The relation between farming practices, ecosystem, and White Spot Syndrome Virus (WSSV) disease outbreaks in *Penaeus monodon* farms in the Philippines

Eleonor V. Alapide-Tendencia

THE RELATION BETWEEN FARMING PRACTICES, ECOSYSTEM, AND WHITE SPOT SYNDROME VIRUS (WSSV) DISEASE OUTBREAKS IN *PENAEUS MONODON* FARMS IN THE PHILIPPINES

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ABSTRACT

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The white spot syndrome virus (WSSV) affecting shrimp aquaculture in most producing countries has caused huge economic losses resulting in bankruptcy to both large and small farmers. Studies done on WSSV epidemiology were mostly tank-based and on species other than *Penaeus monodon*. There is a need to investigate WSSV epidemiology in *P. monodon* in on-farm situations, thus including both risk and protective factors. This thesis aimed to generate knowledge that can improve prevention against WSSV in shrimp culture through better farm husbandry by studying the epidemiology of WSSV in on-farm situations. To achieve this goal data from cross-sectional and case studies were analysed to identify on-farm WSSV risk and protective factors, and longitudinal studies were done to assess factors affecting water quality and causing WSSV infection to result in an outbreak.

The thesis identified the following WSSV risk factors related to the physico-chemical parameters of the water: low and fluctuating temperature, low and fluctuating salinity, and pH fluctuation. The risk of high temperature and high salinity for an outbreak of WSV disease may be related to fluctuations in these two parameters. Risk factors related to farm husbandry techniques were feeding with molluscs, sludge removal and its deposition on the dike, sharing water source with other farms and having the same receiving and intake water. Identified WSSV protective factors were high mangrove to pond area ratio, feeding with natural food or phytoplankton, and higher percentage of beneficial bacteria like the yellow colonies that grow on thiosulphate citrate bilesalt sucrose agar, a *Vibrio* selective medium.

Results of the longitudinal studies demonstrated that WSSV infection may not result in outbreaks in greenwater pond and in ponds with mangroves in the receiving environment. Our results did not provide explanations why the WSSV infection did not result in an outbreak in farms with mangroves in the receiving environment. In greenwater ponds, this was attributed to the better water and soil quality, higher plankton count, and higher heterotrophic bacterial count.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. BACKGROUND

Diseases have affected the shrimp industry worldwide (Wongteerasupaya et al. 1995). Industry estimates suggest annual losses of about 4 billion USD in Asia alone. Most of these pandemic diseases have a viral origin and the most important one is the white spot disease, caused by White Spot Syndrome Virus, WSSV (Flegel 2009). WSSV continues to plague the industry for more than 2 decades without proven control measures (Lightner, 2011; Flegel, 2006; Stentiford and Lightner, 2011). WSSV has been observed in different stages of shrimp, from post-larvae to broodstock with higher susceptibility associated with older stages (Lo et al., 1996). High survivals and large yields were observed in ponds stocked with low-prevalence and WSSV-negative larvae compared to those stocked with high prevalence (Peng et al., 2001). Corsin et al. (2001), however, found no correlation between stocking of WSSV positive fry and outbreak of the disease. Disease outbreaks do not occur if the shrimp contain low-intensity viral infections under low-stress culture conditions (Tsai et al., 1999).

A paper by Belak et al. (1999) reported lower prevalence of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in areas with more mangroves compared to areas with severely degraded mangroves. This suggests that mangroves have some protective effect against viral diseases. In aquaculture, some farmers in the Philippines claimed that the greenwater technology could prevent WSSV outbreak. The greenwater technology has been proven to prevent or control disease outbreaks due to luminous bacteria, there is a possibility that it can also prevent outbreaks due to WSSV. The possible protective effect of the presence of mangroves or of the greenwater technology against shrimp diseases might be by co-incidence. Their potential role in WSSV infection or outbreak needs to be elucidated.

From the foregoing, it may be clear that understanding the epidemiology of WSSV is important for an effective disease control and prevention measures. Epidemiological studies done on WSSV were mostly tank-based and on crustaceans other than *Penaeus monodon*. Literatures on disease prevention through ecological means are rare. Some of the factors that need clarification are: (1) the WSSV risk factors in farmed *P. monodon*; and (2) the ecological means of protection against diseases, especially WSSV, such as the presence of mangroves and the greenwater technology.

1.2. FACTORS ASSOCIATED TO WSSV OCCURRENCE

Not all farms may be affected when the disease occurs in a certain area; and within a farm, not all ponds may be affected (Wongteerasupaya et al., 2003). This could be because of the existence of different WSSV variants/strains (Hoa et al., 2011; Wongteerasupaya et al., 2003) or to the prevailing environmental conditions. A range of environmental conditions related to pond management and pond ecology affect WSSV epidemiology. Complete system dry-out between culture cycles and water filtration through 300 µm mesh screen to fill the water supply channel and ponds could contribute to excellent shrimp survival in spite of the presence of WSSV (Velasco et al., 2002). Corsin et al. (2001) reported lower disease prevalence in ponds infected with WSSV when they were fertilized with phosphorus; WSSV infection was positively correlated with ponds closer to the sea and with the use of commercial feed. Previous authors did not observe an association between stocking density and WSSV infection at harvest. This is contrary to Pienado-Guevara and Lopez-Meyer (2006) who reported that removal of 40 and 50% of the shrimp population at a low level WSSV infection improved shrimp survival, suggesting that at lower density there is less disease prevalence. The effect of stocking density could be explained by the effect of lesser contact between infected and non-infected shrimp and thus reduce disease transmission and avoid mechanical stress that could result to increased tolerance to disease.

Exposure of shrimp to stressors increase the risk of WSSV, since stressors like pH, un-ionized ammonia, water hardness, temperature and salinity, could compromise the shrimp defence system (Takahashi et al., 1995). Corsin et al. (2001) reported that WSSV outbreaks were preceded or coincided with higher pH and un-ionised ammonia in the water. In contrast, Sahoo et al. (2005) found that clinical white spots in shrimps associated with high water pH disappear after molting. Low hardness and low salinity of pond water are stress factors that made shrimp susceptible to *Vibrio* and subsequently to WSSV (Hettiarachchi, 1999). Inversely, Phuoc et al. (2006) reported that prior WSSV infection makes shrimp susceptible to *Vibrio campbelii*.

