Indonesians are similar to many Asian people, and they eat the whole fish and not the fillet. The easier the bones can be removed while eating, and this makes the fish more popular. The Indonesian milkfish bones are smooth in some parts of the fish bodies and therefore not convenient at some point. The milkfish farming in the 1960's relied on the intertidal zone where seawater entered the ponds during the high tides. When the water came, wild shrimp from seawater entered the ponds as well. Shrimp were still considered as a secondary species. When people realized that shrimp are boneless (despite their cuticles and big head), this species became more popular.

Shrimp aquaculture in Indonesia was started in South Sulawesi province with the Giant tiger shrimp (*Penaeus monodon*) and the Banana prawn (*P. merguiensis*). Shrimp were monocultured or polycultured with milkfish. By 1970, extensive (low density) Giant tiger

shrimp farming were distributed in Java, Balikpapan (Kalimantan) and Aceh. In Banda Aceh, both *P. monodon* and *P. indicus* were available in the coastal area. The culture density was about 20,000 – 30,000 shrimp per hectare, and farmers relied on natural feed after fertilization at the beginning of the cycle. After 3 - 4 months, 300 – 400 metric tons of shrimp were harvested in a count size of 30 (size 30 at harvest means 30 shrimp to make up one kilogram biomass or about 33.3 gram each). During the last month of culture, some farmers put milkfish into the ponds (Purnomo, 2001).

About the same year, in 1970, after a successful breeding program from natural broodstock, the first hatchery was started in Makassar, South Sulawesi and followed by one in Jepara, Central Java. The research station in Central Java was first called the Shrimp Research Center and later became the Research Center for Brackishwater Aquaculture Development in 2003, its current name. A significant milestone in shrimp aquaculture was achieved when the eye ablation technology for egg maturation was developed in Jepara in 1975 (Purnomo, 2001).

By 1984-1985, intensive shrimp farming was widely distributed in Banyuwangi and Situbondo (East Java), in Tangerang and Serang (was part of West Java province, and now became part of Banten province), Denpasar (Bali), and Lampung. Intensification and industrialization of shrimp farming led to the operations of feed companies in Indonesia, such as Comfeed Indonesia (1986) and Charoen Pokphand (1989).

Because of shrimp viruses such as Monodon Baculovirus (MBV) in 1986 followed by Yellow Head Virus (YHV) around 1992/1993, and White Spot Syndrome Virus (WSSV) around 1995/1996, shrimp production was affected. Cold storages were closed due to low production. Those viruses all first appeared in East Java and infected *P. monodon*. Several attempts were conducted to overcome the disease outbreaks, for example by dedicating one pond as reservoir. This idea was not preferable for farmers at the beginning because it meant they had less area for grow-out ponds. Probiotics became popular and most farmers use *Bacillus* spp. The use of probiotics is believed by farmers to improve shrimp performance.

Economic losses caused by WSSV led to the introduction of the Whiteleg shrimp (*Penaeus vannamei*), a non native species, which is considered more resistant to WSSV. Sunarto (2011) reviewed the introduction of *P. vannamei* to Indonesia and the associated diseases. *P. vannamei* was illegally introduced to Indonesia in 1999. The non-native shrimp was officially introduced for research (2000) and culture purposes (2001).

The growth performance of *P. vannamei* and its relative resistance to WSSV, despite its higher protein requirement feed, had shifted many farmers to this species instead of *P. monodon*. This increased shrimp aquaculture production, contributing 37.11% to national production in 1999, and 41.2 % in 2003 (Budhiman, et al., 2005). Since then, *P. vannamei* aquaculture had gradually dominated Indonesian shrimp aquaculture, and by 2007, it contributed to about 60% of Indonesian shrimp production (Sunarto, 2011).

Unfortunately, two years after the introduction of *P. vannamei*, the shrimp industry was hit by Taura syndrome virus (TSV), first reported in East Java in 2003 (Sunarto, 2011). Based on the University of Arizona Aquaculture Pathology Lab database, in fact, TSV positive samples from Indonesia were already received in 2002 (corresponds to case 02-318). The availability of shrimp post-larvae with TSV resistance prevented the spread of the disease. TSV has never been reported in area outside Java. The emergence of another virus associated with *P. vannamei*, infectious myonecrosis virus (IMNV) in 2005/2006, first reported in East Java (Nur'aini et al., 2006; Senapin et al., 2007), have affected shrimp production in the country. Total production losses were estimated at $150 – 200 million from 2009 – 2011 (Sunarto, 2011). With the emergence of a new disease, Early Mortality Sydrome, in China, Vietnam, and Malaysia (Lightner et al., 2012), this disease poses a threat to spread to Indonesia as a neighbor country.

4.2.4. Production data and disease outbreaks

The emergence of different shrimp viruses has led to decreased shrimp production overtime. Figure 4.1 was generated based on data from MMAF (2011).

Figure 4.1. Shrimp production in Indonesia (1980 – 2010)

The blue arrows indicated that disease outbreaks have affected production directly. Prior to 2000, *P. monodon* was the most dominant species as well as the viral disease agents with them, for example the Monodon Baculovirus (MBV/1986), the Yellowhead Virus (YHV/1992), and the White Spot Syndrome Virus (WSSV/1996). The collapse of the industry in 1998 because of WSSV, followed by stagnant production in the following year, lead to the introduction of *P. vannamei*. The introduction of the new species led to the introduction of TSV in 2002, and IMNV in 2005/2006. In general, based on the database in the Indonesian Ministry of Marine Affairs and Fishery database, it took 2-3 years for a new disease to impact production significantly following their first

appearances in the country. Early database mainly based on clinical reports, as PCR or other detection methods were not available in the country at that time.

Despite problems caused by different disease agents, Indonesia continues to be one of the main shrimp producers in the world (FAO, 2010). The Indonesian government initiated the development of Indonesian *P. vannamei* lines which were claimed be resistant to different viruses. In 2009, the government named the line as the Indonesian Vannamei Nusantara first generation, or IVN-1, first was developed in Situbondo, East Java. Mass production has been conducted in Karangasem, Bali, with the establishment of Vannamei Broodstock Center in 2010.

In fact, Indonesia is not only a shrimp producing country, but also one of the main producers for tilapia (Fitzsimmons et al., 2011). Farmers have adopted shrimp-tilapia polyculture as it is believed will minimize shrimp disease risk. Most published papers on polyculture have mainly discussed the beneficial effects in reducing vibriosis. However, an extension specialist from the Shrimp Club Indonesia mentioned that in one province (Bangka Belitung in Sumatera island), after white spot disease and Taura syndrome outbreaks, the following year farmers started shrimp-tilapia polyculture. For two years, they did not experience any disease outbreak until IMNV hit the area in 2009 (Prapto Subroto, pers. comm.).

The experimental study presented in this chapter highlights the growth performance of shrimp and tilapia in a polyculture system at brackishwater salinity (25 ppt).

4. 3. Materials and Methods

4.3.1. Experimental tanks

The experimental study was conducted in a greenhouse at the Research Center for Brackishwater Aquaculture, Bangil, East Java, Indonesia for 90 days (three months) to investigate the growth performance of shrimp (*Penaeus vannamei*) and Red Hybrid Tilapia (*Oreochromis niloticus x O. mossambicus*) at 25 ppt. The salinity was achieved by pumping the seawater adjacent to the research center and mixed with well water. There were three treatments (shrimp monoculture, tilapia monoculture and shrimp-tilapia polyculture). Each treatment was done in three replicates in a one cubic meter indoor concrete tank covered with plastic on each side. The depth of the water was adjusted to 80 cm. In shrimp monoculture, the stocking density was sixty (60) shrimp. The tilapia monoculture tanks had ten (10) fish each. In the shrimp-tilapia polyculture group, sixty (60) shrimp and ten (10) fish were combined in one tank. Each tank was supplied with a biofilter to maintain the aeration. Shrimp were placed inside a sinking hapa net in the tank while tilapia freely moved in the water columns outside the hapa net.

4.3.2. Growth parameters

Shrimp post-larvae (average weight of 0.0168 gram) and Red Tilapia (average weight of 97.645 gram) were obtained from local hatcheries. Shrimp were fed *ad libitum* (with recorded feed weight) with commercial sinking pelleted feed (40% protein) for the first two months, and 3% of body weight for the last month. Tilapia received floating feed (18% protein) throughout the duration of study. Shrimp and fish were weighed biweekly

to determine the survival and the specific growth rate (SGR) for each species as well as the feed conversion ratio (FCR).

Formulas used to calculate the survival and growth parameters

$Survival = [\frac{(A-B)}{A}]X\ 100$ (unit in %)

A = number of animals at the beginning of study

B = number of animals at the termination of study

$Specific\ Growth\ Rate = \frac{Ln(WT)-Ln\ (W0)}{T}\ X\ 100$ (unit in % per day)

WT = Weight at the termination of the study

W0 = weight at the beginning of study

T = number of days

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{Total\ weight\ of\ feed\ fed\ for\ each\ species}{Total\ weight\ gain\ by\ each\ species}$$

FCR is unitless

4.3.3. Water quality monitoring

During the trial, dissolved oxygen (DO), temperature, salinity, and pH were measured daily at 8 A.M. and at 4 P.M. Water samples were taken biweekly for analyses of total ammonia nitrogen (TAN), nitrate nitrogen (nitrate-N), nitrite nitrogen (nitrite-N), and

total phosphorus (TP) taken at noon using a kit and following the manufacturer's instructions.

4.3.4 Bacterial count and identification

Prior to the termination of study, the culturable bacteria in the water were counted by taking water samples and plating on Nutrient Agar (NA) with 2.5% salt for total bacteria and TCBS agar for presumptive *Vibrio* spp. Heterotrophic plate count (HPC) were done in three replicates. The BBL Crystal™ Identification System was used to determine the bacteria to species level according to the protocol by the manufacturer.

4.3.5. Microalgae identification

Microalgae composition was identified by taking water samples and observing the microalgae shape and morphology under a light microscope. The identification followed the Marine Plankton Identification Key published by Project Oceanography and GloBallast Monograph Series No. 7 Phytoplankton Identification Catalogue, a joint initiative from the Global Environment Facility, United Nations Development Programme and International Maritime Organization (Botes, 2001).

4.4. Results and discussions

Penaeid shrimp and tilapia rarely meet naturally because of the different habitats they live in. Penaeid shrimp throughout most of their life cycle live at high salinity and tilapia in fresh water (low salinity). In an aquaculture setting, both species can be reared together due to their tolerance to wide range of salinities and their different ecological niches (Fitzsimmons, 2001).

The survival and Specific Growth Rate (SGR) as well as the Feed Conversion Ratio (FCR) are the main parameters of growth performance in any aquaculture species. Table 4.1. summarizes the parameters for both shrimp and tilapia in monoculture and polyculture culture systems.

Table 4.1. Growth parameters of shrimp and tilapia after 90 day of culture

Parameters	**Shrimp**		**Tilapia**	
	monoculture	**polyculture**	**monoculture**	**Polyculture**
Survival (in %)	65.00 ± 4.36*	82.33 ± 4.51*	43.33 ± 15.28*	60.00 ± 10.00*
Specific Growth Rate (SGR in % per day)	7.90 ± 0.05	7.63 ± 0.06	1.06 ± 0.25	1.11 ± 0.12
Feed Conversion Ratio (FCR)	1.29 ± 0.08*	1.46 ± 0.21*	1.90 ± 0.44	1.92 ± 0.77

Data presented is the average of three replicates (mean ± standard deviation).
*significantly different by *t*-test comparison at $p < 0.05$

Based on the survival data, polyculture had higher survival for both shrimp and tilapia, compared to when they were in monoculture system (significantly different by *t*-test). As a consequence of higher survivors, the SGR of shrimp in polyculture was lower, and at the same time the FCR was higher, compared to shrimp in monoculture system, because they had to share the available nutrients. For tilapia, the low survival in this experimental study might due to two reasons. First, the space limit for the fish to move and to grow, as the middle of the tank was occupied with the hapa net. Second, most of the mortalities were recorded after 40 days, indicated that the tilapia strain used in this study could handle high salinity for the first month, but started to decrease the performance beyond that. This finding suggested that the use of salt-tolerant tilapia for polyculture is needed to hold the fish for the duration of the shrimp farming cycle, to give the benefits for shrimp. Another approach which has been implemented in the field for long-time, the fish is stocked for the last month only, no barrier is needed, when shrimp size is already large.

Together, the data on survival, SGR, and FCR show that shrimp performed well in brackishwater system, both in monoculture and polyculture systems, with significant survival increase in polyculture system. On the other hand, performance of tilapia strain used in the study decreased after a month, when mortalities started. This is likely due to salinity tolerance of the strain.

In addition to achieving good growth performance, maintaining water quality is another critical issue in any aquaculture operation. Table 4.2 summarizes the water quality during the experimental study. In general, all the parameters were within the acceptable levels.

Table 4.2. Water quality parameters in different system

	Shrimp monoculture	**Tilapia monoculture**	**Polyculture**
Salinity (ppt)	25	25	25
Temperature (°C)	28.7 ± 0.9	28.7 ± 0.7	28.9 ± 0.5
Dissolved Oxygen (ppm)	4.9 ± 0.2	5.0 ± 0.2	4.9 ± 0.2
pH	7.9 ± 0.4	7.8 ± 0.6	7.6 ± 0.5
Ammonia	0.01	0.02	0.01
Nitrite	0.08	0.10	0.10
Nitrate	0.13	0.15	0.18
Phosphate	1.81	1.03	1.73

Similar to the water quality from low-salinity experiment, pH level in polyculture were lower compared to monoculture. As pH was measured twice daily, in the morning at 8 A.M. and afternoon at 4 P.M, the average presented here was the pH level about noon time. The presence of microalgae in tilapia and polyculture systems would use carbon dioxide and oxygen in the water to do photosynthetic activity. The carbon dioxide balance seemed to lower the pH level for the system, compared to monoculture system where photosynthesis was limited.

In the water, bacteria and microalgae are important biological components which contribute to the pond dynamics. Table 4.3 lists the total heterotropic plate count (HPC) for total bacteria and presumptive *Vibrio* spp. The total heterotrophic plate count in polyculture and tilapia monoculture was about one and half log higher compared to shrimp monoculture. On the other hand, presumptive *Vibrio* spp. in polyculture system was lower compared to shrimp and tilapia monoculture tanks. Together, the data on HPC and presumptive vibrios showed that the higher number and might be diversity of

bacteria would compete and/or inhibit vibrios. During the experiment, it was not observed if the vibrios were beneficial or harmful for shrimp.

Table 4.3. Total bacteria and presumptive *Vibrio* spp. in the water

	Total heterotrophic bacteria on Nutrient Agar (CFU/mL)	**Presumptive Vibrio on TCBS agar (CFU/mL)**
Shrimp monoculture	9.2×10^4	2.5×10^3
Tilapia monoculture	2.6×10^6	2.5×10^3
Polyculture	2.4×10^6	8.7×10^2

CFU = colony forming unit

The green water in tilapia culture and polyculture is a nutrient-rich environment compared to the clearer water in shrimp monoculture. The presence of a higher number and diversity of bacteria, a relatively lower pH, and the presence of microalgae, all together might play synergestic roles in the polyculture system to improve water quality and fitness of the animals. Table 4.4 highlights the most dominant microalgae in each system. The presence of tilapia stimulates microalgae growth and more specifically promotes *Chlorella* sp. dominance which was not found in the shrimp monoculture group. The presence of *Chlorella* does not seem affect the diatom and dinoflagellate composition.

Burford (1997) mentioned that phytoplankton provide shade for organisms, prevent the growth of benthic algae, maintain oxygen level, reduce ammonia levels, and provide food source for zooplankton and other invertebrates eaten by shrimp. Therefore, in the field, it is a common practice to stimulate phytoplankton growth by adding fertilizers which

contain nitrogen and phosphorus before starting shrimp culture cycle. Low N : P ratio will stimulate cyanobacteria, while high ratio will be preferable for diatom.

Table 4.4. Microalgae composition in different system

	Microalgae species	**Abundance (cells/mL)**
Shrimp monoculture	*Nitzchia* sp. (diatom) *Prorocentrum* sp (dinoflagellate)	5×10^4 5×10^4
Tilapia monoculture	*Prorocentrum* sp (dinoflagellate) *Chlorella* sp. (green)	1×10^5 5×10^4
Polyculture	*Prorocentrum* sp (dinoflagellate) *Chlorella* sp (green)	1×10^5 5×10^4

To test polyculture in a larger scale, two ponds (2000 m^2 size) were stocked with shrimp monoculture and polyculture in Situbondo in a parallel study (Wibowo et al., 2010). The density for shrimp was 100 per square meter, and the ratio of shrimp to tilapia ratio was 2000 to 1, which means that the polyculture pond was stocked with 200,000 shrimp and 100 tilapia. The systems were maintained for 100 days. Table 4.5 summarized the growth parameters at harvest, that the presence of tilapia in a polyculture system in the field would improve the survival and final biomass, combined with lower FCR, which means less food was needed.

Table 4.5. Performance of shrimp in polyculture and monoculture systems

	Survival (%)	Size at harvest	Mean Body Weight (gram)	Shrimp Biomass (kg)	FCR
Polyculture	70	66	15.5	2,050	1.5
Monoculture	60	80	14.3	1,729	1.7

Note : size 66 means 66 shrimp to make up one kilogram of biomass at harvest
FCR : Feed Conversion Ratio

Together, the growth data show that shrimp performed well in brackishwater system, both in monoculture and polyculture systems, with higher survival, feed conversion ration, and shrimp biomass, in the polyculture system.

4.5. Conclusions and Future Directions

The data presented in this chapter showed that shrimp-tilapia polyculture in brackishwater is technically feasible. Overall, polyculture resulted in better growth performance by increasing the survival of shrimp and tilapia compared to monoculture. Polyculture, with higher level of bacteria and the presence of microalgae, has the tendency of lower pH but remain in acceptable levels for aquaculture practices. This low pH might have consequences in terms of disease risk as pH affects some virulence factors.

With the relatively lower survival of tilapia after one month in this study, salt-tolerant tilapia strains are needed to maintain tilapia for the duration of shrimp culture cycle (120 days). The availability of salt tolerant tilapia will give better and healthier crops.

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CHAPTER 5

REDUCED LUMINESCENT VIBRIOSIS IN POLYCULTURE SYSTEMS AND LOW SUSCEPTIBILITY OF SHRIMP TO STREPTOCOCCOSIS INFECTION

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5.1. Abstract

A series of microbiology studies and challenge studies were conducted to define if having tilapia in shrimp ponds would minimize luminescent vibriosis risk. The second goal was to examine the susceptibility of shrimp to *Streptococcus iniae* and *S. agalactiae* infections. By mixing luminescent *Vibrio harveyi* UAZ-651 (originally isolated from diseased shrimp in the Philippines in 1990) into shrimp and tilapia feed, the survival of shrimp in monoculture were significantly lower (20%) compared to in polyculture systems (75-95%). Mortality was not found in tilapia suggesting that the risk of vibriosis transmission from shrimp to tilapia was low. Sequencing data from the 16S rRNA gene of the bacterial community in the water revealed that shrimp monoculture was dominated by marine *Vibrio* spp., while the polyculture system water had *Bacillus* spp. and *Vibrio* spp. with high homology to *V. cholerae*. The presence of *Bacillus* spp., Gram-positive bacteria which produce a lactonase enzyme AiiA, seem inhibit Vibrio growth. On TCBS agar, which is a selective media for vibrios, the monoculture system had green and luminescent colony dominance. In the polyculture system, green and luminescent

colonies on TCBS were not found, and yellow colonies became dominant. While providing advantages, on the other hand, shrimp-tilapia polyculture might also contribute to disease transmission. *S. iniae* and *S. agalactiae* which are pathogenic for tilapia and other freshwater fish, andmight be transmitted to shrimp. Injecting shrimp with *S. iniae* and *S. agalactiae* resulted in mortalities. *S. iniae* caused higher mortality in the shrimp cultured in 20 ppt (40%) compared to 10 ppt (20%), and no mortality in 5 ppt. *S. agalactiae* caused higher mortality in 5 ppt (40%) compared to 10 ppt (20%) and 20 ppt (20%). This result provides information that the density of tilapia in polyculture system is critical not only in terms of growth, but also to minimize streptococcus transmission, while maintaining its beneficial role in minimizing vibriosis.