Several studies have reported that fluctuations in salinity and temperature could weaken the shrimp's immune system and enhance viral proliferation and thus increase disease prevalence. Under salinity stress, *M. japonicus* is more susceptible to WSSV due to changes in their immune responses (Yu et al., 2003). Moreover, acute salinity change greater than 4 ppt in 1h or continuous small salinity adjustments could lead to the rapid

WSSV proliferation and decreased disease-resistance ability of *Fenneropenaeus chinensis* (Liu et al., 2006). In farms in Ecuador and Mexico, shrimp mortality was associated with abrupt fluctuations in salinity and temperature, which contributed to, increased viral loads in the shrimps (Rodriguez et al., 2003; Peinado-Guevara and Lopez-Meyer, 2006). WSSV outbreaks seem to occur at temperatures suitable for shrimp farming, while extreme temperatures seem to reduce it. Guan et al. (2003) reported that survival of WSSV- infected *Marsupenaeus japonicus* was low at 23^{o}C and 28^{o}C; and high at 15^{o}C and 33^{o}C in which viral concentration was also lowest (Guan et al., 2003). Hyperthermia could protect *Litopenaeus vannamei* from WSSV because high water temperature completely inhibited or reduced the expression of WSSV genes on shrimp subcuticular epithelial cells which resulted to a decrease in viral replication (Vidal et al., 2001; Rahman et al., 2006; Reyes et al., 2007).

1.3. DISEASE PREVENTION THROUGH ECOLOGICAL MEANS

The disease develops when shrimp are stressed and their immune response is compromised. Providing the shrimp with optimum water and soil quality could prevent stressful conditions. Environmental conditions dictate the shrimp's immunocompetence (Takahashi et al.,1995).

Chemical means of disease prevention, either by improving water and soil quality or by enhancing the shrimp's immunity against diseases, had been the trend in the shrimp industry but is not sustainable. Despite the continuous use of chemicals i.e. antibiotics, probiotics, disinfectants, disease outbreaks continue to bring havoc resulting to economic losses especially to small shrimp farmers. Furthermore, use of antibiotics may lead to antibiotic resistant bacterial strain and antibiotic residues in shrimp; thereby making treatment of both shrimp and human diseases more difficult (Tendencia and dela Pena, 2002; Regidor, pers comm). The effect of the long term use of probiotics on the environment is not studied but prolonged use of probiotics can cause white spots on the shrimp exoskeleton (Wang et al., 2000). An option for an economically and environmentally sustainable method of disease prevention might be through ecological means. Some of the ecological means of disease prevention worth investigating are the presence of mangrove and the greenwater technology.

1.3.1. MANGROVE HABITAT

Mangroves serve as nutrient filters; thus their presence promotes good soil and water quality. Adame et al. (2010) reported that mangroves can improve water quality. Primavera et al. (2007) observed lower N and P concentrations in areas with mangroves. Hyun et al. (2009) reported that vegetated marshes serve as sink against external loading of organic matter. Micro-organisms present in the mangrove ecosystem are responsible for the nutrient cycle contributing to the productivity and food web dynamics and for the well-being of the habitat (Holguin et al., 2001; Vethanayagam, 1991). Among these are the phytoplankton and the bacteria which strip nutrients from shrimp farm effluents and transform toxic substances to less toxic bioavailable form and may serve as nutritional source for other organisms (McKinnon et al., 2002). Furthermore, micro-organisms isolated from mangrove soil have antimicrobial activity and play a critical role in maintaining acceptable water quality by providing oxygen and oxidizing organic matter (Lin et al., 2005). The growth and characteristics of these micro-organisms are maintained by the decomposition and release of nutrients of mangrove roots and rhizomes which are influenced by season, tidal amplitude/elevation, geomorphology, mangrove to pond area ratio, forest type, forest species or a combination of some of the factors (Alongi et al., 1993, Nickerson & Thibodeau, 1985; Thibodeau and Nickerson, 1986; Robertson and Philipps, 1995; Rivera-Monroy, 1999; Shimoda et al., 2007; Shimoda et al. 2005; Saenger et al., 1983; Primavera et al., 2007).

Aside from improving water quality the anti-viral property of extracts of some mangrove plants has been reported. Sudheer et al., (2011) found out that extracts *of Rhizophora mucronata*, *Sonneratia* sp and *Ceriops tanggal* have anti-WSSV activity. Furthermore, the authors reported the suitability of *C. tanggal* extracts as a prophylactic treatment against WSSV.

1.3.2. Greenwater Technology

A green water technology is an innovative technique wherein shrimp are cultured in water collected from a pond where tilapia or other fish species are grown. Two greenwater technologies are used by shrimp farmers in the Philippines (Tendencia et al., 2004): 1) shrimp and finfish are cultured in two separate ponds; water used to culture shrimp comes from the finfish pond; 2) the fish are polycultured with shrimp; finfish are

stocked in an isolated net pen inside the shrimp culture pond. Reports on tank based experiments showed that greenwater technology wherein shrimp are cultured with different fish species such as different tilapia strains, grouper, seabass, snapper and siganid proved effective in controlling luminous bacterial disease (Tendencia et al., 2006a; Tendencia et al., 2006b; Tendencia et al., 2004). The effectiveness of the greenwater culture system against luminous bacteria is attributed to the presence of anti-luminous *Vibrio* factors in the associated bacterial, fungal, and phytoplankton microbiota; the skin mucus and the faeces of tilapia *per se* and the associated bacteria (Tendencia and dela Pena, 2010; Lio-Po et al., 2005).

Antiviral activity of some fishes has been reported. Cain et al. (1996) demonstrated the anti IHNV property of mucus from the skin and gastro intestinal tract of rainbow trout. The antiviral activity of salmonid against infectious pancreatic necrosis virus and salmonid alphavirus has been reported (Sun et al., 2011). Fish mucus contains specific and nonspecific antimicrobial compounds (Alexander and Ingram, 1992) that might be present in the greenwater technology and may contribute to the prevention of WSSV outbreak.

1.4. Hypotheses and objectives

The epidemiology of WSSV remains unclear. Contradictory effects of different stressors like temperature and salinity on WSSV infection have been reported. Most of the studies done are tank-based. Current WSSV control and prevention methods fail, thus there is a need to identify stressors in farmed shrimp, including, how pond ecology affects these stressors. Likewise, ecological means of disease control need to be investigated. Disease occurrence in farm animals greatly depends on the existing environmental conditions that are determined by husbandry and location. The thesis aims to generate knowledge that can improve prevention against WSSV in penaeid shrimp culture through a better farm husbandry.

The project has two working hypotheses:

1) WSSV outbreak is affected by the pond ecology and more specifically by the water/sediment quality.
2) Ponds with a higher ratio of mangrove to pond area and farms using the greenwater technology will have better water quality as far as it relates to factors with potential disease incidence.

The thesis aimed to elucidate the relationship between shrimp disease outbreaks and the influent water quality. The questions we tried to answer were:

1) What are the important risk and protective factors that affect WSSV outbreaks, e.g. factors related to water and sediment bio-physico-chemical parameters, farm culture technique?
2) How does the identified ecological means of disease control affect water and soil quality and WSSV?