Key Words: Vibriosis, Streptococcocis, *Bacillus* spp., disease transmission, salinities

5.2. Introduction

Disease is one of the constraints to the development of shrimp farming. In most, if not all, of the major shrimp farming regions of the world, shrimp diseases continue to cause major losses (Lightner, 1996). A variety of agents, both biological and non-biological have caused shrimp diseases. Viruses, rickettsia/chlamydia, bacteria, fungi, protozoa, metazoa, feed factors (limiting nutrients or toxins), environmental factors (soil and water both physical and chemical, biotoxins and pesticides), and other factors have been found to be contribute to shrimp disease.

Amongst bacterial disease, vibriosis caused by luminescent *Vibrio harveyi*, is considered as the most dangerous and numerous (Lightner, 1993). On the other hand, what was believed at one time, that shrimp are only susceptible to Gram negative bacteria infection, is no longer true. In different places in the world, shrimp have been infected with Gram positive streptococcus and micrococcus (Hasson et al., 2009; Lightner et al., 2009). The disease incidence was found in low salinity, suggesting transmission from freshwater fish (Lightner, 2009), as *Streptococcus* sp. is the main pathogen for different species of fishes (Shoemaker et al., 2001).

As the use antibiotics is not recommended in shrimp aquaculture, several projects have been done to observe ways to overcome the diseases, for example, the use of immunostimulants, probiotics, or phage therapy for shrimp pathogens (Karunasagar et al., 2010). Another approach which seems to be promising is the potential of a tilapia and shrimp polyculture system to reduce vibriosis in shrimp (Fitzsimmons, 2001). Hopefully,

at the same time, the system would not increase the susceptibility of shrimp to streptococcus; or susceptibility of tilapia to luminescent vibriosis.

5.2.1. Vibriosis

The term vibriosis is used to refer all types of infections caused by bacteria of genus *Vibrio.* It has been suggested that the increasing severity has been associated with the degradation of the environment. Cowell (1984) describes the Vibrioceae as a family of facultative anaerobic, Gram negative rods, (0.3 -1.0) µm X (1.0 - 3.5) µm, straight or slightly curved and non spore forming. They are mobile by means of polar flagella or non mobile. Metabolism is chemoorganothrophic with both oxidative and fermentative respiration. Most are oxidize positive. They are found primarily in water and in association with aquatic animals (Austin and Zhang, 2006). Several *Vibrio* species are important in diseases of fish. This genus contains the one of the most significant marine fish bacterial pathogens.

Austin and Zhang (2006) described detailed information on the biology aspect, particularly the pathogenicity mechanism, of *Vibrio harveyi.* As a result of *16S rRNA* gene sequencing, *V. harveyi* is regarded as one of the core species of the genus *Vibrio* although this bacteria was originally named *Achromobacter harveyi.* The hosts of *V. harveyi* range from invertebrates (Japanese abalone, penaeid shrimp, and sea cucumber) to vertebrates (Jack crevalle, various fish species, summer flounder, sandbar shark, and lemon shark).

In terms of phenotypic characteristics, *V. harveyi* grows well in 8% NaCl, ferments glucose and manitol, most produce indole, and are able to use cellobiose as the sole source of carbon. The shrimp brooders, maturation, and spawning facilities are the main source of luminescent *V. harveyi* in the hatchery. Reasons for the outbreak of luminescent bacterial disease in larval shrimp, despite presence of bacteriophages in the larviculture system, remain to be explored (Chrisolite et al., 2008).

Pathogenicity and diagnosis of vibriosis

The pathogenicity mechanism of *V. harveyi* is determined by extracellular products (ECP) such as cysteine protease, phospholipase, and haemolycin; lipopolysachharides, bacteriophage, bacterion-like substance, quorum sensing factors, capacity to bind iron, and the ability to attach and form biofilms (Austin and Zhang, 2006). Nakayama et al. (2006) further explained that wild strains have higher minimum inhibitory concentration (MIC) values for four antibiotics (kanamycin, carbenicillin, oxytetracyclinem and ampicillin), and the shrimp show higher toxicity to wild-type strains (isolated from shrimp farms in Thailand and the Philippines prior to the experiment) compared to vibrios purchased from culture collections.

Diagnosis of bacterial infection in larval shrimp can be made by microscopic demonstration of bacterial rods through internal organs or associated lesions, including melanized cuticular lesions of appendages or small, internal melanized nodules in the gills or other organs (Lightner, 1996). In the pond, vibriosis is characterized by an abrupt onset and rapid course. Affected shrimp have disoriented, weak swimming and gather

along the edge of the pond. *Vibrio* sp. may be isolated in low numbers from the hemolymph of apparently healthy shrimp. The stress of crowding, handling, molting, and capture may result in bacteria being introduced into the hemolymph.

Modern immunological techniques, including ELISA (Enzyme-Linked Immunosorbent Assay), have been developed and proven for the rapid detection of *V. harveyi* from penaeid shrimp and water (Robertson et al., 1998). However, diagnosis of the clinical condition of vibriosis is still problematic, and requires integration of diagnostic information with clinical and production data. PCR diagnostics have also been used to detection of this pathogen (Fukui and Sawabe, 2007), as well as Real-Time PCR (Fukui and Sawabe, 2008).

Treatment and prevention of vibriosis

A variety of strategies have been applied to manage vibriosis in shrimp farming. Hatchery culture practices can vary, although, there are some common practices such as disinfection between stocking, disinfection of water intake, sanitation of live feed and equipment before introduction, low larval stocking density, and control of water temperature. The use of antibiotics in shrimp aquaculture is controversial as it can lead to antibiotic resistance in bacterial populations and it may have an impact on the environment.

The management strategies most commonly applied include: use of medicated feeds and fertilization of ponds with sucrose, through the addition of molasses (Lightner, 1996).

Some other useful preventive measures are a partial harvest to reduce pond biomass, high water exchange and disinfection of the pond bottom between cycles by drying, removal of excessive organic sediment and application of quicklime. In recent years, the use of probiotics has become popular.

The potential of shrimp-tilapia polyculture in reducing vibriosis

Shrimp-tilapia polyculture has been practiced in extensive, semi-intensive and intensive culture systems. In most shrimp-tilapia polyculture systems, shrimp are cultured as the primary species; while tilapia are cultured as the secondary species to reuse shrimp feed wastes and improve water quality (Akiyama and Anggawati, 1999).

Some research has been done to observe the potential of polyculture in reducing vibriosis (Huervana et al., 2006; Tendencia et al., 2010). The use of 'green water', a technique that involves the stocking of tilapia in the reservoir for the production of microalgae. *Chlorella* is believed to help in the reduction of the incidence of luminous vibriosis. Green water from broodstock tanks of tilapia has the ability to inhibit vibriosis, and the broodstock are a better source of green water than juveniles in controlling the disease (Huervana et al., 2006). The effectiveness of the green water in preventing outbreaks of vibriosis can be attributed to the presence of anti-luminous *Vibrio* factors in the bacterial, fungal, phytoplankton microbiota, and the skin mucus of tilapia. Polyculture also improves the feed conversion rate (FCR). Feeding enhances the antibacterial activity, however the efficiency of tilapia at biomass 500g/m^3 is reduced if the shrimp biomass is greater than 80g/m^3 (Tendencia et al., 2006).

5.2.2. Streptococcosis

Tilapia are widely cultured in the world, particularly in tropical regions. In the 1980's, tilapia were considered as equivalent of the aquatic chicken, which meant that they were very popular and common. In 2000, tilapia was already the 10th most popular seafood in the US and its popularity increased to become the fourth most popular seafood, after shrimp, tuna, and Pollock, in recent years (Fitzsimmons et al., 2011).

Based on history, tilapia (*Oreochromis* spp.) is native to Africa and the Middle East. Members of the genus *Tilapia* have been an important source of food for many years, at least since recorded history began. The fish Saint Peter caught in the Sea of Galilee, and those with which Christ fed the multitudes were tilapia. An Egyptian tomb, dated at 2,500 B.C. illustrates the harvest of tilapia and suggests that they may have been cultured since that time or probably before (Lucas and Southgate, 2003).

Other than their long history, it was believed for many years that compared to other fish, tilapia were resistant to disease. Recently, as with other cultured species of fish, tilapia has faced a number of diseases, predominately streptococcocal diseases. The prevalence and severity of disease in tilapia depend on many environmental factors, such as geographical culture system, farming density, salinity and water temperature, and on several factors, such as age, genetics, nutrition and stress (Cedric, et al., 2006). Minimizing disease becomes a crucial issue to prevent mortality, morbidity, and to promote optimal growth.

Streptococcal disease caused by *Streptococcus iniae* and *S. agalactiae* is considered as one of the limiting factors in tilapia aquaculture (Shoemaker et al., 2001). In terms of pathogenicity, the hemolysin of *S. iniae* is a functional homologue of streptolysin S (SLS), demonstrated by complementation of an SLS negative Group A streptococcus (GAS) mutant and inhibition by trypan blue. The hemolysin is regulated by nine genes with high homology to the GAS *sag* operon and it appears to be responsible for soft tissue damage and necrosis (Barnes and Ellis, 2004).

While streptococcal disease in tilapia is widely studied, limited research and publication is found for streptococcal disease in shrimp. The application of shrimp-tilapia polyculture might pose a risk of Streptococcal infection from tilapia to shrimp. To date, streptococcal disease in shrimp is limited to three publications (Hasson, et al., 2009; Lightner et al., 2009, Cuellar-Anjel et al., 2010). Even though none of the reports mentioned *S. iniae* and *S. agalactiae*, the main pathogens for tilapia, some of them had 98% homology to *S. iniae* based on *16S rRNA* gene sequence (Lightner et al., 2009). These findings suggest the potential of disease transmission from tilapia to shrimp, which requires further investigation.

5.3. Materials and methods

5.3.1 Luminescent Vibriosis study

Preliminary study on V. harveyi strain characteristics

Five luminescent *Vibrio* spp. isolates were chosen for preliminary study on optimum temperature, salinity, and luminescent behavior. Table 5.1 summarized relevant

information. These isolates were sequenced based on the *gyrB* gene, and were compared to other *Vibrio* species sequences available in the GenBank database.

Table 5.1. Luminescent *V. harveyi* strains from UAZ collections

Isolate Number	UAZ-case number	Origins	Shrimp disease associated
651	90-166C	Philippines	Yes
796	92-8/A6	Taiwan	NA
1043	97-87/A	Mexico	Yes
1105	98-56/B	Belize	Yes
1112	98-153	Madagascar	Yes
1334	12-260	Princeton collection	No

The first two digits of UAZ case numbers denote the year received.
NA: not available. Isolate #796 has no information on the shrimp disease association.

Preliminary study on tilapia susceptibility to Vibrio infection

The experiment used eight 150 L tanks at the University of Arizona Environmental Research Lab (ERL) with growstones (recycled glass product with high surface to volume ratio) as a filter media. Systems were maintained at 20 ppt and the average temperature was 28°C. Four tanks contained ten (10) tilapia (average weight of 100 gram), and four tanks without tilapia served as the controls. For presumptive vibrio count and total heterotrophic bacteria count, water samples were plated on Thiosulfate-Citrate-Bile salts-Sucrose (TCBS) agar and Tryptic Soy Agar (TSA) with 1.5% NaCl added, and were incubated for 24 hour at 28°C. Plating was done one day prior to *Vibrio harveyi* #651 immersion into water to a final concentration of 10^3 CFU/ml, and repeated every 24 hours for seven days.

Vibriosis challenge study

This experiment used sixteen 150 L tanks at ERL with growstones as a filter media. Table 5.2 summarized the four different groups in the experiment with four replicates: shrimp only, shrimp-tilapia, tilapia only, and tilapia water. Treatment one consisted of ten shrimp (average weight of 2 grams), treatment two with ten shrimp and five tilapia (average weight of 75 gram), treatment three with five tilapia only, and treatment four is tilapia water with ten shrimp. In the tilapia water group, fish were removed just prior to introduction of the vibrio inoculum. All systems were maintained at 20 ppt with an average temperature of 28°C. Shrimp in this experiment refer to Specific Pathogen Free (SPF) *Penaeus vannamei* from the University of Arizona West Campus facility, while tilapia refers to Red Hybrid *Oreochromis niloticus* maintain at ERL.

Table 5.2. Experimental groups and animal numbers combination

Treatment	Group	Shrimp	Tilapia
1	Monoculture (shrimp)	10	-
2	Polyculture (shrimp-tilapia)	10	5
3	Monoculture (tilapia)	-	5
4	Tilapia water	10	5

Twenty hours prior to the challenge study, luminescent *Vibrio harveyi* UAZ #651 was prepared on TSA with 1.5% NaCl added. A full plate of vibrio isolate was transferred into 10 mL of 2% sterile saline and mixed with 10 gram of shrimp feed (1 ml saline : 1 gram feed ratio). The same protocol applied for the tilapia feed. The Vibrio coated feed

was used for the first seven days, after that the shrimp and tilapia were fed a commercially pelleted diet once a day for 28 days.

Water samples were taken every 24 hours from each tank, and plated on TCBS for presumptive Vibrio counts. For bacterial identification, water samples were plated on R2A (low-nutrient) medium, TSA (rich-nutrient) medium, and TSA with 1.5% salt added. Selected isolates from culture were further processed for sequencing, and represented eight isolates associated with shrimp culture water, and ten isolates from polyculture water.

Moribund and freshly dead (i.e. no muscle opacity or difference in color from live shrimp observed) shrimp were collected. Hemolymph (100 µl) was extracted from the ventral sinus using a 25 gauge needle attached to a 1 ml tuberculin syringe, and inserted into the base of the fourth periopod (swimming leg). Hemolymph samples (1 or 2 drops per sample) were immediately streaked on TCBS plates and observed for growth and luminescence after 16-24 hours of aerobic incubation at 28°C. Luminescent and green colonies were transferred onto Tryptic Soy Agar with 2% NaCl. The colonies were then Gram-stained, and observed by light microscopy to confirm the presence of Gram-negative rod shape bacteria, the main characteristic of the original isolate.

After hemolymph extraction, moribund animals were preserved in Davidson's AFA fixative (Bell and Lightner, 1988), to confirm the infection by histopathology analysis

and to verify the disease or health status. For another confirmatory test, alcohol fixed animals were utilized for PCR analysis according to Fukui and Sawabe (2007).

5.3.2. Susceptibility of shrimp to *Streptococcus iniae* and *S. agalactiae*

Streptococcus strains

The *S. iniae* and *S. agalactiae* isolates were obtained from Dr. Phillip Klesius/ Dr. Julia Pridgeon from USDA-ARS (Agricultural Research Service) in Auburn, Alabama, USA. Based on the information given by the senders, the *S. iniae* (ARS-TN-03-SI-28HK) was isolated from the head kidney of tilapia at the ARS research station in Arkansas, 2003. The *S. agalactiae* was originally isolated from the head kidney of wild mullet from Kuwait Bay, 2002.

Both isolates were received on 5% Sheep Blood Agar (SBA). Upon arrival, the isolates were streaked on TSA and Brain Heart Infusion (BHI) agar and incubated at 28°C and 37°C for 36 hours. The colonies were then transferred into TSB with 20% glycerol and distributed into cryovial tubes for long term storage at -80°C.

Preliminary study

Both strains were grown on different media, with NaCl added (0% to 1.5%), and different temperatures (25°C, 28°C, 32°C, and 37°C), to give information on the basic characteristics of the isolates. The isolates were grown on SBA to confirm the hemolytic activity by incubation in aerobic and anaerobic conditions for 36 hours.

An attempt to mimic a natural bacterial infection was done via two different routes, first by immersing streptococcus isolates into water to a final concentration of 10^4 CFU/ml, and second by mixing shrimp feed and tilapia feed with streptococcus isolates for seven consecutive days. Both routes caused no mortalities either in shrimp or tilapia in two salinities tested (5 ppt and 20 ppt) after 14 days.

Streptococcus susceptibility in shrimp

As streptococcus immersion and coated feed resulted in no mortalities for shrimp and tilapia, another route was tested by injection. Considering that streptococcus infections in tilapia have been studied intensively, the challenge study focused only on shrimp susceptibility. Specific Pathogen Free (SPF) shrimp were obtained from the University of Arizona West Campus facilities. Prior to infection, shrimp were adjusted to different salinities according to experimental groups. Three different salinities (5 ppt, 10 ppt, and 20 ppt) were tested for *S. iniae* and *S. agalactiae* susceptibility in shrimp. Each group contained 10 shrimp (average weight 2 gram), and there were nine groups in total. Table 5.3. summarized the experimental groups. Shrimp in each treatment received approximately 10^4 CFU of bacteria by injection in the third abdominal segment. Control groups received 2% saline injections at the same time and at the same site.

Table 5.3. Nine experimental groups for Streptococcus susceptibility test

	S. iniae	*S. agalactiae*	Control
5 ppt	Group 1	Group 4	Group 7
10 ppt	Group 2	Group 5	Group 8
20 ppt	Group 3	Group 6	Group 9

Moribund and freshly dead (i.e. no muscle opacity or difference in color from live shrimp observed) shrimp were collected. Hemolymph (100 µl) was extracted from the ventral sinus using a 25 gauge needle attached to a 1 ml tuberculin syringe, and inserted into the base of the fourth periopod (swimming leg). Hemolymph samples (1 or 2 drops per sample) were immediately streaked on SBA plates and observed for growth and after 36 hours of aerobic incubation at 28°C. Small white colonies, transparent when exposed against light, are an indication of hemolytic activity by *Streptococcus* spp. The colonies were then Gram-stained, and observed by light microscopy to confirm the presence of Gram-positive micrococci, the main characteristic of the original isolate. A PCR detection assay for streptococcus (Zhou et al., 2011) was run as another confirmatory test.

5.4. Results and Discussions

Microbiological characteristics of luminescent Vibrio harveyi

Based on the University of Arizona Aquaculture Pathology Lab (UAZ-APL) bacterial collection database, samples of *Vibrio* spp. that were initially identified using API 20 E® strips, were received since early 1990's. The API database does not contain *Vibrio harveyi*, so the system can only identify *V. harveyi* and closely related species up to genus level. Nevertheless, API identification, which was first launched in 1970, is still a useful tool, combining a strip of biochemistry tests and database based on numerical identification, and now incorporated using a software program on the API website.

The UAZ-APL bacterial collection database provides information that some vibrios associated with shrimp disease are positive for urease by API identification. The reaction

is considered unusual in nature, and therefore the API database has a very limited number of urease positive organisms. The urease positive reaction of vibrios from shrimp ponds seem relate to the practice of fertilizing ponds with urea. Therefore, the urease gene might also be developed as a marker for vibrio diagnostic associated with shrimp disease. Five of the isolates are luminescent, and were used in the preliminary study and showed urease positive by API test.

The first record of luminescent isolates associated with shrimp disease came from Ecuador (case 90-69), and the Philippines (case 90-166), after farmers in both countries reported luminescent vibriosis in shrimp ponds during periods of elevated temperatures (L. Mohney, pers. comm.). By 2012, there were at least 12 luminescent Vibrio isolates in the long-term storage of the Aquaculture Pathology Lab at the University of Arizona (APL-UAZ), and six of them were chosen for preliminary study. While luminescence is an easy marker for study, the relatively low number isolates in two decades might be due to two reasons. First, the luminescent vibrios are generally uncommon, and their emergence might relate to different factors such as elevated temperature or salinity changes in the environment. The second, and by far the most likely possibility is that UAZ-APL might have received limited samples so the collection numbers do not reflect what was happening in the field.

Table 5.4 summarizes the microbiology characteristics of the six luminescent isolates. Although reported as luminescent on original isolation, isolate #651 showed low luminescence. Strongly luminescent organisms were observed with the addition of

glycerol into the TSA media. As growth and luminescence were easily observed, growing bacteria at different salinities and temperature led to different luminescent behavior. Whether or not luminescence is also a marker for virulence gene production needs further studies.

Table 5.4. Luminescence of six different isolatesin five salinities and four temperatures.

Isolates			**Media/Salinities**			**Temperatures (°C)**	
UAZ-651	1		TSA (5 ppt)	1		**25**	**4**
UAZ-796	1		**TSA + 0.5% NaCl (10 ppt)**	**4**		28	3
UAZ-1043	2		TSA + 1.0% NaCl (15ppt)	2		32	2
UAZ-1105	2		TSA + 1.5% NaCl (20 ppt)	3		37	1
UAZ-1112	3		TSA + 2.0% NaCl (25 ppt)	3			
UAZ-1334	**4**						

Note: 1 = less luminescence, 4= greatest luminescence
TSA : Tryptic Soy Agar
ppt : part per thousand

In terms of growth, the five isolates showed similar behavior. Isolate #1334 had the slowest growth for the first 16 hours, the luminescence achieved was slower compared to other isolates (Figure 6.1). But, once full plate growth was achieved, the isolate had the brightest luminescence, which continued up to 72 or even 100 hours. Isolate #1112 maintained its luminescence for 72 hours. The luminescence in other four isolates deceased after 6 – 8 hours. For challenge study, isolate #651 was chosen because this isolate was associated with high mortality of shrimp cultured in the Philippines, which might give an indication of the pathogenicity of the isolate.

For salinity, even though 10 ppt gave the brightest luminescence, for the polyculture study, 20 ppt was chosen as the optimum salinity. Similar for temperature, even though

25°C had the brightest luminescence, 28°C was chosen for challenge study because this is the normal temperature at which shrimp and tilapia are raised. The isolates exposed to the highest temperature (37°C) achieved luminescence faster compared to other isolates, but the effect subsided much faster at this temperature. This might due to the fact that the luciferase enzyme responsible for luminescent does not fold correctly at temperature higher than 30°C (Escher et al., 1989; Meighen, 1991). Therefore, a high level of functional luciferase is found in cells that grow optimally at that temperature. A limited number of eukaryotes have 30°C as their internal body temperature (Hastings and Nealson, 1977; Nealson and Hastings, 1979). This might be one of the reasons behind the interaction of luminescent vibrios and shrimp, as the animals are cultured in temperature around 30°C.

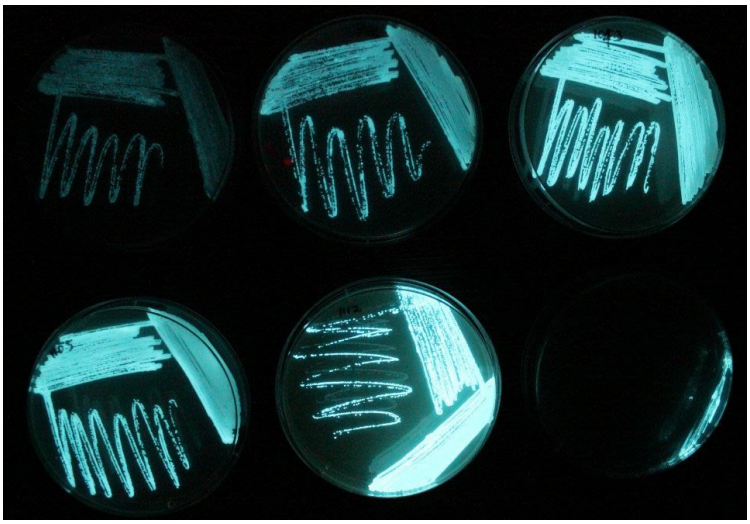

Figure 5.1. Luminescence of different isolates after 20 hour incubation at 28°C.
Top, Left to Right : #651 - #796 - #1043. Bottom Left to Right:#1105 - #1112 - #1334

Other than microbiological characteristics, *gyrB* gene sequencing provides identification at molecular level. Using the GenBank database, which contains information on the same genes from other vibrios and *E. coli*, the relationship was aligned using clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) followed by a Bayesian phylogenetics analysis from http://mrbayes.sourceforge.net/download.php. Figure 5.2 shows that five isolates from UAZ collection fall in either *V. harveyi* or *V. campbellii*, and one isolate (UAZ-1112) groups with *V. alginolyticus*. Separation of *V. harveyi* from *V. campbellii* by one single gene seems not to be sufficient for differentiation. Thompson et al. (2005) used at least three genes (*rpoA*, *recA*, and *pyrH*) to give higher resolution for vibrio identification. The phylogenetic tree also shows that the *V. campbellii* FM202603, seem to be mis-identified as it grouped together with *V. alginolyticus*. For consistency, this chapter refers isolate #651 which was used in the challenge study as *V. harveyi*.

Based on *gyrB* gene only, vibrios associated with shrimp disease share common ancestor to *V. parahaemolyticus*, with the exception of *V. penaeicida*. Three vibrios are known infect humans (*V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus*, the first two share common ancestor). *V. fischeri* live as symbionts with other marine organisms. It would be interesting to do further study to determine, if *V. fischeri* lost their pathogenicity mechanisms because of the long time they took to emerge from the ancestor. The five luminescent isolates from UAZ-APL collection formed a unique group, with the exception of UAZ #1112. This might due to the fact that shrimp culture was not started until early 1980, which might provide favorable environment for vibrio-shrimp interaction and their evolution.

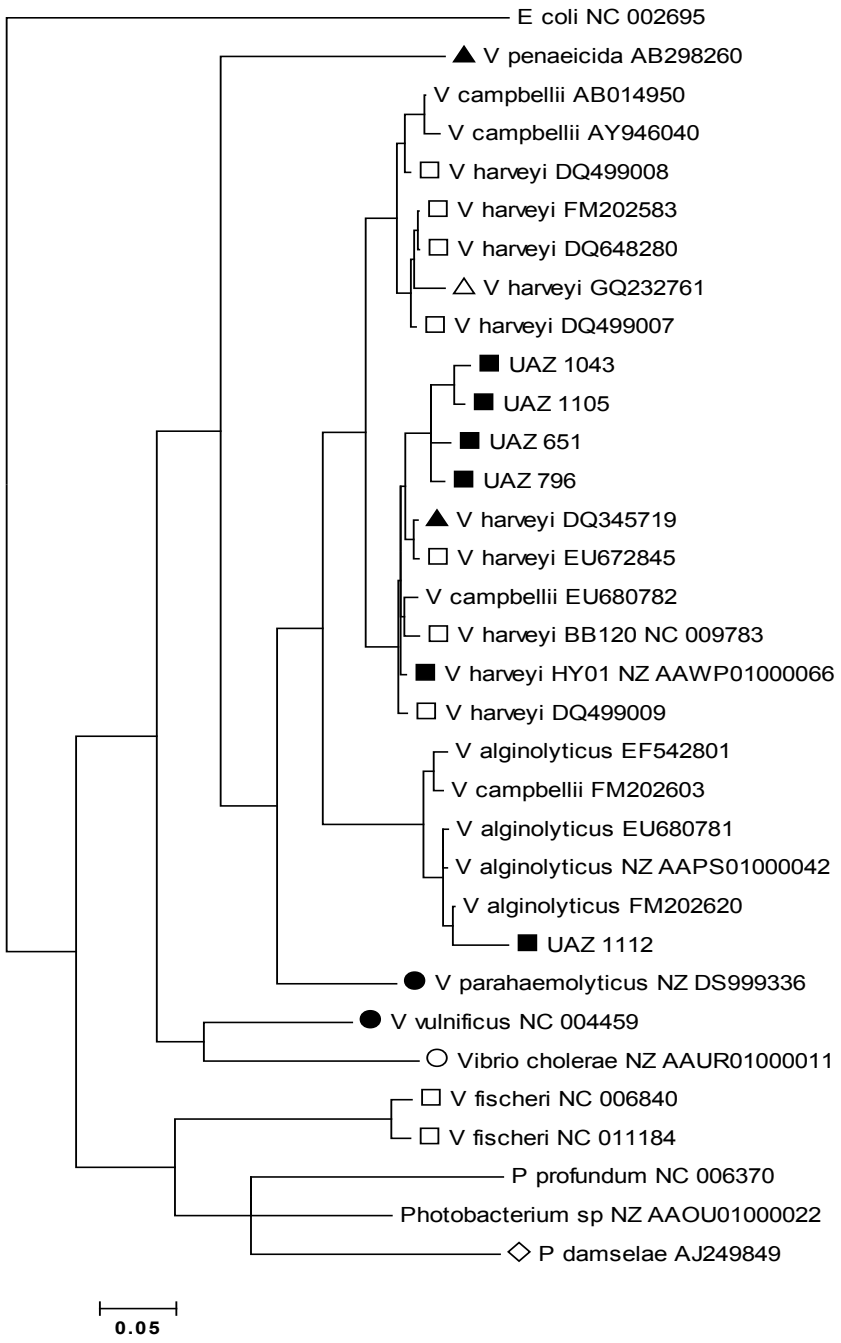

Figure 5.2. Phylogenetic tree of vibrios based on *gyrB* sequence with *E. coli* as an outgroup. Legends = closed triangle : associated with shrimp disease; opened square: luminescent; closed square : associated with shrimp disease and luminescent; closed circle affected humans and marine animals; opened circle: affected humans; opened diamond: infected fish.

Even though UAZ #1112 was grouped amongst the *V. alginolyticus* which caused food poisoning in Japan, this isolate might be a new species based on its position in the far right of the branch. The distance between each isolate to its closest branch represents the accumulation of mutations.

For luminescent vibrio evolution study, the target would be genes responsible for the luciferase enzyme production, the fused LuxCDABEGH, particularly the fused LuxAB (Meighen, 1993). The study might provide information why the genus *Photobacterium* did not maintain its original *Vibrio* designation, when most luminescent marine bacteria are named as *Vibrio*. Based on the *gyrB* gene solely, one would expect that *Photobacterium* deserves to be named as *Vibrio*.

Preliminary study on tilapia susceptibility to Vibrio infection

Figure 5.3 and 5.4 compares total bacteria and presumptive vibrio counts over a seven day period, started a day before the infection as a background/original number. Compared to the controls, the presence of tilapia reduced vibrio counts by two logs after 24 hour, and declined to original level after four days. There were no mortalities after two weeks, suggesting that the isolate was not pathogenic to tilapia. Based on the bacterial counts, vibrio are likely stay in the water for up to 48 hours. The relatively lower vibrio counts in tilapia group and relatively higher total bacteria counts gave an indication that bacteria in the water compete for nutrients, or some bacteria release molecules that inhibit vibrio growth. In the natural environment, microbial colonies are laminated heterotrophic and autotrophic vertically stratified communities (Paniagua-Michel and Garcia, 2003). Every

pond has different microbial activity and nutritional necessities, for example in freshwater environment, sulfate reducing bacteria are not important because sulfate availability is much lower compared to brackishwater.

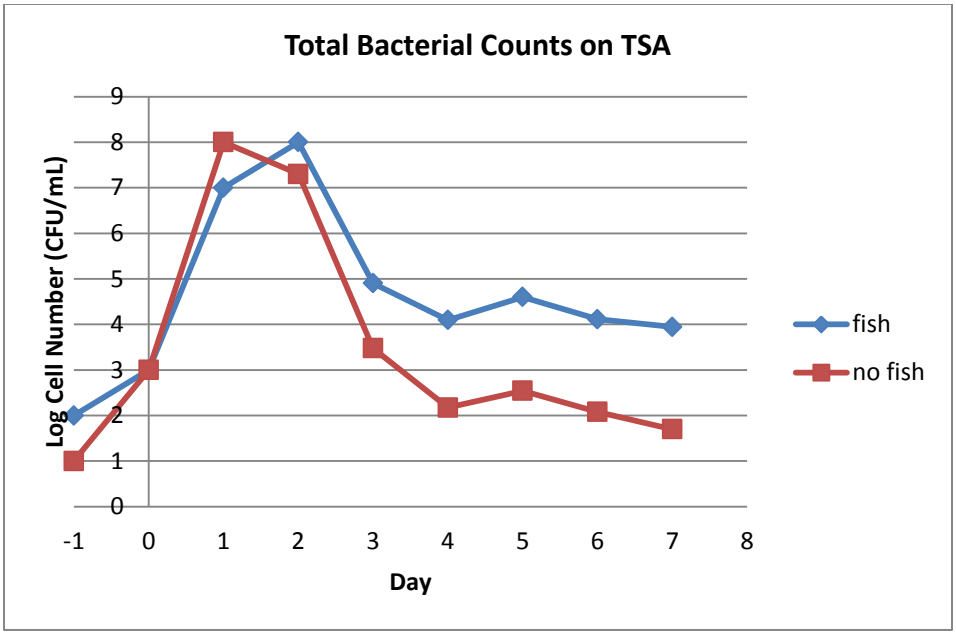

Figure 5.3. Total heterotrophic bacteria on Tryptic Soy Agar (TSA) +1.5% NaCl media

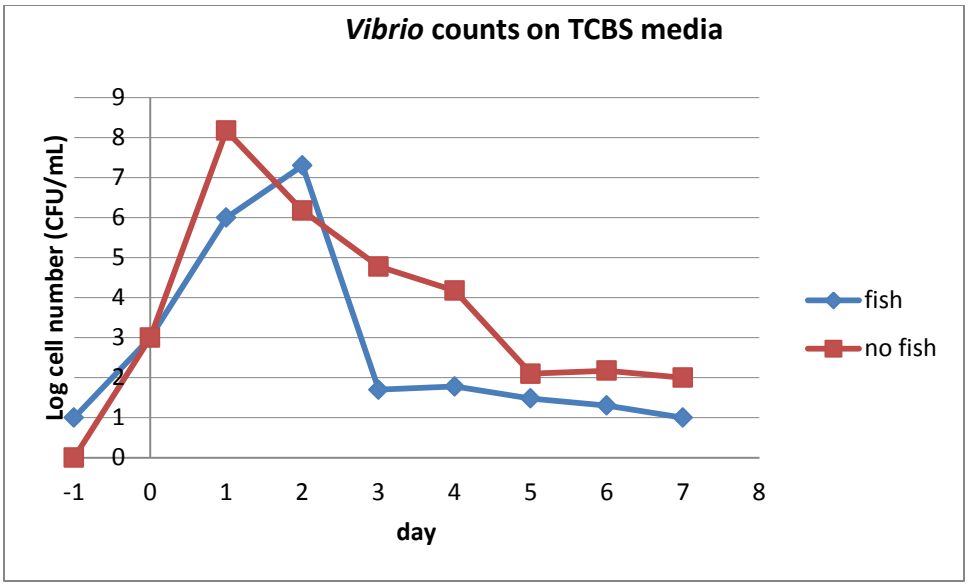

Figure 5.4. Presumptive vibrio counts on TCBS media

Vibrio harveyi challenge study

Table 5.5 presents final survival after four weeks. Mortalities in shrimp were found from day 7 to 15. Comparing the highest survival in tilapia water from this Table and the preliminary data that tilapia reduced presumptive vibrio count, suggested that the presence of tilapia had a direct effect as well as indirect effects. Defoirdt et al. (2008) mentioned that green microalgae can disrupt *V. harveyi* virulence.

The systems were maintained for another two months after the challenge study was terminated. It was observed that in the tanks where fish were removed, the green microalgae declined and changed to brown algae over time, an indication of close relationship between stimulation of green microalgae and the presence of tilapia which secrete nutrient rich waste containing nitrogen, phosphorus, and potassium (N, P, K).

In terms of color, as polyculture water was darker with the microalgae, the shrimp also looked darker compared to in shrimp monoculture where the water was clearer. Shrimp have the ability to reflect and blend with the water color where they live in, possibly to avoid predation.

Table 5.5. Final survival of shrimp and tilapia

Group	Shrimp	Tilapia
Monoculture (shrimp)	(20% ± 5)[a]	NA
Monoculture (tilapia)	NA	100%
Polyculture (shrimp-tilapia)	(75% ± 5)[b]	100%
Tilapia water and algae	(95% ± 5)[c]	NA

NA = not available as monoculture system had either shrimp or tilapia only

In terms of bacterial composition, prior to *V. harveyi* inoculation, water samples on TCBS agar in all groups showed yellow colony dominance in the range of 10^2 CFU/ml in shrimp culture water, while tilapia and polyculture had very limited colonies to none. Total heterotrophic bacterial count on TSA was in the range of 10^3 CFU/mL. In nature, interaction with pond bottom would likely give more bacterial diversity. After the introduction of the pathogenic bacteria (luminescent green colonies), shrimp monoculture groups were dominated by green colonies, while the other groups were able to repress the green colony growth. In tilapia monoculture and polyculture, the water was dominated by yellow colony bacteria on TCBS agar, even after the introduction of green and luminescent colonies (Figure 5.5).

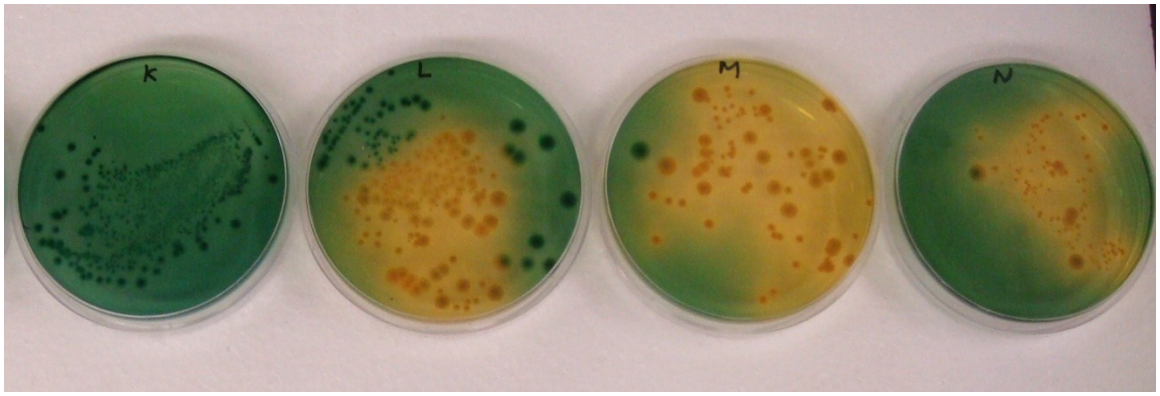

Figure 5.5. Water samples on TCBS agar from different groups after 24 hour. Left to Right: shrimp monoculture, shrimp/tilapia polyculture, tilapia water, tilapia monoculture

Gram staining revealed that in shrimp monoculture, Gram negative bacteria were dominant, while tilapia monoculture and polyculture systems had a mix of rod shaped Gram positive and Gram negative bacteria (Table 5.6). Several bacteria were selected for sequencing to give an idea of the different communities. This selection was based on different morphology and color on the plates, not necessarily the abundance, and it does not account for unculturable and anaerobic bacteria from the water.