To answer the first question, several activities were implemented. To assess the effect of water quality on WSSV epidemiology, physicochemical water parameters of 91 shrimp production cycles were gathered from stocking until harvest. A case study was done for a more detailed information of the identified stress factors. To determine how farm management affects WSSV epidemiology, a questionnaire type of survey was conducted.

To answer the second question, six *P. monodon* monoculture farms using the greenwater technology with different mangrove to pond area ratio (0:1, 1:1, 4:1) were monitored monthly before stocking until 120 days of culture, when most of the farms harvested. In addition, a farm using the greenwater technology with no mangrove and three farms not using the greenwater technology with no mangrove were also monitored. These 4 farms were added to determine the effect of greenwater technology on pond ecology. Soil and water samples were taken from the intake channel, reservoir, culture pond and drainage channel of the culture pond and processed for plankton profile, microbiological study and physicochemical analyses. Shrimp samples were also collected and analyzed for determination of WSSV load.

Elaborate discussions of the activities are included in the subsequent chapters. Chapters 2, 3 and 4 focus on the epidemiology of WSSV. Different risk and protective factors related to the water physicochemical parameters are discussed in Chapters 2 and 3, and farming practices in Chapter 4. Chapters 5 and 6 differentiate the ecology of different farms in relation to the identified WSSV protective factors. How mangrove to pond area ratio affect pond ecology and WSSV prevalence was analysed and discussed in Chapter 5, and the effect of greenwater culture technique in Chapter 6. Chapter 7 synthesizes the most important results of the study, as well as recommendations and insights for future research.

CHAPTER 2

WSSV RISK FACTORS RELATED TO WATER PHYSICO-CHEMICAL PROPERTIES AND MICROFLORA IN SEMI-INTENSIVE *PENAEUS MONODON* CULTURE PONDS IN THE PHILIPPINES

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(Corrections in Results and Discussion regarding significance of salinity effect)

ABSTRACT

Whitespot syndrome virus, WSSV, is the most important among the shrimp diseases. One of the suggested WSSV risk factors is the occurrence of stress since stressors could compromise the shrimp defence system thus increasing the risk of WSSV infection. Stressors are usually related to the physico-chemical properties of both water and pond bottom. This paper investigates the effect of some biotic and abiotic components of the pond ecosystem on WSSV infection and outbreak. Water physico-chemical properties and microflora of 91 production cycles of 8 semi-intensive shrimp farms were analyzed to determine WSSV risk factors, using factor analysis and logistic regression. Fluctuations of temperature and pH are important risk factors that will result to an infection but not necessarily to an outbreak. Exposure to high salinity and high temperature are important factors for an infection to result to an outbreak. The risk of an infection is reduced when the water temperature is high, salinity fluctuations are small, and percentage of yellow *Vibrio* colonies is greater than the green ones. Further studies are needed to clarify the effects of water depth, water transparency, and various bacterial counts; these factors could be individual or interactive.

2.1. INTRODUCTION

Whitespot syndrome virus, WSSV, is the most important among the shrimp diseases. One of the suggested WSSV risk factors is the occurrence of stress (Chou et al., 1995; Lo et al., 1996; Mohan et al., 2008). Stressors are usually related to the physico-chemical properties of both water and pond bottom. Stressors could compromise the shrimp defence system thus increasing the risk of WSSV infection (Takahashi et al., 1995). Furthermore, some physico-chemical conditions might stimulate the rapid replication of WSSV, which could subsequently cause the death of the shrimp (Lo and Kou, 1998). In the following paragraphs we give an overview of present knowledge on the effect of water parameters on WSSV infection; starting with evidence on abiotic factors acquired from tank experiments and farm surveys, an intermezzo on effects of bottom soil, and ending with biotic factors in water.

Tank-based studies have reported that fluctuations in salinity and temperature could weaken the shrimp's immune system and affect viral replication. In *Marsupenaeus japonicus*, the immune response becomes weaker as the deviation from the original salinity they were kept in becomes greater (Yu et al., 2003). Acute salinity change of greater than 4 ppt in 1 h, as well as continuous small salinity adjustments, could lead to rapid WSSV proliferation and decreased disease-resistance ability of Fenneropenaeus chinensis which makes the shrimp more susceptible to viral infection than they previously were, resulting in WSSV outbreak (Liu et al., 2006). Furthermore, a decrease in temperature increases the viral load, while hyperthermia could protect *Penaeus vannamei* from WSSV (Rahman et al., 2006; Reyes et al., 2007; Granja et al., 2003). Guan et al. (2003) reported that viral concentration was lower at 15 °C and 33 °C than at 23–28 °C. WSSV DNA load in WSSV-infected shrimp is reduced at 32 °C (Granja et al., 2006).

A few authors reported farm based WSSV risk factors. Corsin et al. (2001) reported that WSD outbreaks were preceded or coincided with higher pH and un-ionised ammonia in the pond water. This was contradicted by Sahoo et al. (2005), who reported that clinical white spots in shrimps associated with high water pH disappear after molting. Low salinity and low hardness of pond water are stress factors that made shrimp susceptible to *Vibrio* and subsequently to white spot disease (Hettiarachchi et al., 1999). On farms in Ecuador, Rodriguez et al. (2003) showed temperature being associated with mortality. In Mexico abrupt fluctuations in temperature and salinity due

to heavy rain were reported to contribute to increased viral loads in the shrimps, which resulted in 80% mortality (Peinado-Guevara and Lopez-Meyer, 2006).

Water microflora also affects WSSV infection. Hettiarachchi et al. (1999) reported that prior exposure to Vibrios make shrimp susceptible to WSSV infection. Phuoc et al. (2008) reported accelerated mortality in WSSV-infected Littopenaeus vannamei after infection with *V. campbellii*. Dela Peña et al. (2003) reported *V. harveyi* and WSSV dual infection in *Penaeus monodon* in the Philippines. Planktons such as *Chlorella* sp, *Scrippsiella troichoidea* and rotifer serve as vector in WSSV transmission (Liu et al., 2007; Yan et al., 2007). On the other hand, *Spirulina* could delay the onset of clinical signs but has no effect on the final mortality (Rahman et al., 2006).

Mohan et al. (2008) reported no direct relationship between the condition of pond sediment and risk of disease outbreaks or production. However, in other studies the occurrence of black and toxic bottom sediments has been shown to adversely affect shrimp health and lead to disease outbreak or poor survival. Sludge removal, ploughing of pond bottom and liming reduces the risk of WSSV and other infections as it re-establishes a healthy pond bottom (Avinimelech and Ritvo, 2003).

Summarising, most papers on the effect of physico-chemical water parameters on WSSV are tank-based experiments and not on *P. monodon*. Papers on WSSV epidemiology in ponds are mostly on farm management practices, which are assessed by the authors in another type of survey (Tendencia et al., 2011). Little is known how the pond ecology characterised by both physico-chemical and bacteriological parameters affects WSSV epidemiology. This paper investigates the relation of bacterial and algal counts, bacterial characteristics and water main physico-chemical parameters to WSSV infection and outbreak in pond-cultured *P. monodon* in the Philippines.