Based on *16S rRNA* gene sequencing (Table 5.6), shrimp monoculture water had marine vibrio species (originally isolated on TSA + 1.5% NaCl), and other marine species (on R2A agar). The bacteria identified from the polyculture system had vibrios closely related to *V. cholerae* (on TSA) and *Bacillus* spp. (on TSA+). The findings suggest that *Bacillus* spp. and normal microflora seem contribute in protecting shrimp from luminescent vibriosis.

Several papers mentioned different *Bacillus* species as candidates for probiotics (Balacazar and Rojas-Luna, 2007; Ravi et al., 2007; Hill et al., 2009; Nakayama et al., 2009). Other than *Bacillus*, normal microflora vibrio are also believed have similar effects (Ninawe, 2009). Most of these probiotic candidates have been isolated from shrimp culture water, or from the intestine of different penaeid species. At a genetics level, Bassler and Losick (2006) proved that *Bacillus* spp. produces lactonase enzyme AiiA, which inhibits acyl homoserine lactone in the HAI-1 circuit of the *Vibrio harveyi* quorum sensing, and therefore inhibits its virulence.

To prove that the isolated *Bacillus* spp. inhibits *Vibrio* spp. Growth, a series of experiments were run that included cross streaking and modified minimum inhibitory concentration methods. Both methods did not show any inhibition. It seems some natural interactions could not be replicated with either method. Another approach was taken, in which 10µL of *Bacillus* spp. and 10µL of *Vibrio harveyi* #651 from long term storage were mixed and diluted by 1 : 100, and then plated on TSA + 1.5% agar, and incubated for 24 hours at 28°C. The results showed that the *Bacillus* spp., which is an orange color on TSA, overgrew *V. harveyi* which is creamy. Luminescent colonies were not detected. This observation needs further confirmation.

Table 5.6. Relevant information for selected bacteria from shrimp culture water (S1-S8) and polyculture water (P1 – P10) as defined by *16S rRNA* gene sequencing

Isolate	Isolation media	Gram staining	Shape	%homology	Species
S1	TSA+	Negative	Rod	99	*Vibrio sp*. PaH3.41
S2	TSA+	Negative	Rod	98	*Vibrio sp.* V794
S3	TSA+	Negative	Rod	96	*Vibrio communis*
S4	R2A	Negative	Rod	84	*Shewanella haliotis*
S5	R2A	Negative	Rod	92	*Ruegeria pelagia*
S6	R2A	Negative	Rod	96	*Vibrio sp*
S7	R2A	Negative	curve-rod	98	*Bowmanella denitrificans*
S8	R2A	Negative	curve-rod	97	*Bowmanella denitrificans*
P1	TSA+	Negative	Rod	96	*Vibrio sp.*
P2	TSA	Negative	Rod	98	*Vibrio cholera*
P3	**TSA+**	**Positive**	**Rod**	**94**	***Bacillus sp.* BJGMM-B1**
P4	R2A	Negative	Rod	94	*Vibrio sp.* GDLAMI-1210
P5	R2A	Negative	Rod	98	*Vibrio cholera*
P6	TSA	Negative	Rod	99	*Vibrio cholerae* LMA3894-4
P7	**TSA+**	**Positive**	**Rod**	**96**	***Halobacillus sp.* NT-B**
P8	**TSA**	**Positive**	**Rod**	**94**	***Bacillus sp.* 85-4**
P9	TSA	Negative	Rod	97	*Vibrio cholerae* LMA3894-4
P10	**TSA+**	**Positive**	**Rod**	**91**	***Bacillus sp.* JL1082**

TSA : Tryptic Soy Agar, rich nutrient media
TSA+ : TSA + 1.5% NaCl
R2A : Reasoner´s 2A agar, low nutrient media

Histopathology analysis

Shrimp presented low to severe bacterial infections of the lymphoid organs, hepatopancreas and gills (Figure 5.6). The bacteria responsible for these lesions appeared to be small rods. Multifocal melanized hemocytic nodules were also commonly observed during outbreaks of bacterial infections. The vacuolization levels of hepatopancreas, an indication of nutrient absorption and digestion, were found to be below a normal range. The absence of the biofouling in the shrimp suggested that the water quality was within normal parameters.

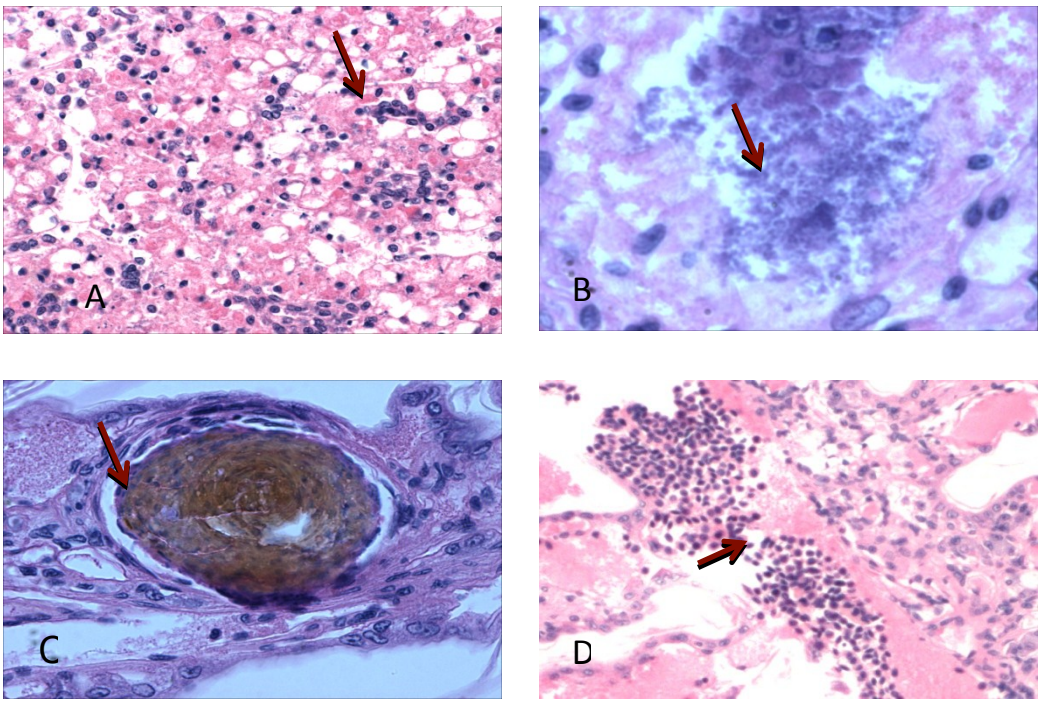

Figure 5.6. Histopathological changes after *Vibrio harveyi* #651 introduction.
Absence of normal arteriole in the lymphoid organ (A); bacterial plaque in the hepatopancreas (B); multifocal melanized hemocytic nodules and colonization in the gills (C and D). Mayer-Bennett's hematoxylin/eosinphloxine (H&E) stain. Total magnification (A = 100X; B = 250X, C=150X, D = 25X)

Susceptibility of shrimp to Streptococcus iniae and S. agalactiae infections

Streptococcus iniae and *S. agalactiae* are important pathogens of both fish and humans. Pier (1976) reported the first isolation of *S. iniae* from the Amazon dolphin (*Inia geoffiensis*). Since then, it has been considered to be among the most important bacterial pathogens in the aquatic environment, infecting a variety of hosts, including tilapia (Shoemaker et al., 2001). The clinical signs of streptococcus usually include meningoencephalitis, which destroys the nervous system, followed by eye and skin lesions, and septicemia. Since 1995, *S. iniae* has caused disease in humans after direct handling of infected fish. Hence, it is an example of a zoonotic disease.

Streptococcus agalactiae, commonly known as group B streptococcus (GBS), is an important disease agent responsible for bovine mastitis (Pattison et al., 1955). The bacteria also infected humans during the 1960's–1970's (Wilkinson, 1978). Multilocus sequence typing (MLST) demonstrated that many bovine GBS isolates do not share Sequence Types (STs) with human GBS isolates (Jones, et al., 2003). GBS is also widely distributed in different species of fish include tilapia, and aquatic mammals such as the bottlenose dolphin (Evans et al., 2006), confirming the wide range of GBS hosts. Evans et al. (2008) mentioned that genomic diversity existed between dolphin and GBS isolates in fish, and were largely unrelated to human and bovine GBS. But, GBS isolated from human neonatal meningitis caused disease and death in Nile Tilapia (*Oreochromis niloticus*), which would have been considered alarming if the transmission happened the other way around (Evans et al., 2009).

Table 5.7 summarizes the microbiological characteristics at different salinities and temperatures, under aerobic and anaerobic conditions. In general, both isolates grew better at lower salinity (without additional salt) compared to high salinity. On the other hand, higher temperature (37°C) seems preferable for streptococcus, and the isolates grew better under anaerobic condition. The data confirmed how the bacteria have adapted well in both terrestrial hosts (37°C) and aquatic hosts (28°C). For challenge study purposes in shrimp, the temperature used was approximately 28°C and salinity ranged from 5 ppt to 20 ppt.

Hemolytic activity on SBA media is one of the main methods of identification for *S. iniae* and *S. agalactiae* isolates. Both isolates were reported as having β-hemolytic activity, which means that the red blood cells in the SBA media completely lyse. Incubating in aerobic conditions confirmed the characteristic for *S. iniae*, but the *S. agalactiae* showed no hemolytic activity, as if this isolate belonged to γ-hemolytic group. Because many streptococcus are facultative anaerobic organisms and grow better with oxygen limitation, both isolates were placed in an anerobic incubator at 37°C in Dr. Gayatri Vedantam Lab, Veterinary Science and Microbiology Department, University of Arizona. Anaerobic incubation confirmed that both isolates had β-hemolytic activity. The fact that *S.agalactiae* showed hemolytic activity only under anaerobic incubation suggests that the isolates had a weak β-hemolysis compared to *S. iniae* isolate.

Table 5.7. Growth of Streptococcus at different salinities and temperatures after 36 hours.

Growth Media	*Streptococcus iniae*		*Streptococcus agalactiae*	
	28°C	**37°C**	**28°C**	**37°C**
TSA	3	3	2	3
TSA + 0.5% NaCl	2	2	2	3
TSA + 1.0% NaCl	2	3	1	3
TSA + 1.5% NaCl	2	3	1	3
5% SBA (aerobic)	3	4	2	3
5% SBA (anaerobic)	NA	4	NA	3

Note : scale 1 – 4, the lowest growth is 1.
TSA : Tryptic Soy Agar
SBA : Sheep Blood Agar
NA, not available : anerobic incubator was set for 37°C and there was no data for 28°C

Survival data of shrimp

The first mortality occurred 30 hour post infection and the last mortality after 4 days. Small circular white colonies bacteria (approximately 1.0 mm diameter), suggestive of *S. iniae* and *S. agalactiae* were observed on SBA plates from the hemolymph samples of the moribund shrimp. Gram-staining confirmed the coccus shape which formed chains as a Gram positive bacteria. Both isolates showed β-hemolytic activity. PCR analysis confirmed the presence of streptococcus (data not shown).

Table 5.8 summarizes survival data in different groups after 3 weeks. Moribund shrimp showed massive white muscle at the sites of injections (3rd abdomen), possibly caused by toxin accumulation. The response is common following bacterial injection, for example

with vibrios, but this response was not common with viral injection (personal observation), suggesting that bacterial infection would likely be associated with a toxin.

Table 5.8. Survival data of shrimp following *Streptococcus* injection.

Salinities	*S. iniae*	*S. agalactiae*	Control
5 ppt	100 %	60%	100%
10 ppt	80%	80%	100%
20 ppt	60%	80%	100%

ppt : part per thousand

As mortalities occurred at 10 and 20 ppt, the data provides an alarm for shrimp-tilapia polyculture raised around those salinities. While there were no mortalities for *S. iniae* at 5 ppt, prevention at low salinities is still recommended, as most cases of streptococcus infection in shrimp in the field were found at salinities close to freshwater (Hasson et al., 2009; Lightner, 2009), particularly following an extended period of high temperatures. This might also explain the experimental result which was conducted around 27 - 28ºC (a normal temperature for shrimp), and which might not be the optimum temperature for streptococcus. As the microbiology study showed that the isolates grew better at 37ºC, it would be interesting to test the interaction between salinity and temperature. For example, running an experimental study at 32ºC in the same salinity range (5, 10, and 20 ppt) might give different mortality data.

Infections by streptococcus and other Gram-positive bacteria typically occur in the rainy season or during extended periods of high temperature at very low salinity, which was found in the Middle East, Madagascar, French Guiana (South America), and Central

America (Hasson et al., 2009; Lightner et al., 2009). This suggests that at low salinity, streptococcus might occur across a broad temperature range.

The data presented in this chapter does not directly reflect the possibility of streptococcus transmission from tilapia to shrimp. In the field, streptococcosis is more common in fish than in shrimp. To better understand if streptococcus would likely be transmitted from tilapia to shrimp, an improved susceptibility assay, which would mimic the natural setting, is needed. One approach would be to inject tilapia intramuscularly or intraperitoneally as described by Evans et al., 2009. This method would cause lesions and mortalities in tilapia. If *Streptococcus* spp. would likely to be horizontally transmitted from tilapia to shrimp, it would come from the fact that shrimp are carnivorous animals. Shrimp will start to consume dead fish as is commonly seen in shrimp-milkfish polyculture (pers. observation). An experiment in which shrimp can consume dead streptococcus infected tilapia would be a better method for determining if shrimp can become infected, and then a better conclusion might be derived.

An attempt to develop *in-situ* hybridization for streptococcus was conducted, but the probe did not bind to pathogens. This might due to the cell membrane thickness in *Streptococcus* spp., and in Gram-positive bacteria in general. Therefore, the Proteinase-K enzyme was not able to degrade the cell membrane, and the probe was unable to bind to the target DNA.

Histopathology analysis

Shrimp responded to *S. iniae* infection by forming multifocal melanized hemocytic nodules in the antennal gland free space, gills, hepatopancreas, and muscle (Figure 5.7). The bacteria were encapsulated in the nodules in an attempt to localize the infection. At high magnification, very small cocci forming chains were apparent in most tissues.

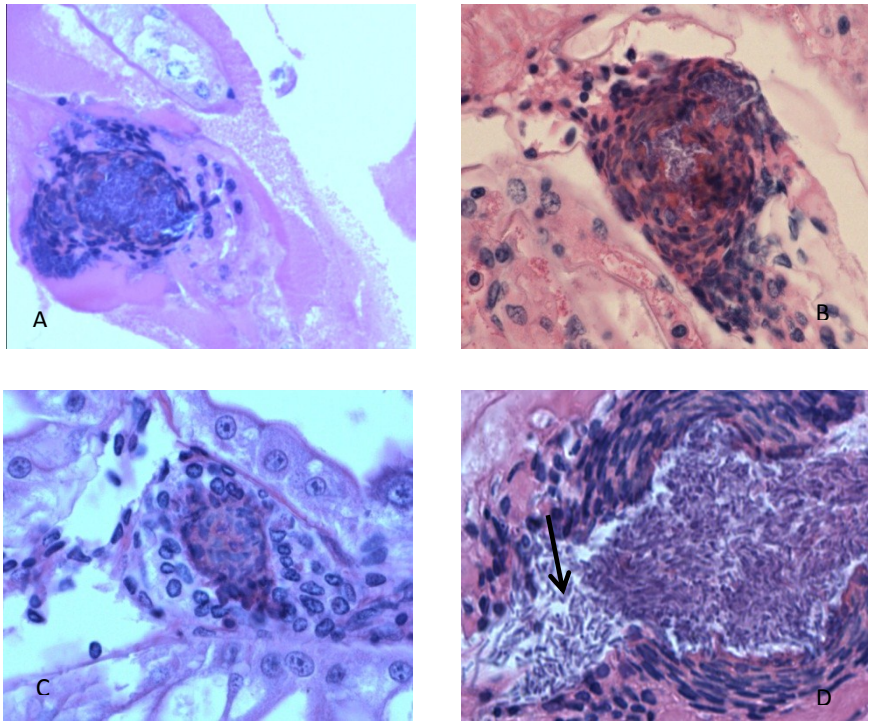

Figure 5.7. Bacterial colonization caused by *S. iniae* in the antennal gland (A), gills (B), hepatopancreas (C), and pleopod muscle (D). Mayer-Bennett's hematoxylin/eosin-phloxine (H&E) stain. Total magnification (A = 150X; B, C = 100X; D=300X)

Streptococcus agalactiae caused systemic infection and can be seen in the muscle, heart, hepatopancreas and the gills. Nodules were not found indicated that the infection might overwhelm shrimp so there was not enough time to activate the defense systems.

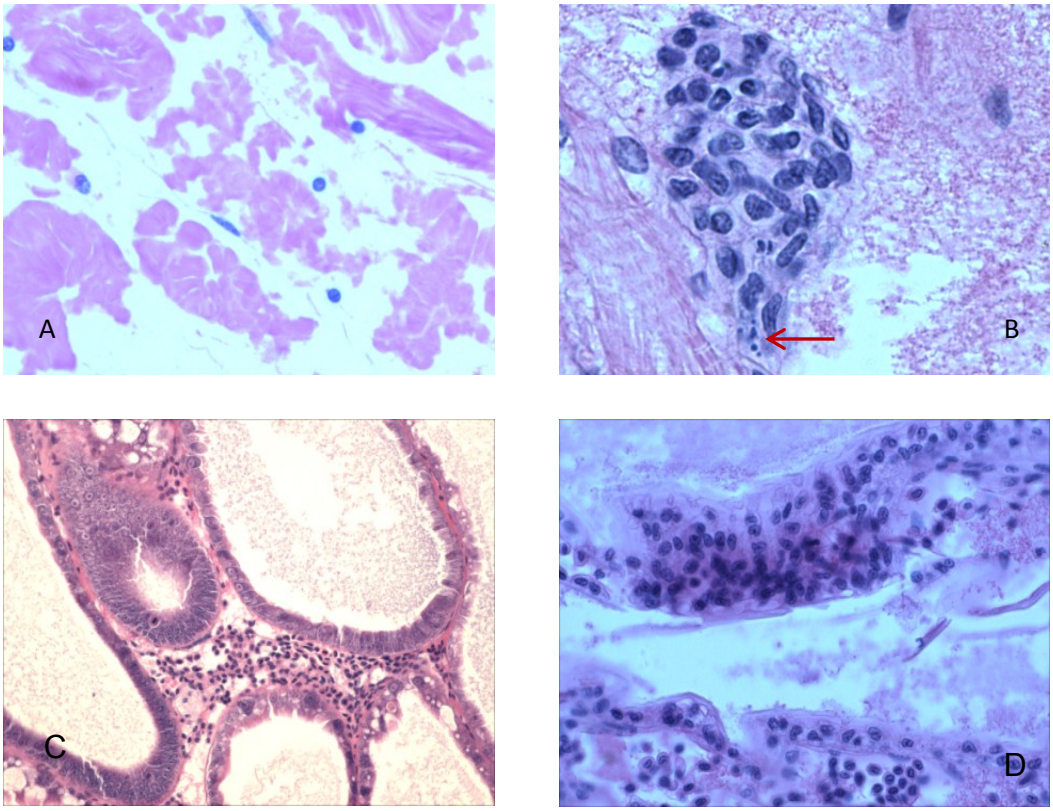

Figure 5. 8. Histopathological changes after *S. agalactiae* introduction. Muscle necrosis (A); heart (B), bacteria in the free-space between hepatopancreas tubules (C), and gills (D). Mayer-Bennett's hematoxylin/eosin-phloxine (H&E) stain. Total magnification (A = 50X; B = 250X; C = 50X; D = 100X)

5.5. Conclusions and Future Directions

Shrimp-tilapia polyculture seems promising, not only by improving the fitness and growth of both species, but also for disease management. The lower risk of vibriosis compared to in shrimp monoculture is a result of the higher diversity of bacteria associated within the polyculture system, the presence of green microalgae, and the antimicrobial properties which can be secreted by tilapia, microalgae, or bacteria (Tendencia et al., 2010). Compared to shrimp culture water, which is dominated by Gram-negative marine vibrios, the polyculture systems tested had a mixture of Gram negative bacteria, which are closely related to *V. cholerae*, and Gram-positive bacteria with the highest homology to *Bacillus* sp., which is known to produce lactonase enzymes AiiA that inhibit vibrio virulence.

Streptococcus iniae and *S. agalactiae*, while causing less than 50% mortalities to the shrimp in different salinities, the pathogens have a potential zoonosis for humans who work in fish handling industries. The disease can affect the nervous system in humans and fish, and in fish the clinical signs are sometimes very dramatic, for example losing eyes or skin. With a wide tolerance of salinities, streptococcus posses a major risk for different aquatic hosts, both in fresh and marine water. Therefore, streptococcosis management either in tilapia ponds or shrimp-tilapia ponds is crucial.

Natural disease transmission from shrimp to tilapia, or *vice versa*, might also require intermediate hosts or other reservoirs, for example higher crustaceans, other arthropods, or mollusk. To date, none of the shrimp viral diseases have been reported being

transmitted to fish. Only *Vibrio parahaemolyticus* infect both shrimp and fish (and humans), probably due to wide salinity tolerance of the bacteria, and not necessarily transmission from shrimp to tilapia.

5.6. Acknowledgement

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CHAPTER 6

CqsS GENE IN CAI-1 CIRCUIT DETERMINES *Vibrio harveyi* VIRULENCE IN SHRIMP *Litopenaeus vannamei*

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6.1. Abstract

Quorum sensing (QS) is a density dependent cell to cell communication process in bacteria that requires extracellular chemical signal molecules termed as autoinducers. A series of challenge studies using shrimp (*Penaeus vannamei)* was conducted to confirm the *in vitro* findings from the last two decades, that QS regulates virulence of *Vibrio harveyi*. The wild-type BB120 strain caused 75-90 % mortality through injection route of 10^6 CFU/shrimp in three different challenge studies. The mutants locked at low cell density (LCD) mode showed immediate virulence pattern, while the high cell density (HCD) mutants exhibited delayed virulence behavior. These results suggest that QS defined, when specific virulence genes were expressed or repressed. The final mortality rates of the wild-type (75%), LCD (80%), and HCD (90%), show that *V. harveyi* mutants that are unable to appropriately control the timing of QS gene expression are nonetheless, virulent. As QS in *V. harveyi* consists of three different circuits, further experiments deployed six mutants lacking either a synthase or a receptor for each circuit. The wild-

type and CqsA- caused the highest mortality (80%), followed by LuxM- and LuxN- (70%), LuxS- and LuxPQ- (40-50%), and CqsS- (20%), indicating that the CAI-1 circuit is the most crucial for virulence, followed by AI-2 and HAI-1. Microbiology, histopathology, and PCR analysis confirmed the presence of *V. harveyi* in the hemolymph of dead shrimp. To the best of the authors' knowledge, this is the first report that the CqsS gene in the CAI-1 cascade is the most important determinant for the virulence of *V. harveyi* in shrimp.

Key Words: *Vibrio harveyi*, quorum sensing, autoinducers, CAI-1, *cqsS* gene

6.2. Introduction

6.2.1. Connecting the dots: *Vibrio harveyi* genetics and shrimp disease study

In the last two decades, the pathogenicity mechanisms of bacterial diseases have been studied extensively by geneticists. Bacteria act as multicellular organisms, they need to be in a high number to cause disease (Bassler et al., 1993), or to provide beneficial effects for other organisms. Quorum sensing (QS), the process of cell to cell communication enables bacteria to do many tasks they cannot accomplish as individuals, and QS allows bacteria to collectively control processes including biofilm formation and the secretion of virulence factors (Fuqua et al., 1994). The roles of QS in pathogenicity of *Vibrio harveyi* has been one of the most well studied areas in the *in vitro* scale (reviewed in Waters and Bassler, 2005; Ng and Bassler, 2009).

Unfortunately, in terms of *in vivo* studies, the manifestation of *V. harveyi* infection in its host (shrimp) is still limited. The current state-of-the-art in QS study has been focused on screening and formulating potent anti-QS molecules to disrupt the communication in harmful bacteria, and discovering pro-QS molecules for beneficial bacteria (Geske et al., 2007; Li et al., 2008; Ni et al., 2008; Choi et al., 2012; Ng et al., 2012; Wei et al., 2012). At the same time, shrimp disease studies should benefit from the cutting edge technology widely used in the human disease studies. Challenge studies in shrimp to test potential drugs may be simpler, compared to clinical trials for new drugs for humans which require more strict regulations. In fact, most of the QS studies in aquaculture have been conducted at Gent University in Belgium in the last five years, and mainly used artemia, rotifers, or larviculture as animal models (Defoirdt et al., 2007; Natrah et al., 2011). The importance of QS seems underestimated by aquaculture scientists. Most of the disease management strategies focus on the use of immunostimulants, probiotics, or phage therapy for shrimp pathogens (Karunasagar et al., 2010). Limited research has been done related to blocking QS for harmful bacteria, and pro-QS molecules for beneficial bacteria, which would provide alternatives to antibiotics (Bjarnsholt and Givskov, 2007; Defoirdt et al, 2012).

Considering that QS is important in aquaculture disease studies, this chapter provides a review of QS from an historical perspective, and its scientific significance. Preliminary results from challenge studies in shrimp defined which QS circuit is the most important for virulence.

6.2.2. QS in pathogenic bacteria: to kill or not to kill

In nature, as bacteria grow and divide, they all make and release autoinducer molecules through the outer membrane (Dunny and Winans, 1999). The autoinducers increase in proportion to cell number. The bacteria have receptors on their surface to detect these molecules, and they elicit changes in the behavior as a group, when the autoinducer molecules reach a threshold level. Using these autoinducers, bacteria have the ability to detect, when other bacteria are around them. This cell to cell communication process enables bacteria to achieve benefits they could never accomplish as individuals. This behavior was coined as QS, in a paper by Fuqua, Winans, and Greenberg (1994). At that time, Steven Winans from Cornell University, had studied the phenomenon in *Agrobacterium tumefaciens*. He tried to explain his work during the Thanksgiving feast. Rob Johnston, his brother in law who was not a science major, came up with the term 'QS' (Dunny and Winans, 1999).

Prior to 1994, the QS phenomenon was termed autoinduction. The story started when J.W. Hastings and Kenneth Nealson from Harvard/ Scripps Institute of Oceanography, discovered that a marine bacterium *Vibrio fischeri* produced light (bioluminescence), when its population reached a certain density. They speculated that the bacteria released a signal they termed an autoinducer (Nealson et al., 1970). There was no clue that the phenomenon would later have relevance in the study of bacteria group behavior in general, and not be limited to bioluminescence. Michael Silverman and his group at the Agouron Institute in La Jolla, achieved a major accomplishment, when they defined the genes responsible for *V. fischeri*'s QS, *luxI* and *luxR* (Engebrecht et al., 1983; Engebrecht

and Silverman, 1984). The word 'lux'is derived from *luxor*, the God of Lights (Bassler, 2010).

The story continued as Bonnie Bassler, a young postdoc fellow (28 years old) in Silverman's Lab, decided to investigate another glowing marine bacterium, *V. harveyi* (Bassler et al., 1993; 1994a; 1994b). Bassler (pers. comm.) had at least two reasons to focus on *V. harveyi*. First, *V. harveyi* are similar to *V. fischeri*, in that they are bioluminescent, and therefore provide a powerful genetic read-out that can be easily observed. Second, while *V. fischeri* live in a symbiotic relationship with the Hawaiian Bobtail squid (*Euprymna scolopes*), *V. harveyi* are free living. She speculated that free-living organisms would require more complex systems to communicate in mixed microbial communities, having to cope with the changing environment of the open sea.

Soon, Bassler figured out that the signaling system of *V. harveyi,* which behaves much like *V. fischeri* in culture and light production, is also density dependent. Interestingly, the autoinducer molecule that controls bioluminescence in *V. fischeri* has no effect on light production in *V. harveyi,* and *vice versa*. This suggests that in nature, there is more than one autoinducer molecule. The complexity of *V. harveyi* led to the discovery of another autoinducer and circuit (Bassler et al., 1993). She coined it as autoinducer-2 (AI-2), and the one defined by Hastings became AI-1 (Bassler et al., 1994a). As Bassler moved to Princeton for a faculty position, *Vibrio harveyi* had become well studied, and perhaps the best model to study QS. Since then, more than anyone else, Bassler has been the linchpin of QS study. Over time, the Bassler Lab has figured out the roles of the

genes involved in QS, and one of the most important genes associated with pathogenicity is LuxS (Surette et al., 1999).

By 1999, LuxS genes had been identified in many species of bacteria by sequencing projects, but no functions had been described. The wide distribution of LuxS suggested that QS is a behavior used by most bacteria, not only by the bacteria that produce light when they have enough cell number. LuxS synthesizes the AI-2 signal, and is found in both Gram negative and Gram positive bacteria, suggesting that the gene existed before the split of the two groups (Schauder et al., 2001).

For pathogenic bacteria, AI-2 is important in regulating the transition from a non-pathogenic existence outside a host to a pathogenic state once inside a host (Surette et al, 1999). In some, if not most pathogenic bacteria, causing disease in the host is considered as an unintentional consequence. From the bacterial point of view, they need something in or from the host, with or without causing infections.

The search for the elusive AI-2 structure in the QS field was accomplished in 2002, when Bassler and collaborators defined the structure of the AI-2 as a furanosyl borate diester (Chen et al., 2002). Using crystallography, it was the first time anyone had solved the structure of a small molecule by trapping the ligand inside its receptor. They also revealed that AI-2 uses the element boron as a cofactor to carry its biological role. Even though boron has been used in chemical industries for long time, its biological functions and its abundance in the ocean were not understood before (Ahmed, 2008). Furthermore,

they proved that AI-2 could interconvert, and therefore *V. harveyi* AI-2 would be recognized by receptors in *E. coli* or *Salmonella*. The finding that AI-2 is a universal chemical language used by most bacteria (Chen et al., 2002), was published in Nature magazine. It took nine years for Bassler, to fill the question marks in the circuit she drew back in the 1993/1994 papers. Since the Nature paper, QS, the idea that bacteria talk to each other, is officially accepted. Other than simple growth and division, QS behavior is one of the factors that contribute to the successful life of bacteria over a long period of time.

6.2.3. QS in *Vibrio harveyi*: A tale of two lifestyles in three circuits

In *Vibrio harveyi,* QS is accomplished by three different circuits (Henke and Bassler, 2004b), summarized in Table 6.1 and Figure 6.1. The first circuit consists of HAI-1 (harveyi autoinducer-1) as the autoinducer, which is synthased by LuxM, and recognized by the receptor LuxN. The second circuit uses AI-2 as the autoinducer, LuxS as synthase, and LuxPQ as the receptor complex. The third circuit has CAI-1 (cholera autoinducer-1) as the autoinducer, CqsA (cholera QS Autoinducer) as the synthase, and CqsS (cholera QS Sensor) as the receptor. The third circuit name indicates that the system was first found in *V. cholerae*, and later it was widely found in other vibrios.

Table 6.1. Three systems in QS of *Vibrio harveyi*

System	Structure	Synthase	Receptor
HAI-1	Acyl homoserine lactone	LuxM	LuxN
CAI-1	(S)-3-hydroxytridecan-4-one	CqsA	CqsS
AI-2	Furanosyl borate diester	LuxS	LuxPQ

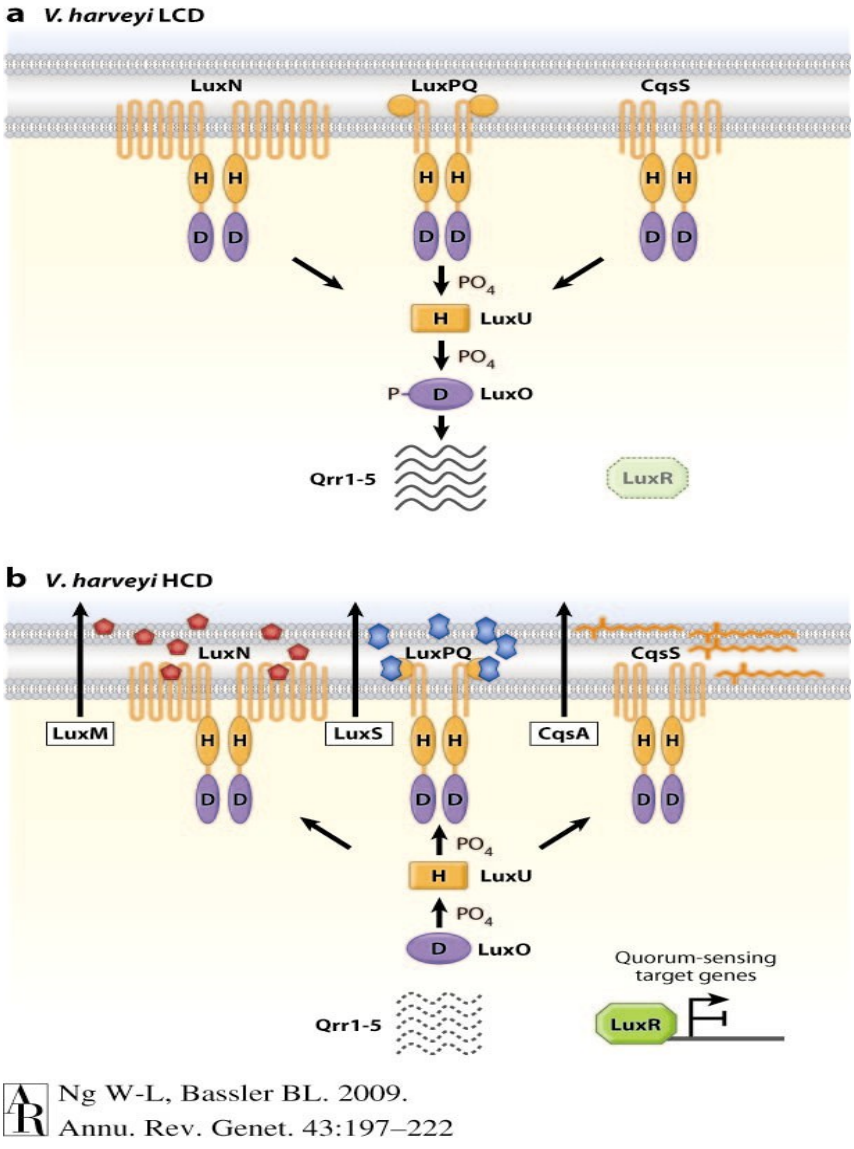

Figure 6.1. Three QS circuits in *Vibrio harveyi*. (a) At low cell density (LCD), small RNAs (Qrr1-5) activate AphA gene production that represses LuxR. (b) At high cell density (HCD), LuxR is the master regulator for gene expression that regulates QS target genes. Arrows describe phosphate flow, note the difference in direction between the LCD and HCD. Reproduced with permission from B. L. Bassler.

The complexity of the circuits led to the idea, that three different signaling systems were used by bacteria. *Vibrio harveyi* uses HAI-1 to communicate within its own species (Mok et al., 2003). The CAI-1 is the language used by most vibrios (Ng and Bassler, 2009), so *V. harveyi* can communicate to closely related species which have CAI-1, for example *V. campbellii* (Defoirdt et al., 2008), or *V. parahaemolyticus* (Henke and Bassler, 2004a). The AI-2 is the chemical signal found in many bacteria, and is considered as the universal language, as bacteria live and continuously interact with other bacterial species in nature (Schauder et al., 2001; Federle and Bassler, 2003).

In vitro studies have shown that the HAI-1 (the intra-species communication) signal is the strongest compared to the other two QS signals (Waters and Bassler, 2006), but all circuits are needed (Henke and Bassler, 2004b). The second circuit which has the AI-2 and boron in it, is needed at the early stage of infection, when bacteria switch from a non-pathogenic (free living style in the water), to a pathogenic state once they enter the hosts (Waters and Bassler, 2006). The pathogenicity function of the third circuit (CAI-1) for *V. harveyi*, other than for communication with other vibrios, has not been widely studied.

Recently, Henares et al. (2012) revealed that bioluminescence in *V. harveyi* is also dependent on nitric oxide (NO) concentration, regulated by HnoX gene which acts as a NO sensor. Based on the finding, they proposed the fourth circuit, a nitric oxide (NO) responsive QS, which seems responsible for bacteria and host (eukaryotic) communication. The finding is quite new, and therefore the discussion in this Chapter 1s limited to the three circuits widely known prior to 2012.

Austin and Zhang (2006) reviewed pathogenicity mechanisms of *V. harveyi*, which are determined by extracellular products (ECP), such as cysteine protease, phospholipase, and haemolycin, lipopolysachharides, bacteriophage, bacterion-like substance, QS factors, capacity to bind iron, and ability to attach and form biofilms. However, the authors did not elucidate which of these factors are the most important determinants. Possibly, there are strain differences and virulence may be attributable to any of a number of factors. Interestingly, most of the factors are controlled by QS.

QS controls some virulence factors in *V. harveyi* by negatively regulating chitinase A (Defoirdt et al., 2010), siderophore (Lilley and Bassler, 2000), and T3SS (Henke and Bassler, 2004a). On the other hand, QS positively regulates the production of metalloprotease (Mok et al., 2003), and an extracellular toxin (Manefield et al., 2000). Two other virulence factors (lipase and hemolysin) are independent of QS (Natrah, et al., 2011). The findings indicate, that different virulence factors are responsible at different stages during infection. Virulence factors that are negatively regulated by QS (expressed at low cell number), most likely are needed early during infection, to switch life styles from low density as free living bacteria in the water, to attach to the hosts, and to multiply inside. Virulence factors that are positively regulated by QS (expressed at high cell numbers), are required at later stages to cause disease, and to escape from the hosts. From the bacterial point of view, as in other organisms, the main goal is to grow and to reproduce. It would be considered a suicide act, to kill hosts or at least to cause disease, without the ability to escape and find other hosts.