2.2. MATERIALS AND METHODS

2.2.1. POND CHARACTERISTICS

Ponds used in the study are *P. monodon* ponds in Negros Island, Philippines. Pond size ranged from 0.2 to 0.8 ha. Ponds were stocked with WSSV PCR negative post larvae (PL) 13–20 at an average density of 10–16 ind/m2. Shrimp were fed with commercial pellets from stocking until harvest. In some cases, shrimp culture was aborted after 19 days of culture (DOC), in other cases shrimp were harvested at DOC

184; next to market demand the day of harvest depended on the health status of the shrimp.

2.2.2. DATA GATHERING

Data were gathered from 75 ponds in 8 farms over 100 production cycles. Data from 2 production cycles were taken from 21 of the 75 ponds, and 3 production cycles from 2 ponds. To avoid power reduction due to missing values, only 91 of these production cycles were used in the analysis. Forty-eight culture periods did not have WSSV infection, 15 had infection but did not result in an outbreak, and 28 had infection that resulted in an outbreak.

Physico-chemical water parameters, namely, temperature, pH, salinity, transparency and water depth were measured twice daily at 08:00 h and 17:00 h from DOC 1 until the culture was aborted or until the shrimp were harvested. Bacterial and algal flora in pond water were counted every three days. The spread plate and dilution techniques were used for total bacterial count (TBC), luminous bacterial count (LBC) and presumptive *Vibrio* count (PVC). TBC and LBC were plated and counted on nutrient agar; for PVC we used thiosulfate citrate bilesalt sucrose agar (TCBS). All plates were incubated for 24 h at 30 °C. Luminescence for the LBC was observed in a dark room. Green and yellow *Vibrio* colonies were counted separately on TCBS. Algal flora was counted using a haemocytometer.

Shrimp samples (n = 5) were analyzed for WSSV detection weekly, when abnormalities in behaviour/appearance and when mortalities were observed using the SBBU kit, a test kit for the molecular diagnosis of WSSV developed by the Shrimp Biotechnology Business Unit (SBBU) of Mahidol University, Thailand (Withyachumnarnkul, 1999). Positive and negative controls were included in all tests. WSSV infection was reported when shrimp tested positive for WSSV. An outbreak was reported when mass mortalities were observed in WSSV positive shrimp.

2.2.3. DATA ANALYSIS

Averages and daily fluctuations of the observed physico-chemical water parameters were computed, as well as averages of the counts of the microflora. To avoid dilution of the data, series of repeated measures in the culture period were cut-off to a

limited number of days of culture (DOC) before data analysis. For culture periods with outbreak, DOC cut-off was set at 4 days before outbreak and for those with infection not resulting in outbreak at 4 days before infection. The cut-off of 4 days was based on previous observations that 4 days of exposure to multi stressors could lead to WSSV infection (Tendencia and Verreth 2011b). To determine the cut-off for culture periods without infection, the median of the DOC at which WSSV outbreaks occurred and the DOC at which highest WSSV incidence was observed were considered (see Results). We used the average of the values observed between these periods to characterise production cycles without incidence.

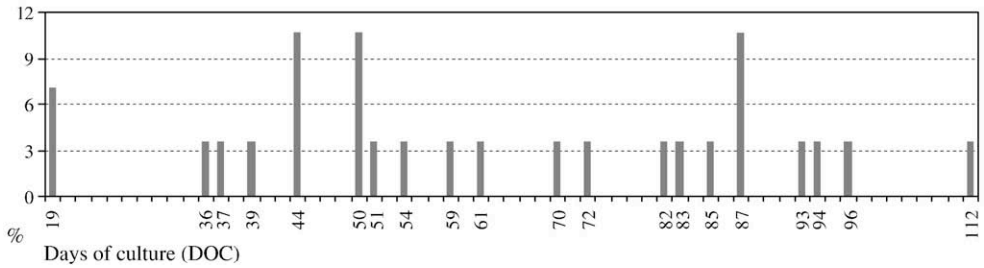
The data analysis was done in two steps: variable reduction and binary logistic regression in SPSS V. 17, with both complemented with correlation analysis. The number of variables was reduced using factor analysis. Before the second step the number of explanatory variables within a category was reduced by excluding correlated variables; the highest positively and negatively correlated factors were maintained. WSSV risk factors were identified using binary logistic regression's backward stepwise method with a likelihood ratio test. Not all WSSV infection resulted in an outbreak. To determine which factors were important for an outbreak to occur, two analyses were done: one on the entire dataset and one on the cases where infection occurred whether or not resulting in an outbreak. In the entire dataset, WSSV infection whether resulting or not resulting in an outbreak was used as the dependent variable. In the smaller dataset with cases where infection was confirmed, WSSV outbreak was considered the dependent variable. The model which gave a lower negative log likelihood value, a R^2 value closer to one, and a higher percentage correctness in predicting WSSV incidence was retained. We used Spearman correlation to check the direction of the impact by the variables in the model, and the multicollinearity diagnostics from the linear regression analysis to assess if the model parameters are biased. The predictor with lesser correlation with WSSV was omitted in cases when collinearity was observed.

2.3. RESULTS

Highest incidence of WSSV infection was observed before DOC- 51 and the last outbreak occurred at DOC-115 (Fig. 2.1). The median of the DOC at which WSSV outbreak was observed is DOC-60. DOC-60 was considered the cut-off and the averages

from the values measured between DOC 51 to DOC 60 were used to characterise ponds without infection.

Figure 2.1. Frequency distribution of days of culture at which WSSV outbreak is observed in semi-intensive *P. monodon* farms in the Philippines.



Observed physico-chemical parameters, including their fluctuations, and microflora characteristics of ponds without infection and with infection resulting or not resulting in an outbreak, are presented in Table 2.1.

Observed lowest temperature (26°C) in ponds with infection was 1°C lower than those without infection. Mean temperature observed in ponds with infection (27°C) was 2°C lower than those without infection (29°C). Temperature fluctuations as large as 9.75°C were observed in ponds with infection compared to those without infection (max: 4.75°C). Greatest pH fluctuation was larger in ponds with infection (1.1) than those without infection (0.75). Observed salinity was lower in those with infection (6.5–20 ppt) compared to those without infection (6.6–31.4 ppt). Larger salinity fluctuation was observed in ponds with infection (4.25) than in those without infection (1.1). Mean water depth inside the pond was higher in ponds with infection (>100 cm) compared to those without infection (<100 cm). Water transparency observed in ponds with infection was lower (<30 cm) than those in ponds without infection (>30 cm).

Total bacterial counts in ponds with and without infection were comparable. Luminous bacterial counts in ponds without infection were not significantly higher in ponds without infection. Presumptive *Vibrio* counts in ponds with infection were higher than in those without infection with higher percentage of the green colonies. Total algal counts were comparable in ponds with and without infection.

Table 2.1. Physico-chemical parameters including fluctuations observed during pond culture without infection and culture with infection resulting or not resulting to an outbreak.

Parameters	Without inf		With inf without outbreak		With inf with outbreak	
	Range	mean	range	mean	range	mean
Tpam (^oC)	27-31	29^b	26- 31	27^a	26-31	28.42^b
Tpm (^oC)	27.57-33.31	31^a	27 – 33	30.1^a	27.5- 33	30.65^a
Tfab(^oC)	0-2.9	1.86^a	0.67 – 4	2.53^b	1.25-3	2.09^a
Tfpab(^oC)	.56-4.75	1.99^a	0.67- 9.75	3.04^b	0.75-9.6	2.18^a
pHam	7.45- 8.64	8.06^b	7.38 – 8.51	7.88^a	7.38-8.48	8.03^{ab}
pHpm	7.54-9.07	8.36^a	7.6 – 9.48	8.23^a	7.6-9.48	8.34^a
pHfapb	0.7- 0.75	0.3^a	0- 1	0.33^a	0-1	0.3a
pHfpab	0.06- 0.64	0.3^a	0.1 – 1.1	0.41^b	0.1-1.1	0.32^{ab}
sal (ppt)	6.6 - 31.4	14.88^b	6.5- 20	11.11^a	6.5-20	13.52^{ab}
salfpa(ppt)	0-1.1	0.27^a	0- 4.25	0.32^a	0-4.25	0.46^a
tran (cm)	16.2 – 80	39.57^b	14.25- 49.2	28.64^a	15.5- 45	28.60^a
depth (cm)	62.1 – 124.2	98.76^a	85 – 139	108.95^b	85-118.25	103.51^{ab}
TBC (cfu/ml)	$1.0x10^3$-$2.66x10^4$	5.0×10^{3a}	6.58×10^2-9.3×10^4	5.6×10^{3a}	$6.77x10^3$ 9.3×10^4	$8.86x\ 103^a$
LBC (cfu/ml)	$0–8.06x10^4$	1.8×10^{3a}	$0 – 9 \times 10^2$	1.3×10^{2a}	$0- 9 \times 10^2$	1.28×10^{2a}
PVC (cfu/ml)	1.0×10^1 – 2.8×10^3	8.8×10^{2a}	5.0×10^1 – 4.45×10^4	1.5×10^{3a}	$2.15x10^2$ 4.45×10^4	3.40×10^{3a}
Yellow Vibrios (%)	1 – 98	61^a	11- 98	60^a	11- 98	51^a
Green Vibrios (%)	2- 99	39^a	2 – 89	40^a	2 – 89	49^a
TCC (cells/ml)	$1.94x10^5$ – 2.35×10^6	5.9×105^a	2.32×10^5 – 1.21×10^6	6.5×10^{5a}	$2.32x\ 0^5$ – 1.21×10^6	6.9×10^{5a}

pHam=pHmorning; pHpm=afternoon; sal=Salinity; depth=Water level inside the pond; tran= transparency; Tpm=Temperature, afternoon; Tpam=Temperature, morning; Tfap=Temperature fluctuation, morning to afternoon; Tfpa=Temperature fluctuation, afternoon to morning of the following day; pHfpa=pH fluctuation, afternoon to morning of the following day; salfpa=Salinity fluctuation, afternoon to morning the following day; pHfap=pH fluctuation, morning to afternoon; TCC=total algal count; LBC=Luminous bacterial count; TBC=Total bacterial count; PVC=Presumptive *Vibrio* count; yellow= yellow Vibrio colonies; green=green *Vibrio* colonies; mean with the same superscript in the same row are not significantly different ($P<0.05$).

The factor analysis identified 6 sets of variables explaining 77% of total variance (Table 2.2). Variables with an Eigenvalue >0.5 were water transparancy and depth, total bacterial count, green and yellow *Vibrio* colonies, algal count, and parameters related to temperature, pH and salinity.

Table 2.2. The component matrix of the factor analysis of the data on WSSV incidence in semi- intensive culture *P. monodon* farms mentioning the factors with B N 0.5 only, and between brackets the cumulative explained variance.

1 (21.99)	2 (37.96)	3 (53.25)	4 (61.8)	5 (69.99)	6 (77.22)
Tfap	Tpam	Tpam	salfpa		pHpm
Tfpa	pHam	Tfap	TBC		tran
pHfap	pHpm	TBC	PVC		TCC
pHfpa	depth	PVC			
Yellow	LBC	Tpm			
Green		Sal			

pHam=pH,morning; pHpm=pH, afternoon; sal=Salinity;depth=Water level inside the pond; trantransparency; Tpm=Temperature,afternoon;Tpam= Temperature, morning; Tfap=Temperature fluctuation, morning to afternoon; Tfpa=Temperature fluctuation, afternoon to morning of the following day; pHfpa=pH fluctuation, afternoon to morning of the following day; salfpa=Salinity fluctuation, afternoon to morning the following day; pHfap=pH luctuation, morning to afternoon; TCC=total algal count; LBC= Luminous bacterial count; TBC=Total bacterial count; PVC=Presumptive *Vibrio* count; yellow=yellow *Vibrio* colonies; green=green *Vibrio* colonies

Only four of the 8 variables included in the model predicting WSSV infection resulting or not resulting in an outbreak contributed significantly to its predictive value. The risk of an infection was reduced when the water temperature was high, transparency was high, and the percentage of yellow *Vibrio* colonies was greater than the green ones and the luminous bacterial count was high (Table 2.3). Yellow colonies had a significant negative influence in the model and a non-significant negative correlation with WSSV infection. There was no collinearity among the predictors in this

Table 2.3. Variables included in the model for WSSV infection and outbreak risk factors associated to water environmental parameters in semi-intensive shrimp culture systems in the Philippines (figures in bold were maintained in the final model).

Factor	WSSV infection (n = 91)	WSSV outbreak (n = 43)
Temperature fluctuation	**+1.087** (p<0.05)	-0.747
pH fluctuation	+ 2.104	-5.025
Salinity fluctuation	- 0.748	-6.12
Temperature	**- 0.819** (p<0.01)	**+1.847** (p<0.05)
Transparency	**- 0.088** (p<0.01)	
Water level inside pond	+ 0.036	-0.135
Yellow Vibrios	**- 2.979** (p<0.05)	
LBC	- 0.001	
TBC		-0.001
pH		-1.907
Salinity		**+0.17**(P<0.01)

LBC=luminous bacterial count; TBC=total bacterial count

model. In the model the coefficient for transparancy was close to zero but the likelihood ratio of the final model with the three other variables was not as strong as the complete model (Table 2.4). Temperature and transparency were significantly

Table 2.4. Characteristics of the binary logistic regression of retained WSSV risk factors on infection and outbreak within infection (for variables considered in the model see Table 2.3).