6.2.4. *Vibrio harveyi* BB120: what is in a name

Recent genomic studies (Lin et al., 2010), proposed that the *V. harveyi* BB120 strain, and also *V. harveyi* HY01 strain, both are *V. campbellii*. If *V. harveyi* BB120 and HY01 strains, which have been fully sequenced, and the sequences are available, really are *V. campbellii*, then that means there is no full genome sequence of a *Vibrio harveyi* bacterium. Richter and Rosello-Mora (2009) mentioned that the cut off for whole genome species separation is 96%. An effort is currently undergoing to sequence *Vibrio* sp. CAIM 1792 strain isolated from diseased shrimp in Mexico, which is believed by the authors will be the first full genome for *V. harveyi* (Espinoza-Valles et al., 2012). At the same time, another effort to sequence the first whole genome sequence of *V. campbellii* is done for strain DS40M4 (Dias et al., 2012). For consistency, this dissertation refers the BB120 strain as *V. harveyi*, because whole genome sequence comparison for BB120, HY01, CAIM 1792, and DS40M4 strains could not be established at this moment.

Based on the full sequence, BB120 (also known as ATCC BAA-1116 strain), has a relatively larger circular DNA genome for both chromosomes (Table 6.2), but lack of integrons, when compared to other vibrios. The BB120 has 3.8 Mbp for chromosome I (GenBank accession number CP000789.1), and 2.2 Mbp for the chromosome II (GenBank accession number CP000790.1).

Table 6.2. Comparison of BB120 genome size to other vibrios

Species	Chromosome I (Mbp)	Chromosome II (Mbp)	References
V. harveyi BB120	3.8	2.2	GenBank accession numbers CP000789.1 and CP000790.1
V. vulnificus	3.2	1.8	Park et al., 2011
V. fischeri	2.9	1.3	Ruby et al., 2005
V. parahaemolyticus	3.3	1.9	Makino et al., 2003
V. cholerae	3.0	1.1	Heidelberg et al., 2000

Irrespective of whether the BB120 strain remains *V. harveyi* after the completion of the CAIM 1792 and DS40M4 strains sequencing, the roles of BB120 and its derivatives in the QS study will not change. What would be interesting is, if *V. harveyi* and its closely related species (*V. campbellii* and *V. parahaemolyticus*) share similar pathogenicity mechanisms. They have HAI-1, CAI-1, and AI-2 systems, but the virulence factors such as the secretion systems might be different. The BB120 strain, which was isolated from the marine environment and is not associated with any disease, has a type III secretion system (Henke and Bassler, 2004a). On the other hand, the shrimp pathogen CAIM 1792 strain has type I, II, III, IV, and VI secretion systems, and more than one hundred genes encode for putative virulence, for example hemolysins, proteases, chitinases, collagenases, iron acquisition, RTX toxins, and vibriolysin (Espinoza-Valles et al., 2012). The DS40M4 strain, similar to BB120, was also isolated from the open sea. The strain has type II, III, IV, and VI secretion systems, and genes related to iron transport, virulence, and environmental fitness, such as those encoding anguibactin and

vanchrobactin biosynthesis proteins, and proteorhodopsin (Dias et al., 2012). But, as BB120 is larger compared to other vibrios, it is interesting to figure out what extra features are present within its genome compared to other vibrio strains.

6.3. Materials and methods

The experimental studies were run in three different stages and used different *V. harveyi* strains summarized in Table 6.3. The strains were received in Luria Marine stabs, provided by Dr. Bonnie L. Bassler, a Howard Hughes Medical Institute Principal Investigator at Princeton University. Upon arrival, the strains were transferred onto Tryptic Soy Agar with 2% NaCl added. For long term storage in -80°C, a full plate of bacteria were then further transferred into Tryptic Soy Broth with 2% salt and 20% glycerol, and distributed in small cryovial tubes.

Table 6.3. *Vibrio harveyi* strains used in the study and relevant information

Strains	UAZ #	Phenotype	References
BB120	1334	Also known as ATCC BAA-1116 strain, wild-type from which other strains were derived.	Bassler et al., 1997
JAF548	1341	LuxO D47E linked to Kan^R, LuxO locked in LCD conformation (QS is repressed).	Freeman and Bassler, 1999
BB721	1342	ATCC 700106, LuxO :: Tn5 linked to Kan^R, LuxO locked in HCD conformation (QS is constitutively expressed).	Bassler et al., 1994b
BH005	1343	LuxPQ-	unpublished strain
BH006	1344	LuxN-	unpublished strain
BH113	1345	LuxS-	unpublished strain
BH114	1346	LuxM-	unpublished strain
JMH598	1347	CqsS-	Henke and Bassler, 2004b
JMH603	1348	CqsA-	Henke and Bassler, 2004b

6.3.1 Pathogenicity of BB120 strain in shrimp

The first experiment was run to define if *V. harveyi* BB120 strain is pathogenic to shrimp. The challenge test was conducted in the University of Arizona Aquaculture Pathology Lab (UAZ-APL). Specific Pathogen Free (SPF) shrimp (1 - 2 gram) were used in the study. Ten shrimp were placed into each of six 90-L tanks, with oyster shell, charcoal, and floss as biofilter media. All tanks were supplied with dechlorinated tap water and air stones to maintain desired dissolved oxygen (DO) levels. Three tanks served as treatments, and three tanks served as respective controls. The three routes of infection in the treatments were: injection, reverse gavage, and feeding. Temperature and salinity were adjusted to 27°C and 25 ppt, respectively. All tanks were fed twice a day with commercial pelleted feed (Rangen 35% protein) at 10% body weight/day.

In each treatment, *V. harveyi* BB120 dose was approximately 1 X 10^6 CFU/shrimp, modified from an infection method for the same strain on *Artemia franciscana* larvae and shrimp by Phuoc et al. (2009). *V. harveyi* BB120 previously stored at -80°C, were aseptically inoculated by streaking on Tryptic Soy Agar (TSA) with 2% salt added. After 18 – 24 hours aerobic incubation at 28°C, one full plate would generate approximately 10^8 - 10^9 CFU. The bacteria were then harvested from plates, and transferred into 5 mL of sterile 2% saline, and diluted to achieve the desired concentration. The concentration was reconfirmed by plating and OD600 readings prior to the challenge study in shrimp (OD600 = 0.3 is approximately 10^8 CFU/ml).

For reverse gavage, a commercial green dye was added to the inoculum to verify the presence of bacteria in shrimp hindgut and midgut. Reverse gavage is a modified gavage, by which an infectious material is introduced using pipette tip through the anal cavity and moves into the shrimp's anterior midgut, hepatopancreas, and gastric region (Aranguren et al., 2010). Injection treatment was done intramusculary in the 3rd abdominal segment using a 1 ml tuberculin syringe containing 50 - 100 µL bacterial solution to achieve 10^6 CFU/shrimp. For the feeding route, the bacteria were diluted in 2% saline, and mixed with the commercial pellet (1 gram pellet : 1 mL of saline). Three controls received 2% saline solution by reverse gavage, injection, and feeding respectively.

Moribund and freshly dead (i.e. no muscle opacity or difference in color from live shrimp was observed) shrimp were collected. Hemolymph (100 µl) was extracted from the ventral sinus using a 25 gauge needle attached to a 1 ml tuberculin syringe, and inserted into the base of the fourth periopod (swimming leg). Hemolymph samples (1 or 2 drops per sample) were immediately streaked on TCBS plates and observed for growth and luminescence after 24-30 hour aerobic incubation at 28°C. Luminescent and small green colonies were transferred on TSA with 2% NaCl, and kept for long term storage and further PCR analysis. The colonies were then Gram-stained, and observed by light microscopy to confirm the presence of Gram-negative rod shaped bacteria, the main characteristic of the original isolate.

After hemolymph extraction, moribound animals were preserved in Davidson's AFA fixative to confirm the infection by histopathology analysis, to verify the disease or health

status (Lightner, 1996). As there were no mortalities in the groups other than in the injection treatment group, representatives from each treatment were preserved for histopathological analysis at day 14. PCR analysis (Fukui and Sawabe, 2007) was also used to confirm the luminescent colonies in the hemolymph from different samples.

As feeding and reverse gavage resulted in no mortality, another experiment was added with a smaller number of shrimp. Considering that autoinducer-2, one of the main components in the QS cascade requires the element boron for its biological functions, an additional experiment was conducted to determine, if the difficulty in establishing a *V. harveyi* infection model through feeding was due to the limitation of boron in the artificial saltwater. Boron is abundant in the ocean, and might not be replicated in a lab setting. The artificial salt used in the experiment contained boron, but no information is provided on the concentration. Four aquaria (three treatments and one control) with five animals each were set-up. Artificial salt was added to reach the desired salinity (25 ppt). The water was also supplemented with boric acid to a final concentration of 100 μM. The first treatment was with bacteria immersion into water to a final concentration of 10^6 CFU/mL. In the second group, the shrimp were fed with the vibrio coated feed. The third treatment used reverse gavage as route of infection. Control group aquarium consisted of five shrimp with no additional treatment. The concentration of *V. harveyi* in feeding and reverse gavage treatments were approximately 10^6 CFU/shrimp.

6.3.2. QS experiment

The first experiment confirmed that BB120 strain was pathogenic for shrimp by injection, and followed by the second experiment to define if QS controls the virulence. The study employed two different mutants, locked at low or high cell density conformation. As a result, both strains do not respond to the presence of autoinducers. The JAF548 strain was a mutant locked at low cell density (LCD), where QS is repressed (Freeman and Bassler, 1999). This strain does not produce light regardless of the cell number. The BB721 strain was a mutant locked at high cell density (HCD) mode, and QS genes are constantly expressed. Regardless of the cell number, this strain produces light (Bassler et al., 1994b). The challenge study protocol was similar to the first experiment, and the wild-type (BB120) and 2% saline served as control. Injection was used as the route of infection.

6.3.3. Defining the most crucial cascade(s) for the virulence of *V. harveyi*

The second experiment was performed to determine if there was a difference in terms of mortality pattern and final mortality rate. The mortality pattern and timing between the wild-type, LCD, and HCD mutants indicated that QS controls the expression of specific genes at specific times. As *V. harveyi* QS consists of three circuits, a third experiment further investigated if specific pathways are the most important determinants. This challenge study used six different mutants: LuxM-, LuxN-, LuxS-, LuxPQ-, CqsA- and CqsS-. Each mutant lacked either the synthase or receptor gene for each autoinducer. The wild-type and 2% saline groups served as control.

6.3.4. Statistical analysis

Statistical analysis was conducted using SPSS 20.0. The survival behavior (pattern) analysis were determined by Kaplan Meier survival analysis followed by overall comparisons and pairwise comparisons using Log Rank (Mantel-Cox), Breslow (Generalised Wilcoxon), and Tarone Ware (Appendix 6A). In the overall comparisons and pairwise comparisons, value of less than 0.05 in alpha (or Sig. as in Appendix 6A Table) shows a significant difference. By having control group in the overall comparisons, it was expected that there would be a significant difference. Therefore, pairwise comparisons were run to confirm the differences between two groups.

In Kaplan Meier survival analysis, mortality events were defined as 'completed' or 'dead', while the survivors were termed as 'censored'. For large samples, Kaplan Meier survival analysis is useful in estimating the overall survival pattern (and indirectly the survival) for the remaining samples when the observation is terminated. For example, in a challenge study of one thousand shrimp with probiotics treatment, the survival pattern can be estimated by observing the performance of the first one hundred shrimp. The remaining nine hundred shrimp are censored, and the estimated pattern will be provided.

As this experiment presented in this chapter used a relatively small number of animals, and it was terminated when it was likely no more mortalities would occur, the Kaplan Meier analysis for the survival/mortality behavior was based on actual events, and not an estimated one.

6.4. Results

6.4.1. Pathogenicity of BB120 strain in shrimp

The final survival after 21 days post challenge were 10% for the injection route, and 90% for both the reverse gavage and feeding routes. Mortalities in the injection tank occured within 36 hours, and in other groups after 15 days. In the negative control, the survival were 90% for the injection, and 100% for both the reverse gavage and feeding groups.

In the reverse gavage and feeding treatments, the bacteria remained in the gut/digestive system. The luminescent material was observed only in the digestive system within 4 hours, and was not spread immediately to the whole body. After 3 – 4 hours, the luminescence could not be seen, and the colonization cannot be observed. This observation was different for the injection group, where bacteria spread immediately through an open circulatory system (Figure 6.2).

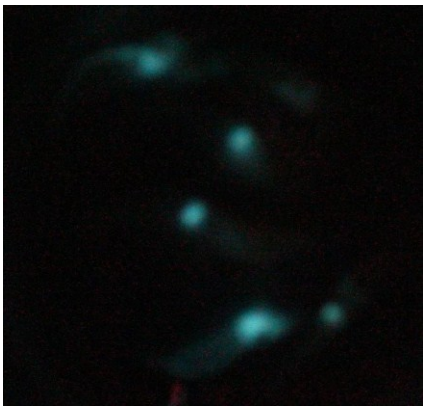

Figure 6.2. Glowing shrimp 4 hours after the injection with *Vibrio harveyi* BB120. The most luminescent regions were the hepatopancreas and the gills

Boric acid experiment

There were no mortalities in all groups, suggesting that boron supplementation had no effect on the experimental infection. This confirmed the nature of *Vibrio harveyi* infection which is considered as a secondary and opportunistic pathogen (Lightner, 1993). To cause an infection, there would be other factors either biotic (for example viruses), or abiotic such as bad water quality prior to vibrio colonization.

One interesting point to note, in the dark condition, shrimp consumed the regular feed faster compared to the glowing feed (vibrio coated feed). When the glowing part subsided from the feed, the shrimp started to consume it faster. This observation was consistent with their cannibalistic behavior. Cannibalism did not begin immediately when the dead shrimp had glowing material in their bodies (most of them in the gills and appendages). When the luminescence disappeared, cannibalism began. From a bacterial point of view, once bacteria destroyed the shrimp's immune system, and eventually the shrimp became sick and died, the bacteria would either escape and find new hosts, or stay in the dead shrimp but possibly turn-off the light production. By turning off the luminescence, other shrimp would not be able to differentiate if the dead ones have harmful material in them, and thus the cannibalistic behavior would begin. Horizontal transmission started because of consuming vibrio infected shrimp.

Microbiology Characterization

Small circular green and luminescent bacterial colonies (approximately 1.0 mm diameter), suggestive of *Vibrio harveyi* were observed on TCBS plates from the

hemolymph samples of the moribound shrimp (Figure 6.3A). The colonies grew well on Tryptic Soy Agar (TSA) and in Tryptic Soy Broth (TSB) with 2% salt added. Gram-staining confirmed the rod shape Gram negative bacteria (Figure 6.3B). Compared to the original isolates, in general, the colonies derived from the hemolymph of the diseased shrimp looked darker, larger, and maintained the luminescent behavior. PCR analysis (data not shown) confirmed the presence of vibrio.

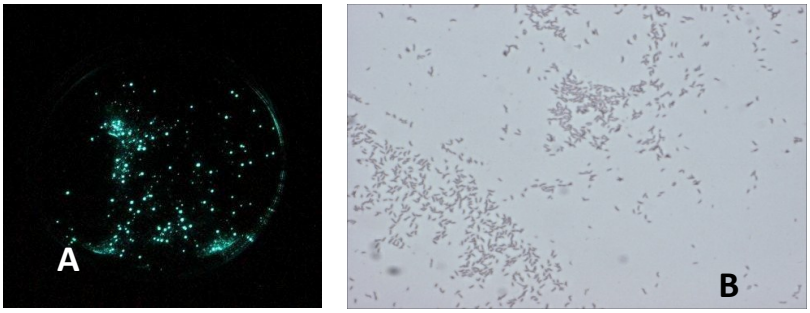

Figure 6.3. Confirmatory tests for *V. harveyi* BB120 presence. (A) Luminescent bacteria on TCBS agar; (B) Small rod shaped Gram negative bacteria based on Gram staining from the hemolymph of infected shrimp.

Compared to other luminescent *Vibrio harveyi* in the UAZ collection, the BB120 isolate grew slower and had the smallest colony size on TCBS and TSA. The luminescence was observed after 6 hours and remained up to 72 hours (3 days). In five other luminescent isolates from the UAZ collection, they grew well and very fast on plates. The luminescent behaviors were optimum between 16 – 20 hours after the inoculations and remained for about 4 – 6 hours, with the exception of UAZ-1112 isolate which had the closest character to BB120 in terms of luminescence. The UAZ-1112 was originally isolated from pond water in Madagascar in 1998. Based on the *gyrB* gene sequencing, the vibrio

phylogenetic tree presented in Chapter 5 showed that BB120, and four other luminescent isolates, were either *V. harveyi* or *V. campbellii*, and the UAZ-1112 seemed to be *V. alginolyticus*.

Histopathology analysis

Samples from the control group showed normal histology in all organs observed. The reverse gavage and feeding groups were considered normal with very low hemocytic infiltration or congestions in the gills, and some low vacuolisations in the hepatopancreas (data not shown).

In the injected group which resulted in high mortality, hemocytic congestion was found in the gills (Figure 6.4A), heart (Figure 6.4B), and antennal gland (Figure 6.4C). When infection occurred, shrimp would form hemocytic nodules in an attempt to confine the bacteria in specific tissues. In the case of severe infection, shrimp were not able to form the nodules as shown in the injection group. Massive amounts of bacteria were found in the hepatopancreas (Figure 6.4D), and occupied the hemolymph in the free space in between heart and hepatopancreas (6.4E), and in between central nerve cord and abdominal muscles (Figure 6.4F).

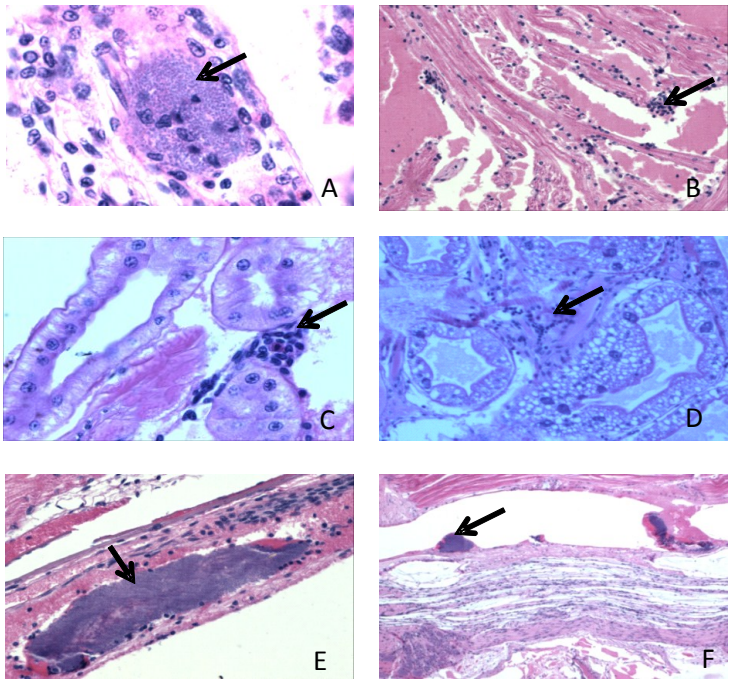

Figure 6.4. Systemic infection in different organs caused by *Vibrio harveyi* BB120. Hemocytic congestion was found in the gills (A), heart (B), and antennal gland (C). Massive amounts of bacteria were found in the hepatopancreas (D), in the free space in between heart and hepatopancreas (E), and in between central nerve cord and abdominal muscles (F). Hematoxylin and Eosin (H&E) stain. Total Magnification (A = 100X; B = 50X; C = 150X, D = 20X; E = 50X, F = 25X).