				Percentage correctness		
		-2LL	Nag. R^2	0	1	overall
Infection	extensive model	68.546	0.547	85.4	73.5	80.5
	final model	74.748	0.484	79.2	64.7	73.2
Outbreak (within infection)	extensive model	21.584	0.699	86.7	89.5	88.2
	final model	29.862	0.522	78.9	80.0	79.4

Legend: -2LL=- 2 log likelihood; R2=Nagelkerke; 0=without WSSV infection/ outbreak; 1=with WSSV infection/outbreak

negatively correlated with WSSV infection indicating that high temperature and high transparency were reducing infection (Table 2.5).

Among the eight variables included in the model predicting an outbreak to occur in an infected pond, temperature and salinity contributed significantly to its predictive value (Table 2.3). Unlike the temperature fluctuation, temperature and salinity levels were significantly correlated with the occurrence of an outbreak among the infected ponds (Table 2.5). The predicting values of the final model were lower than for the complete set. Collinearity existed between temperature and pH but was too weak to consider exclusion of either (rho = 0.224).

Table 2.5. Spearman rho's correlation analysis of variables considered in the model with WSSV infection (n = 91) and WSSV outbreak (n = 48).

	Tf	pHf	salf	Tpam	tran	depth	yellow	pH	sal
Infection	+0.20	-0.05	-0.27*	-0.30**	-0.43**	+0.17	-0.13	-0.13	-0.18
Outbreak	-0.20	+0.03	+0.19	+0.05**	+0.04	-0.23	-0.20	+0.19	+0.31*

Tf=temperature fluctuation; pHf=pH fluctuation; salf=salinity fluctuation; Tpam=temperature, morning; Tran=transparency; sal=salinity; * = $p<0.05$; ** = $p< 0.01$

2.4. DISCUSSION

This study confirms previous reports on tank-based studies stating that temperature, pH and salinity are important WSSV risk factors. Temperature, pH and salinity affect the immune response of shrimp (Le Moullac and Haffner, 2000; Vargas-Albores et al., 1998; Cheng and Chen, 2000; Lu-Qing et al., 2005; Yu et al., 2003). Furthermore, virus replicate more rapidly at low temperature (Rahman et al., 2006).

Salinity fluctuation of 4.25 ppt was observed in the ponds with infection in this study; correlation confirms the effect of this fluctuation on WSSV infection but not on outbreak. Though salinity persé was not retained in the model for infection but only for outbreak, according to Vargas-Albores et al. (1998), Cheng and Chen (2000), Yu et al. (2003), Lu-Qing et al. (2005), low salinity is correlated with infection. The model and

correlation show that high salinity level significantly increases the risk of an outbreak, thus partially confirming the cited results of Liu et al. (2006). Salinity fluctuation was positively correlated with temperature and pH. Furthermore, increase in temperature was highly correlated with outbreak. This correlation could have masked the effect of salinity fluctuation on WSSV outbreak and prevented its representation in the model for WSSV infection and outbreak risk factors. However, according to farmers salinity fluctuation is not a major causal factor.

Temperature fluctuation and low temperature are identified as risk factors for WSSV infection, while an increase in temperature can be a risk factor for an outbreak in pond-cultured *P. monodon*. In tank-based experiments Vidal et al. (2001) confirmed the effect of low temperature and sudden temperature decreases; 100% mortality was observed in WSSV-infected *L. vannamei* transferred from 32 °C to 25.8 ± 0.7 °C. The contrasting effect of temperature on WSSV infection could be explained by the fact that an increase in temperature strengthens the immune response of the shrimp, thereby making them less susceptible to WSSV infection, while viral replication and load is increased when the temperature is decreased (Rahman et al., 2006; Reyes et al., 2007).

Pond water transparency is a minor factor in reducing WSSV risk in the model, contrary to its observed strong negative correlation to infection. Water transparency is used to monitor the abundance of algae, which are natural foods present in the aquaculture system. Moreover, water transparency is affected by the suspended solids present in the water. It should be noted that total algal count in ponds with and without infection are comparable, thus implying that the observed low water transparency in infected ponds could be attributed to the suspended solids. Suspended solids could clog the gills thereby stressing the shrimp and making them susceptible to infection. This might explain for the strong negative correlation between water transparency and WSSV infection. The minor protective effect against WSSV infection of transparency might be explained by its negative correlation with water depth, which is a minor risk factor for WSSV infection.

Deep water is a risk factor for infection but a protective factor for outbreak. The contrasting effect of deep water on infection and outbreak could be due to the interaction of water depth and transparency. Water transparency is lower in ponds with infection. This lower transparency could be attributed to suspended solids as earlier explained. So in less transparent deep ponds, the shrimp are exposed to a more stressful

environment than those in less transparent shallow ponds as in the case of the non-infected ponds. Water depth is negatively correlated with fluctuations in salinity and pH, which are positively correlated with outbreak. This means that in deeper water, there is less fluctuation in salinity and pH, thus, reduced risk on an outbreak.

In addition to water transparency, yellow *Vibrio* colonies, reduce the risk of WSSV infection to occur. Some Vibrios that form yellow colonies on TCBS like *V. alginolyticus* have a probiotic effect and found to give some protection against disease (Austin et al., 1995; Garriques and Arevalo, 1995). However presence of yellow colonies is not a significant protective factor for an outbreak, while the total bacterial count is. Total bacterial count includes both the harmful and beneficial bacteria. The beneficial bacteria could have overwhelmed the number of harmful ones thus making the environment less stressful to the shrimp or prevented the harmful ones from infecting the shrimp.

Fluctuations of temperature and pH are important risk factors that will result in an infection but not necessesarily in an outbreak in pond-cultured *P. monodon*. The risk of an infection is reduced when the water temperature is high, salinity fluctuations are small, and percentage of yellow *Vibrio* colonies is greater than the green ones. Exposure to high temperature and high salinity are significant factors for an infection to result in an outbreak. Contradictory effects of factors resulting in infection but not resulting in an outbreak were observed for water depth and transparency, and for level of, and fluctuations in temperature, pH and salinity. The initial exposure to fluctuations and low levels of these parameters could have stressed the shrimp and made them susceptible to infection. Further sudden changes in these parameters from low to high in infected shrimp could have stressed them more thus resulting to an outbreak. Further studies are needed to clarify the effects of water depth, water transparency, and various bacterial counts; these factors could be individual or interactive.