The findings based on microbiology characterization, histopathology analysis, and PCR confirmatory tests, suggest that *V. harveyi* BB120 is pathogenic for shrimp during experimental infection, by injection of 10^6 CFU/shrimp, with 90% mortality within 36 hours.

6.4.2. QS experiment

The final survival after 7 days post challenge were 20% for the wild-type, 25% for LCD group, and 10% for HCD group summarized in Figure 6.5, generated from SPSS 20.0 Kaplan Meier survival analysis. Figure 6.6 described the duration of mortalities in three different groups. The mortalities in the LCD group occurred between 6 – 32 hours post infection (h.p.i), in the wild-type from 8 – 40 h.p.i, and for the HCD from 12 – 26 h.p.i. In the negative control, the survival was 100%. For virulence, QS is about dictating the time, at which genes are optimally expressed. Particular genes turn-on and off, but some genes are up regulated and down regulated overtime through QS control.

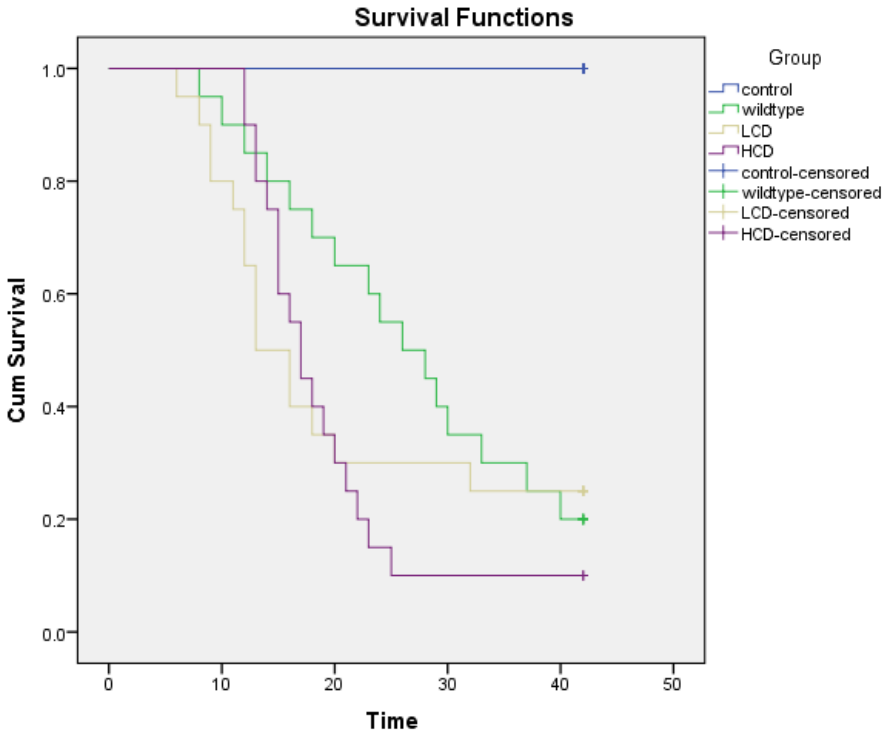

Figure 6.5. Mortality behavior of shrimp exposed to different *V. harveyi* mutants.

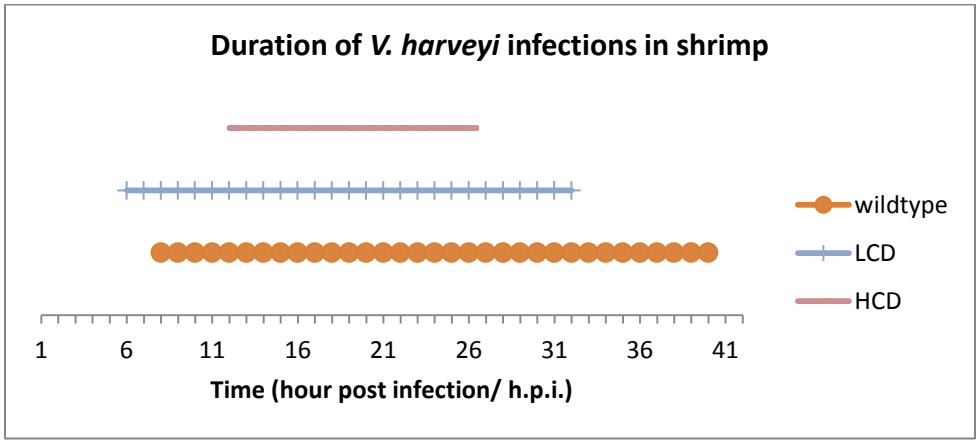

Figure 6.6. Timing of shrimp mortalities in different groups challenged by injection.

Histopathology analysis

Histopathology analysis for the vibrio infections focused on five main organs, which were the lymphoid organ, gills, hepatopancreas, antennal gland, and heart (Figures 6.7A to 6.7F). In these organs, the hemolymph is continuously circulated, and therefore those are excellent sites for the bacteria to infect their hosts. When there was a bacterial infection, the shrimp's immune system responds by forming a nodule to localize the infection, which was seen in the gills, antennal gland, hearts, and lymphoid organ. Depending on the severity of the infection, if the shrimp was too overwhelmed, the shrimp could not form the nodule as the bacteria would have already destroyed the shrimp's immune system. The histopathology also displayed edema and spheroids in the lymphoid organ of the wild-type and LCD mutants. In the hepatopancreas, the sick shrimp displayed low vacuolization, B-cell dominance, and the absence of, or limited R-cells. The more R-cells found in the hepatopancreas, the healthier the shrimp (Lightner, 1996).

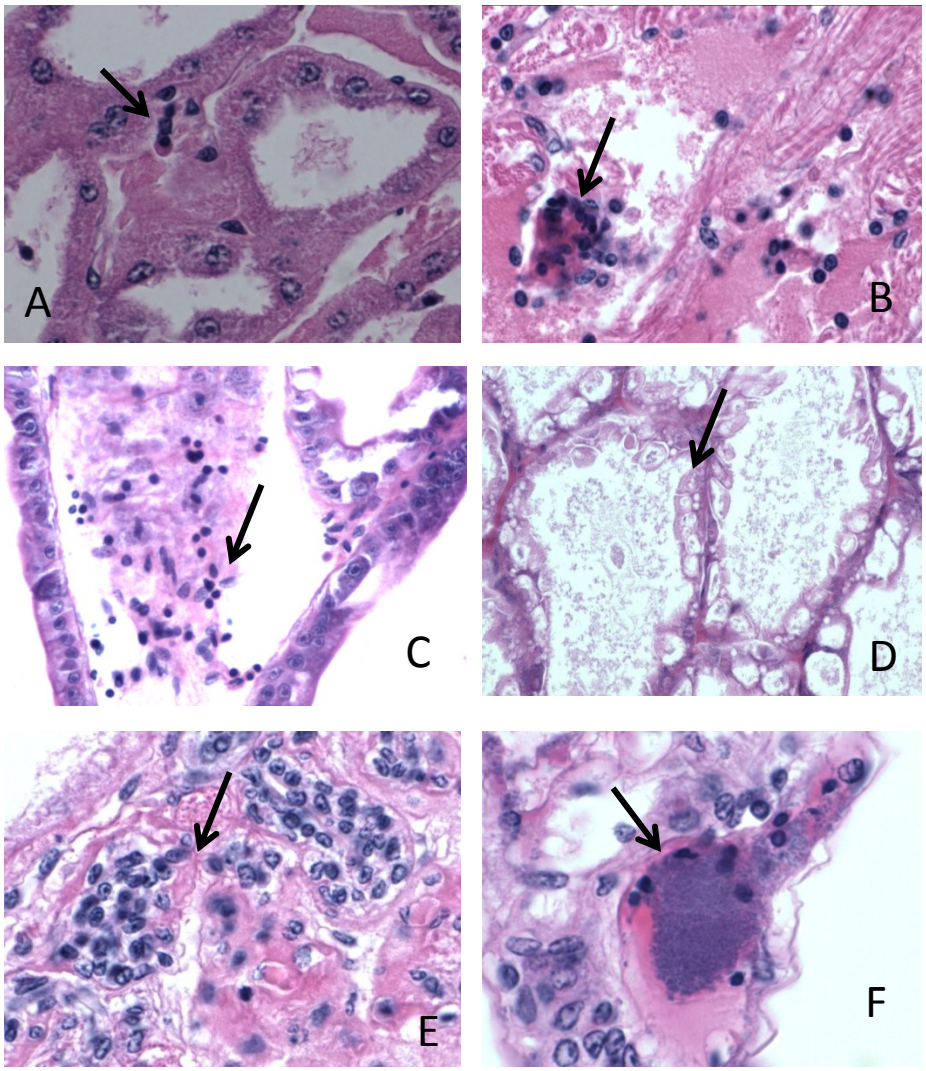

Figure 6.7. Histopathological changes after *V. harveyi* BB120 introduction.

Antennal gland (A), heart (B), hepatopancreas (C) with low vaculolization (D), edema and spheroid in lymphoid organ (E), and nodules in gills (F). Hematoxylin Eosin (H&E) stain. Total magnification = 100X.

6.4.3. Defining the most crucial cascade(s) for the virulence of *V. harveyi*

Mortality data showed that the wild-type and CqsA- (CAI-1 system) caused the highest mortality (80%) followed by LuxM- and LuxN- (HAI-1 system) with 70%, LuxS- and LuxPQ- (AI-2 system) with 40-50%, and CqsS- (CAI-1 system) with 20%.

As synthase mutants (LuxM-, LuxS-, and CqsA-) would lock the bacteria in LCD mode, and the sensor mutants (LuxN-, LuxPQ-, and CsqS-) are similar to HCD mode, the mortality pattern was consistent with the LCD-HCD experiment. The synthase mutants had immediate virulence compared to the sensor mutants, even though the final mortalities were similar to the receptor mutants (with the exception in CqsS- group).

While transferring from agar to broth for long term storage, the CqsS- colonies attached better on agar surface, while other strains were easier to be picked up with microbial loop. CqsS- expressed light at the latest compared to the other strains, while the CqsA- had the brightest luminescence at the beginning.

Microbiology and PCR analysis confirmed the presence of *V. harveyi* in the hemolymph of dead shrimp (data not shown). Histopathology analysis was similar to the findings presented in Figures 6.7A to 6.7F, with additional finding on vibrio colonization in the hindgut, suggesting close relationship between vibrio and chitin acquisition (Figures 6.8A to 6.8D). Chitin is the component of shrimp cuticle and cuticle epithelium which lined the stomach and hindgut (Bell and Lightner, 1988).

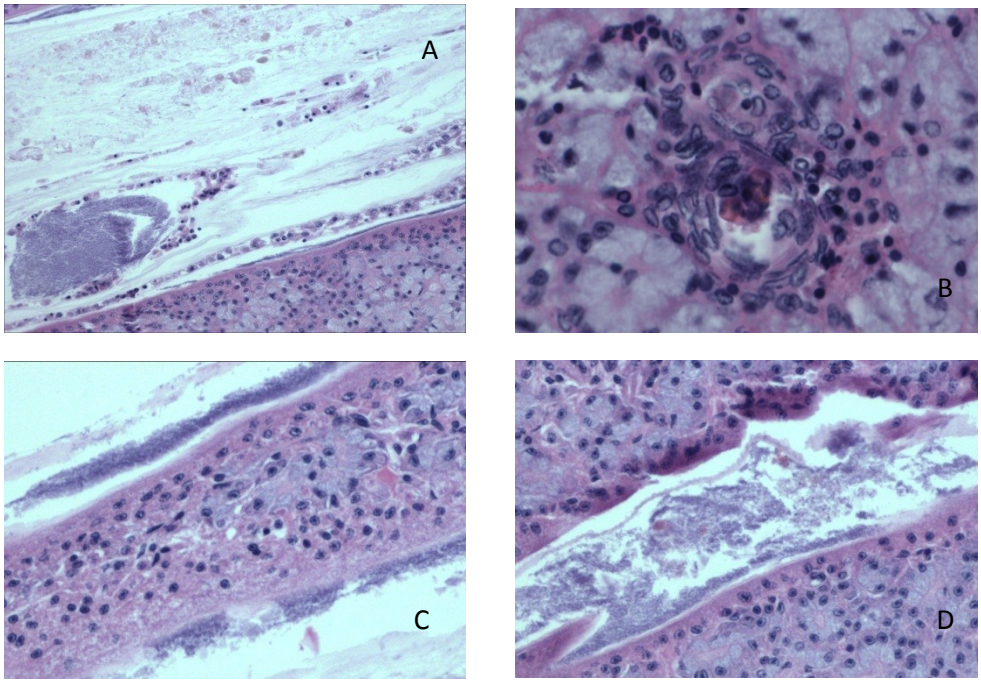

Figure 6.8. Vibrio colonization in the hindgut lumen (A), hemocytic nodules (B), forming plaques on to the epithelium lined with chitin (C and D). Hematoxylin Eosin (H&E) stain. Total magnification (A, C, D =50X, B = 100X).

6.5. Discussion

In the first experiment, shrimp in the control group showed high survival, suggesting that the three routes of infection through injection, reverse gavage, and feeding can be used for experimental purposes. Intramuscular injection had the disadvantage that this route bypasses the shrimp's primary defense mechanisms such as the cuticle, cuticular epithelium, and midgut mucosa where resistance factors might be located (Smith, 1991). With reverse gavage, shrimp received the infectious material via the anal cavity, which is opposite from the natural digestive system. The green-dye showed that the bacteria were introduced into the midgut and the hepatopancreas.

The high survival in the control injected group, and very low survival in the vibrio injected group suggest that both stressful conditions and the presence of pathogens are needed to cause vibrio infections. It is difficult to conclude which route would cause more stress for the shrimp, the injection or the reverse gavage. Bacterial injection might cause immediate shock in shrimp, when the dose was too high or mis-handling of the inoculating needle. The same did not happen in the reverse gavage group. However, even though each shrimp received approximately the same amount of pathogens (10^6 CFU) in the three treatments, only the injection route caused high mortalities. Observation within 6 hours after the infection of the bacteria showed that in the injected group, the bacteria spread very rapidly through the open circulatory system. This confirmed the systemic infection caused by vibrio which affected different organs. The luminescence was seen clearly in the gills, the hepatopancreas, and the muscles. In the hemolymph of the shrimp,

the bacteria might find favorable conditions or nutrients to reproduce, and to cause infections when their numbers are high enough.

The histopathology analysis showed that massive amounts of bacteria were located in the hemolymph in the free space between the heart and the hepatopancreas, in the hepatopancreas tubules, and in other free spaces where hemolymph is located. The bacteria might also produce toxins because the shrimp were dead within 36 hours, and 80% had already died between 12 – 22 hours. The dead shrimp showed white areas at the site of injection within 24 hours possibly indicating a toxin response (Smith, 1991). In contrast, some survivors displayed dark colorization (melanization) at the site of injection, an indication of immune response.

In the LCD-HCD experiments, the terms "low cell density (LCD)" and "high cell density (HCD)" mutants do not refer to cell numbers, but rather to their constitutive QS states. They have different mutations in LuxO, a negative regulator of QS, and thus, the LCD and HCD mutants do not respond to the presence of autoinducers. Compared to the wild-type, the growth of both mutants was not affected. The LCD mutants act as individuals, and no group behavior is initiated regardless of cell number. These mutants do not produce light even though they have high cell numbers. In contrast, the HCD mutants always behave as if they are in proximity to other bacteria, and they express luminescence constitutively. The wild-type produces light when they reach at least 5 X 10^4 CFU/ml so they can switch between the LCD and HCD states.

Using 10^6 CFU/shrimp for injection, the final survival of shrimp infected with the wild-type and both mutants were in the range of 10 – 25%. Based solely on the survival, QS seems to have no role in pathogenicity. If QS was the key for virulence, one would expect that the LCD mutant and the HCD mutant would cause different mortality rates, for example low mortality in the LCD, and high rate in the HCD modes. But, the mortality timing/patterns gave a different clue, that QS was actually affecting the timing of the outcome of the challenge experiment. Kaplan Meier analysis confirmed that the survival or mortality behavior of the HCD group was significantly different compared to the wild-type (Appendix 6A).

QS is about two things: cell number and timing of expression of specific genes. Figure 6.5 described the mortality events, and showed that the timing (Figure 6.6) was skewed in the different groups. Compared to the wild-type, the LCD mutants displayed immediate infection, while the HCD mutants had delayed virulence. In terms of final survival, the short infection duration in HCD caused highest mortality rates compared to the wild-type and LCD groups (Figure 6.5). As all mortalities happened within 40 hours, the time difference might not seem obvious. An improved experimental infection for vibrio with longer duration is needed, for example by lowering the dose, or by modifying the routes of infection.

In the LCD group which does not express light, the LuxN, LuxPQ, and CqsS sensors act as kinases. The sensors autophosphorylate on conserved histidine residues, and transfer the phosphoryl group to the conserved aspartate residues in their attached response

regulator domains. Phosphorylation of LuxO activates the protein to cause repression of LuxR, and eventually the LuxCDABEGH operon. Therefore, at low cell density, the bacteria make no light (Mok et al., 2003).

The immediate infection in the LCD mutants was a result of constitutive virulence caused by the presence of activated LuxO D47E variant in the JAF548 strain (Freeman and Bassler, 1999). The mutation activates phospho-LuxO, and increases siderophore production in the presence of σ^{54} factor (Lilley and Bassler, 2000). At low cell density, redundant small non-coding RNAs (sRNAs) activate production of AphA, a transcription factor, a master regulator of QS. Both sRNAs and AphA repress production of LuxR (Lenz et al., 2004; Tu and Bassler, 2007; Rutherford et al., 2011). The JAF548 strain causes a massive attack, by having its virulence factors on all the time. In this situation, the LCD caused a shortened virulence scheme (Figure 6.6).

In contrast, at high cell density, the LuxN, LuxPQ, and CqsS switch from being kinases to phosphatases, and drain phosphate out of the QS system. These activities result in rapid elimination of LuxO-phosphate, and the dephosphorylated form of LuxO is inactive. Therefore, at high cell density, LuxR and LuxCDABEGH are not repressed, and the bacteria emit light (Ng and Bassler, 2009).

The HCD mutant strain used in the experiment was the BB721, and is a null mutant for LuxO because it harbors a Tn*5* insertion in the *luxO* gene (Bassler et al., 1994b). This HCD strain experienced delayed virulence because it cannot turn on LCD virulence

genes. Based on the mortality pattern of the LCD group, the virulence genes at low cell density are obviously potent. The results show that the HCD strain maintained virulence, but expressed it at a different time compared to the LCD strain. In HCD mode, LuxR is the master regulator for gene expression that represses AphA (Rutherford et al., 2011).

The findings from the LCD and HCD mutants indicated that QS regulates graded virulence in *V. harveyi,* which contrasts to *V. cholerae,* which expresses virulence only at low cell density (Zhu et al., 2002). QS is about dictating the time at which genes are optimally expressed. Particular genes turn on and off, but some, such as perhaps virulence genes, are up or down-regulated, but not all the way on or off over time through QS control. AphA and LuxR reciprocally control QS in *V. harveyi*. At low cell density, production of AphA is maximal, and at high cell density, LuxR is maximal (Rutherford et al., 2011).