CHAPTER 3

TEMPERATURE FLUCTUATION, LOW SALINITY, WATER MICROFLORA: RISK FACTORS FOR WSSV OUTBREAKS IN *PENAEUS MONODON*

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ABSTRACT

White spot syndrome virus (WSSV) has been devastating the shrimp industry for almost a decade. This study compares water parameters, alkalinity, and microflora in three ponds on a farm on Negros Island (Philippines) during two production cycles where WSSV infection resulted in an outbreak in 2006 but not in 2005. The total bacterial count of the pond water in 2005 was about twice as high as in 2006. However, luminous bacterial counts were twice as high in 2006 than in 2005 and total presumptive *Vibrio*, as counted on *Vibrio* selective thiosulfate citrate bilesalt sucrose (TCBS) agar, was over ten times higher, with a greater percentage of green colonies. More green colonies might indicate a higher concentration of harmful *Vibrio* bacteria. Total alkalinity for both production cycles was within the normal range while temperature, salinity, pH, and dissolved oxygen varied and sometimes fell below or exceeded the acceptable range. In 2006, there were more instances during which the temperature fluctuated 3-4°C within the period of 7h, and salinity more often dropped below 15 ppt. Our survey suggests that WSSV outbreak might be triggered by water temperature fluctuations of 3-4°C, coupled with low salinity and a high presumptive *Vibrio* count.

3.1. INTRODUCTION

White spot syndrome virus has been devastating the shrimp industry for almost a decade. It was probably introduced in Asia by careless importation (Flegel, 2009). Exposure of shrimp to stressors increases the risk of WSSV, since stressors compromise the shrimp immune system (Takahashi et al., 1995). Consequently, under stressful conditions, WSSV can proliferate rapidly and cause mortality (Lo and Kou, 1998; Doan et al., 2009). WSSV outbreaks may be preceded by or coincide with high pH and un-ionized ammonia in shrimp pond water (Corsin et al., 2001). Clinical white spots associated with high water pH disappear after molting (Sahoo et al., 2005). Ammonia-N at 5 mg/l appears to reduce the immunocompetence of *Penaeus japonicus* but may also decrease the virulence of WSSV (Jiang et al., 2004). Temperature is associated with mortality in Ecuadorian farms (Rodriguez et al., 2003). Abrupt fluctuations in temperature and salinity due to heavy rains contribute to increased viral loads in shrimp in Mexico, resulting in 80% mortality (Peinado-Guevara and Lopez-Meyer, 2006).

A WSSV outbreak occurred on the west coast of India when the monsoon peaked and salinity approached 0 ppt (Karunasagar et al., 1997). Fluctuations in salinity and temperature can weaken the shrimp's immune system and affect viral replication. Low salinity and low hardness of pond water are stress factors that increase the susceptibility of shrimp to V*ibrio* and, subsequently, white spot disease (Hettiarachchi et al., 1999). Acute salinity changes of greater than 4 ppt within one hour can lead to rapid WSSV replication and decreased disease-resistance in *Fenneropenaeus chinensis* (Liu et al., 2006). Continual small salinity adjustments may also result in increased WSSV replication and the loss of self-adaptive ability after a long period of salinity stress (Liu et al., 2006).

Water temperature affects WSSV infection and the outbreak of clinical disease. Survival of WSSV-infected M. japonicus was low at 23°C and 28°C and high at 15°C and 33°C (Guan et al., 2003) while shrimp maintained at or transferred from a lower temperature to 33°C after WSSV challenge did not show signs of disease (Rahman et al., 2006). Mortality was 100%, however, in WSSV-infected shrimp transferred from 32°C to 25.8±0.7°C (Vidal et al., 2001).

Most of the above reports are tank based. The current study compares and correlates water parameters measured daily, and alkalinity and microflora observed every 2-3 days, in three ponds in a *Penaeus monodon* farm in the Philippines where a

disease outbreak associated with WSSV infection was observed in 2006 but not in 2005.

3.2. MATERIALS AND METHODS

3.2.1. FARM SITE

The farm is located on Negros Island, Philippines. It has a total area of 32 ha with 29 ponds ranging 5996-11000 m^2 and a reservoir of 10.5 ha. Water is supplied mainly from the sea and irrigation systems.

3.2.2. SHRIMP

Penaeus monodon were purchased for both production runs (2005 and 2006) from the same hatchery, located in a different province. Postlarvae were acclimatized in unopened plastic bags allowed to float in the pond in which the shrimp were to be stocked and stocked as shown in Table 3.1.

Table 3.1. Stocking of *Penaeus monodon* on a farm in 2005, when there was no WSSV outbreak, and in 2006, when there was a WSSV outbreak.

	Pond					
	2005			2006		
	A	B	C	A	B	C
Stocking density (ind/m^2)	12	13	15	20	13	15
Age at stocking (postlarvae stage)	19	20	18	19	20	18
Stocked in:	Jun	Jul	Jun	Jul	Jun	Aug

3.2.3. Farm inputs

The three ponds were managed identically during both production cycles. The shrimp were fed a commercial pellet diet to which probiotics, vitamin C, immune enhancers, and molasses were added. Bio-remediation products were applied to the water weekly. Water was not changed for the first 30 days, but as required between

days 31 and 90, and every 2-5 days after day 90 until harvest.

3.2.4. MONITORING

Monitoring of physico-chemical parameters and microflora in the water was identical during 2005 and 2006. Temperature, salinity, and pH were measured twice daily, at 8:00 and 18:00. Dissolved oxygen (DO) was measured before dawn at 5:00 and at 18:00. Alkalinity was measured every 2-3 days (APHA, 1995). Total bacteria, luminous bacteria, and presumptive *Vibrio* were counted on *Vibrio* selective thiosulfate bilesalt citrate sucrose agar (TCBS) every 2-3 days using the plate count method. Water samples (1 l) were collected in sterile bottles and serially diluted using autoclaved seawater. Representative dilutions of (0.1 ml) were plated onto nutrient agar (NA) and TCBS agar plates, in duplicate. Inoculated plates were incubated at room temperature (approx. 30°C) for 24 h. After incubation, total bacterial counts were determined on NA plates, presumptive Vibrio on TCBS plates, and luminous bacteria on NA plates in a dark room.

3.3. RESULTS

Total alkalinity was within the normal range during cycles but temperature, salinity, pH, and dissolved oxygen varied and sometimes fell out of the acceptable range (Table 3.2). Generally, temperature, DO, and pH were lower in the morning than in the afternoon. The water temperature fluctuated more than 3-4°C within a 7 hour period more often during 2006, when there was an outbreak of disease due to WSSV infection, than in 2005. Likewise, salinity dropped below 15 ppt more often in 2006. The total bacterial count in the pond water was twice as high in 2005 than in 2006, but the luminous bacteria count was twice as high in 2006 as in 2005 and the total presumptive *Vibrio* count was over ten times higher in 2006 than in 2005, with a greater percentage of green colonies.