During the experiment, it was also noticed that the bacteria had a kind of memory for either LCD or HCD mode. By diluting the bacteria immediately after the luminescence disappeared, the first thing the bacteria did once they entered the shrimp bodies was to produce light. This observation suggests that HCD mode is preferable when the *V. harveyi* is inside the hosts, as the condition is more favorable, compared to when they live in a bigger space in the water column. Inside the hosts, luminous bacteria can grow in a confined, nutrition-rich environment, in which the autoinducer can be better accumulated compared to situation in the experimental tanks.

The final experiment suggested that CAI-1 circuit is the most crucial for virulence, followed by AI-2 and HAI-1. The CqsA- mutant showed similar behavior to the wild-type. This was due to the mutant possessing the other two systems, and it possibly used CAI-1 or CAI-1-like molecules from other vibrio in the water, which were recognized by CqsS receptor. This is consistent with the findings from Chapter 5, in that, the most dominant bacteria associated with shrimp culture water are vibrios. Other than vibrio in the water, Sinderman (1990) mentioned that vibrio species are part of the natural microflora of wild and cultured shrimp.

As most vibrios have CAI-1 or CAI-1 like activity (Ng and Bassler, 2009), the CqsA-mutant can utilize CAI-1 produced by other bacteria via CqsS to activate the QS system, and therefore resulted in high mortality similar to the wildtype (80%). On the other hand, the CqsS- mutant resulted in low mortality (20%), because even though the CAI-1 molecule is available in the water, the molecule has no function in the absence of the CqsS receptor.

To the best of the authors' knowledge, this is the first report that the CqsS gene in the CAI-1 cascade is the most important determinant for the virulence of *V. harveyi* in shrimp, which is the same crucial pathway for *V. cholerae* infection (Ng and Bassler, 2009). This finding is different from the *in vitro* result that showed that HAI-1 is the most crucial pathway for *V. harveyi* virulence (Henke and Bassler, 2004b). The difference is not unexpected as *in vitro* studies did not account for the host's innate immune response, while the challenge study considered host-microbe interactions.

The findings also indicate that vibriosis, caused by different species of vibrio in different hosts, is likely accomplished at the genus level, by the ability of vibrio to recognize CAI-1 or CAI-1 activity secreted by other vibrios. This result has a significant relevance in the study of vibriosis in shrimp, as closely related pathogenic species (*V. campbellii* and *V. parahaemolyticus*) share the same pathogenicity pathways (HAI-1, CAI-1, and AI-2) (Henke and Bassler, 2004a; Defoirdt et al., 2008).

An additional observation, based on the histopathology analysis, revealed that the bacteria colonized the hindgut lumen (Figures 6.8A – 6.8D). The clinical signs showed whitish hindgut, an indication of inflammation. Johnson et al. (2008) compared the bacterial community in the foregut, midgut and hidgut of shrimp, and found that *Vibrio* spp. colonized the hindgut as this area is lined with chitin. Vibrios have the unique ability to survive on chitin, and their association with chitin may be a key to the evolution of why they colonize shrimp, which have chitin in different areas such as the cuticle and cuticle epithelium in the stomach and hindgut (Bell and Lightner, 1988). Huq et al. (1986) demonstrated that *V. cholerae* attach exclusively to crab hindguts, which are lined with chitin, as opposed to the crab midguts which are endodermal and not lined with chitin. C. Pantoja (pers. comm) mentioned that by histopathology analysis, natural infection of vibrio in shrimp showed degradation of cuticle by chitinolytic enzymes, which also confirmed the close relationship between chitin and vibrio colonization.

Other than chitin acquisition, luminescence seems to be related to virulence, either directly or indirectly. Light expression takes up about 20% of the energy, which may

affect bacterial growth (Chen, 2002). When bacteria sacrifice or compromise their growth and energy for luminescence, there must be something beneficial for the bacteria in return. During the experiment, it was noted that gills and legs/abdomen which had direct exposure to oxygen in the water were the brightest parts in shrimp. It seems that glowing in the dark may be one of the *Vibrio harveyi* strategies to compete with other organisms. By taking up high amount of oxygen from the environment to produce light, it would possibly limit the availability of oxygen for others.

Dissolved oxygen availability is dependent on respiration and photosynthetic activities, and therefore is minimum in the morning (around 6 A.M.), increases gradually during the day when photosynthesis produces oxygen, and reaches a peak in the afternoon (around 6 P.M.). The time frame between 6 P.M. to 6 A.M. is when the oxygen level gradually decreases. For shrimp, which are physiologically active both during the day and at night, oxygen limitation would be a significant stress factor as oxygen availability is already minimal, compared to day time when oxygen is more available by photosynthesis activity. For luminescent *V. harveyi*, the simplistic immune system in shrimp, the presence of chitin, which is the main carbon source for vibrios, and the oxygen limitation in the water, are favorable factors, which might contribute to their preferences to infect shrimp.

Understanding luminescence behavior in *V. harveyi* is important, as the bacteria express toxin and luminescence only when QS is achieved, and luminescence is easily observed compared to toxin production. Hammer and Bassler (2008) mentioned that as the *V.*

harveyi cells grow and reach a critical cell density, they deplete the available iron, oxygen, and carbohydrate and under these conditions, in the presence of toxin and DNA damaging agents. In nature, several of these conditions are met simultaneously.

There is no report available if *V. harveyi* express luminescence during the day in natural environments, for example in shrimp ponds. But, luminescence behavior which is well studied in dinoflagellate might give a clue. The luciferase gene responsible for luminescence in the dinoflagellates is transcribed only once a day at night in a circadian rhythm fashion (Bae and Hastings, 1994; Mittag et al., 1994). Cell division occurred about the same time as luminescence during night phase, and both are optimum early in the morning before sunrise. During day phase, photosynthesis is the main activity in dinoflagellate. However, as photosynthesis is not part of *V. harveyi* activities, it would be interesting to figure out if circadian rhythm exists and if it controls luminescence.

In a lab setting, luminescent *V. harveyi* expressed light when QS was achieved, both during the day and night, by introducing at least 10^4 CFU/mL of bacteria into aquarium. *V. harveyi* maintained the luminescence up to 4 hours in a room without light, and up to 2 hours in a room with light. When the bacteria were inside shrimp bodies, they maintained the luminescence up to 6 hours. On the other hand, bacteria grew one log higher in tryptic soy broth under continuous agitation and light, compared to when the same one was done without light.

A direct interview with Prapto Subroto from Shrimp Club Indonesia, who observed the luminescent vibriosis in shrimp ponds since early 1990's, provided information that when the pond is glowing, either by vibrios or dinoflagellates, shrimp do not consume the feed given at night, and may be an indication of stress. TCBS plating for vibrio count is one of the routine practices in the field to monitor water quality. Yellow colony dominance is an indication of good water quality, green colony is an alarm, and green and luminescent colonies of more than 10^3 CFU/ml is an indication of pathogen presence. As shrimp become stressed, they gather in the pond edge, and become more susceptible to diseases, either by vibrios or viral diseases. High counts of green colony vibrios are common prior to viral infection. Therefore, minimizing vibrio infection might also be useful from a practical point view to indirectly prevent viral infection. When viruses were not present, and vibrio become the only disease agent found in the water, after several mortalities during the first week, high mortalities occurred 7 days after high luminescent green colonies were observed on TCBS plate.

6.6. Conclusions

The CqsS gene in the CAI-1 system of *Vibrio harveyi* QS is the most dominant QS gene for virulence. As the CAI-1 system is widely distributed in different species of vibrios, the finding indicates that vibriosis in different hosts might be accomplished at genus level, and not at species level. It is understandable that each species will maintain fidelity and preference for specific CAI-1 structure as part of their evolution (Ng et al., 2011).

The mortality data from the LCD group, and the bacterial colonization in the hindgut from the histopathology analysis, indicated a close relationship of vibrio infection with siderophore production and chitin acquisition, in which both are expressed at low cell density and regulated by QS. On the other hand, based on the mortality data which mostly occurred within 24 hours, the later stage of *V. harveyi* infection is likely involved toxin production, and the toxin is what kills shrimp.

Other than chitin acquisition which is general in most vibrios, luminescence behavior in pathogenic *Vibrio harveyi* and related species might be another key factor in vibrio-shrimp relationship. Shrimp have a simple immune system, and they require oxygen to support their physiological activities. In response to those two features of shrimp, Vibrio have the strategy to release virulence factors, and take up high amount of oxygen to emit light, so that the oxygen availability for shrimp and other organisms become limited. Survival of the brightest, a smart strategy from a luminescent vibrio point of view. They search for chitin and oxygen to survive, and unintentionally, they kill shrimp: A happy aquatic world for vibrio, an unfair one for the shrimp. Indeed, life offers symbiotic relationships (as in *V. fischeri* and the Hawaiian bobtail squid), as well as parasitic ones. In the story of luminescent *Vibrio harveyi* and shrimp, the first party takes advantage of the second. ‘All that glitters is not gold’.

6.7. Future Directions

As potent CAI-1-type molecules that agonize QS are already available (Ng et al., 2012; Wei et al., 2012), it would be interesting to test if the molecules would block vibrio infection in shrimp. Prior to that, it would be wise to reconfirm the roles of CqsS gene by testing the double mutants lacking two of the systems. Another confirmatory test would be difficult to establish, in which sterile aquaria are used, so there are no CAI-1 or CAI-1 activities secreted by other bacteria, or possibly from shrimp feed in the system. Another effort would be to develop gnotobiotic shrimp, that is even more difficult. Gnotobiotic organisms are free of bacteria or contaminants, or into which a known microorganism or contaminant has been introduced for research purposes. The use of gnotobiotic aquatic animals is an excellent tool to extend the understanding of the mechanisms involved in host–microbe interactions, and to evaluate new treatments of disease control (reviewed in Marques et al., 2006 and Nayak, 2010), and have been demonstrated in rotifer (Tinh et al., 2006) and Artemia (Ruwandeepika et al., 2010).

Other than testing available synthetic molecules, screening natural anti-QS molecules would be another important key for disease management in aquaculture. In the natural environment, beneficial bacteria or microalgae work together with the host immune system to overcome pathogens. For example, *Bacillus* spp. produces lactonase enzyme AiiA, which inhibits acyl homoserine lactone in the HAI-1 circuit of the *Vibrio harveyi* QS (Bassler and Losick, 2006). In fact, most shrimp farmers around the world have added *Bacillus* spp. or *Lactobacillus* spp. as probiotics to minimize disease risk, without knowing the scientific mechanisms behind it.

Researchers around the world are searching for potential natural anti-QS molecules. The main concern would be resistance issues similar to those from antibiotic use. Anti-QS might lead to some kind of resistance for bacteria which occur due to evolution. Another concern would be, how bacteria would adjust themselves in hosts, because different from antibiotics, anti-QS would not kill the bacteria, and only inhibit their chemical conversations. From host point of view, the inability to recognize and to clear the pathogens happens when the infection is so massive and overwhelms immune system. By cutting the communication routes between bacteria, anti-QS would give longer time for host immune system to recognize the infection, and to respond, hand in hand with the beneficial bacteria.

6.8. Acknowledgement

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6.9. Appendix 6A

Kaplan-Meier Analysis for LCD-HCD experiments

Case Processing Summary

Group	Total N	N of Events	Censored	
			N	Percent
control	20	0	20	100.0%
wildtype	20	16	4	20.0%
LCD	20	15	5	25.0%
HCD	20	18	2	10.0%
Overall	80	49	31	38.8%

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
	1	42.000	censored	.	.	0	19
	2	42.000	censored	.	.	0	18
	3	42.000	censored	.	.	0	17
	4	42.000	censored	.	.	0	16
	5	42.000	censored	.	.	0	15
	6	42.000	censored	.	.	0	14
	7	42.000	censored	.	.	0	13
Control	8	42.000	censored	.	.	0	12
	9	42.000	censored	.	.	0	11
	10	42.000	censored	.	.	0	10
	11	42.000	censored	.	.	0	9
	12	42.000	censored	.	.	0	8
	13	42.000	censored	.	.	0	7
	14	42.000	censored	.	.	0	6
	15	42.000	censored	.	.	0	5

	16	42.000	censored	.	.	0	4
	17	42.000	censored	.	.	0	3
	18	42.000	censored	.	.	0	2
	19	42.000	censored	.	.	0	1
	1	8.000	dead	.950	.049	1	19
	2	10.000	dead	.900	.067	2	18
	3	12.000	dead	.850	.080	3	17
	4	14.000	dead	.800	.089	4	16
	5	16.000	dead	.750	.097	5	15
	6	18.000	dead	.700	.102	6	14
	7	20.000	dead	.650	.107	7	13
	8	23.000	dead	.600	.110	8	12
	9	24.000	dead	.550	.111	9	11
	10	26.000	dead	.500	.112	10	10
Wildtype	11	28.000	dead	.450	.111	11	9
	12	29.000	dead	.400	.110	12	8
	13	30.000	dead	.350	.107	13	7
	14	33.000	dead	.300	.102	14	6
	15	37.000	dead	.250	.097	15	5
	16	40.000	dead	.200	.089	16	4
	17	42.000	censored	.	.	16	3
	18	42.000	censored	.	.	16	2
	19	42.000	censored	.	.	16	1
	20	42.000	censored	.	.	16	0
	1	6.000	dead	.950	.049	1	19
	2	8.000	dead	.900	.067	2	18
	3	9.000	dead	.	.	3	17
	4	9.000	dead	.800	.089	4	16
	5	11.000	dead	.750	.097	5	15
	6	12.000	dead	.	.	6	14
LCD	7	12.000	dead	.650	.107	7	13
	8	13.000	dead	.	.	8	12
	9	13.000	dead	.	.	9	11
	10	13.000	dead	.500	.112	10	10
	11	16.000	dead	.	.	11	9
	12	16.000	dead	.400	.110	12	8
	13	18.000	dead	.350	.107	13	7

	14	20.000	dead	.300	.102	14	6
	15	32.000	dead	.250	.097	15	5
	16	42.000	censored	.	.	15	4
	17	42.000	censored	.	.	15	3
	18	42.000	censored	.	.	15	2
	19	42.000	censored	.	.	15	1
	20	42.000	censored	.	.	15	0
HCD	1	12.000	dead	.	.	1	19
	2	12.000	dead	.900	.067	2	18
	3	13.000	dead	.	.	3	17
	4	13.000	dead	.800	.089	4	16
	5	14.000	dead	.750	.097	5	15
	6	15.000	dead	.	.	6	14
	7	15.000	dead	.	.	7	13
	8	15.000	dead	.600	.110	8	12
	9	16.000	dead	.550	.111	9	11
	10	17.000	dead	.	.	10	10
	11	17.000	dead	.450	.111	11	9
	12	18.000	dead	.400	.110	12	8
	13	19.000	dead	.350	.107	13	7
	14	20.000	dead	.300	.102	14	6
	15	21.000	dead	.250	.097	15	5
	16	22.000	dead	.200	.089	16	4
	17	23.000	dead	.150	.080	17	3
	18	25.000	dead	.100	.067	18	2
	19	42.000	censored	.	.	18	1
	20	42.000	censored	.	.	18	0

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	35.913	3	.000
Breslow (Generalized Wilcoxon)	32.088	3	.000
Tarone-Ware	34.195	3	.000

Test of equality of survival distributions for the different levels of Group.

Pairwise Comparisons

	Group	control		wildtype		LCD		HCD	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	control			28.039	.000	24.703	.000	35.861	.000
	wildtype	28.039	.000			.574	.449	4.291	.038
	LCD	24.703	.000	.574	.449			.027	.870
	HCD	35.861	.000	4.291	.038	.027	.870		
Breslow (Generalized Wilcoxon)	control			25.600	.000	22.957	.000	31.211	.000
	wildtype	25.600	.000			2.490	.115	4.221	.040
	LCD	22.957	.000	2.490	.115			.860	.354
	HCD	31.211	.000	4.221	.040	.860	.354		
Tarone-Ware	control			26.897	.000	23.892	.000	33.605	.000
	wildtype	26.897	.000			1.536	.215	4.671	.031
	LCD	23.892	.000	1.536	.215			.190	.663
	HCD	33.605	.000	4.671	.031	.190	.663		

CHAPTER 7

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Polyculture system, integration of tilapia and shrimp in the same water bodies has gained attention in the last decade. The system is not only beneficial for shrimp health, but also provides opportunities for a better economic return. Across most shrimp farming regions, tilapia are increasingly being produced in cages or hapa nets inside shrimp ponds, or are produced in supply channels or head ponds. The increasing interest in integrated multi-trophic aquaculture systems for tropical production is certain to further contribute to overall tilapia production as most systems consider tilapia to be a key component to the systems. Indonesia is an example, how tilapia production has increased rapidly in the last decade, to meet the demands both in domestic and international markets.

Based on experimental studies with insights into growth performance, shrimp-tilapia polyculture is technically feasible, both in low salinity and brackishwater. Overall, polyculture resulted in better growth performance by increasing the survival of the shrimp when compared to monoculture. The green water in tilapia culture and polyculture, is a nutrient-rich environment compared to the clearer water in shrimp monoculture. The presence of a higher number and diversity of bacteria, a relatively lower pH, and the *Chlorella* dominance, all together might play synergestic roles in the polyculture system to improve water quality and fitness of the animals. Shrimp line and tilapia strain selection is critical for survival in the system, and the performance will largely depend on animal densities and pond management.

Shrimp-tilapia polyculture seems promising not only by improving the fitness and growth of both species, but also for disease management. The lower risk of luminescent vibriosis compared to what often occurs in shrimp monoculture is a result of the higher diversity of bacteria associated within the polyculture system, the presence of green microalgae, and the antimicrobial properties which can be secreted by tilapia, microalgae, or bacteria. Compared to shrimp culture water which is dominated by Gram negative marine vibrios, polyculture systems had a mixture of Gram negative bacteria which are closely related to *V. cholerae*, and Gram positive bacteria with the highest homology to *Bacillus* sp., which is known to produce enzymes that inhibit vibrio virulence. While offering advantages, polyculture system might lead to natural disease transmission from shrimp to tilapia, or *vice versa*. The relatively low susceptibility of shrimp to *Streptococcus iniae* and *S. agalactiae*, which are pathogenic to tilapia, indicating that direct transmission from tilapia to shrimp, if it is going to happen, might require intermediate hosts or other reservoirs, for example higher crustaceans, other arthropods, or mollusk. To minimize the disease risk, maintaining appropriate densities for shrimp and tilapia is crucial, as most bacterial diseases occur at high density culture.

In terms of *Vibrio harveyi* virulence, the CqsS gene in the CAI-1 system is the most important determinant. As the CAI-1 system is widely distributed in different species of vibrios, the finding indicates that vibriosis in different hosts might be accomplished at genus level, and not at species level. As potential anti CAI-1 (and also anti HAI-1) circuits are already available, it is advisable to test the molecules in shrimp.

The mortality data, and the bacterial colonization in the hindgut from the histopathology analysis, indicated a close relationship of vibrio infection with siderophore production and chitin acquisition, in which both are expressed at low cell density and regulated by QS. On the other hand, based on the mortality data which mostly occurred within 24 hours, the later stage of *V. harveyi* infection is likely involved toxin production, and the toxin is what kills shrimp. Luminescence behavior in pathogenic *Vibrio harveyi* and related species might be another key factor in vibrio-shrimp relationship. Shrimp have a simple immune system, and they require oxygen to support their physiological activities. In response to those two features of shrimp, luminescent vibrio have the strategy to release virulence factors, and to scavenge oxygen to emit light, so that the oxygen availability for shrimp and other organisms become limited.

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