3.4. DISCUSSION

Total alkalinity during both production cycles was above 80 ppm and suitable for *P. monodon* culture (Chanratchakool et al., 1995; Tookwinas, 2000). During the 2005

Table 3.2. Physico-chemical parameters and microflora of culture water in ponds without (2005) and with (2006) WSSV outbreaks.

Parameter	Accept-able range	Time of day	Without WSSV infection (n=3)			With WSSV outbreak (n=3)		
			Range	mean	sd	range	mean	sd
Temperature (^oC)	28-32	0800h 1500h	23-31 26-33	28.56 29.72	1.25 1.45	26- 31 26.2-34	28.4 30.2	1.1 1.6
Salinity (ppt)	15-25	0800h 1500h	7-25 7-25	16.11 15.89	4.45 4.48	9-22 10-22	15.9 15.8	3.4 3.4
DO (ppm)	>4	0500h 1500h	3.2-12.6 4.6-6.2	4.71 7.6	0.63 1.4	3.6- 5.8 5.4-12.1	4.4 7.7	0.5 0.9
pH	7.5-8.5	0800h 1500h	7.4-8.9 7.5-8.9	8.11 8.34	0.25 0.28	7.6- 8.4 7.4-8.6	8.1 8.3	0.2 0.2
Alkalinity (ppm)	>80		108-222	155.8	29.87	110-180	147.4	19.6
Total bacterial count (cfu/ml)	10^3-10^4		$1.1x10^2$-$2.7x\ 10^4$	3.4E±3	3.4E±3	$2.5x10^3$-$2.1x10^2$	$8.48x10^2$	$4.01x10^2$
Luminous bacterial count (cfu/ml)	10^2		$<1.0x10^1$-$2.5x10^3$	$3.14x10^2$	$4.27x10^2$	$<1x10^1$-$9.4x10^4$	$6.87x10^2$	$1.2x10^3$
Presumptive *Vibrio* count (cfu/ml)	$<10^2$		$<1x10^1$-$1.8x10^3$	$3.14x10^2$	$3.15x10^2$	$6x10^1$-$8.8x10^3$	$5.39x10^3$	$1.99x10^4$
Green *Vibrio* colonies (%)			0-100	25.77	25.76	0- 96.55	50.00	30.00
Days of culture				143			95	
Culture days wherein T fluctuated 3-4^{o}C (%)				0.005			11.5	
Culture days wherein salinity was <15 ppt (%)				34.8			45.1	

cycle, the mean total bacterial count of the shrimp ponds was within the prescribed range of 10^3-10^4 cfu/ml (Tookwinas, 2000), but it was lower during the WSSV-associated disease outbreak in 2006.

Total bacterial count includes all kinds of beneficial as well as harmful bacteria. The beneficial bacteria might have conditioned the pond water, making environmental conditions less stressful to the shrimp and rendering them less susceptible to WSSV infection. Further, probiotics encourage the proliferation of bacteria that inhibit the colonization of pathogens (Das et al., 2006; Ganguly et al., 2010). The presumptive *Vibrio* count was over 10 times higher in the 2006 cycle than the maximum 10^2 cfu/ml recommended for shrimp culture (Baliao, 2000). Further, more green colonies, believed to be pathogenic, were recovered in 2006 than in 2005, which may have stressed the shrimp, making them more susceptible to WSSV infection. *Vibrio* infection due to poor environmental conditions makes shrimp susceptible to WSSV (Hettiarachchi et al., 1999).

The poor conditions may have been attributable to sub-optimal temperature and salinity. Temperature and salinity affect the immune response of crustaceans (Vargas-Albores et al., 1998; Le Moullac and Haffner, 2000). Temperature changes are one of the factors that trigger WSSV infection in shrimp culture (Kautsky et al., 2000). Temperature shifts can induce an outbreak of WSSV disease in lightly infected shrimp (Hsu et al., 2000). There were a greater number of 3-4°C temperature fluctuations during the WSSV outbreak in 2006 than during 2005. The temperature fluctuations could have stressed the shrimp, lowering their immune response and making them susceptible to WSSV infection. Temperature effects survival of WSSV infected shrimp (Rodríguez and Sonnenholzher, 2001; Guan et al., 2003).

Salinity affects the immune response of *Marsupennaeus japonicus*; the further from the original salinity the shrimp are maintained, the weaker their immune response (Yu et al., 2003). The ideal salinity for shrimp culture is 15-25 ppt (Baliao, 2000). Salinities dropped below 15 ppt in both production cycles but more instances were observed during 2006. Acute salinity stress is more significant at low salinity than at high, affecting the immunocompetence of *P. monodon* and resulting in increased susceptibility to WSSV infection (Joseph and Philip, 2007). Although their immune defense and metabolic response overwhelmed the pathogen during the early stages of infection, delaying the onset and pace of mortality, shrimps maintained at 15 ppt did not

completely eliminate virus particles from circulation and thwart infection (Joseph and Philip, 2007).

Results indicate that temperature fluctuations of 3-4°C within a 7 hour period, salinity below 15 ppt, high presumptive Vibrio counts, and presence of 50% green *Vibrio* colonies might be important risk factors in WSSV outbreaks. Our work suggests that appropriate management measures such as the use of reservoirs should be adopted in *P. monodon* culture ponds to minimize acute salinity stress or salinity below 15 ppt, temperature fluctuations, and dominance of green *Vibrio* colonies. Whether induced dominance of yellow *Vibrio* colonies would limit WSSV outbreaks might be a subject of study.

CHAPTER 4

WHITE SPOT SYNDROME VIRUS (WSSV) RISK FACTORS ASSOCIATED WITH SHRIM FARMING PRACTICES IN POLYCULTURE AND MONOCULTURE FARMS IN THE PHILIPPINES

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ABSTRACT

White spot sydrome virus (WSSV) is one of the most important viral diseases of shrimp. Several studies to control the disease have been done. Tank experiments identified WSSV risk factors related to the physico- chemical properties of the water. A few studies reported pond level WSSV risk factors. This study identifies the risk factors associated with essentially two different farming systems: polyculture and semi-intensive monoculture of *Penaeus monodon*. Data were gathered from a total of 174 shrimp farmers in eight provinces of the Philippines using a structured questionnaire. Forty-seven variables related to pond history and site description, period of culture, pond preparation techniques, water management, culture methods, feed and other inputs, and biosecurity measures were investigated. In the analysis for combined monoculture and polyculture farms, feeding live molluscs was identified as important WSSV risk factors. In addition to feeding live molluscs, sharing of water source with other farms, having the same receiving and water source, larger pond size, and higher stocking density were identified as important WSSV risk factors in monoculture farms. Climate, i.e. stocking during the cold months and sludge removal and its deposition on the dikes were identified as WSSV risk factors in polyculture farms. Protective factors, listed in decreasing significance, were feeding with planktons and high mangrove to pond area ratio, both observed in the dataset with both monoculture and polyculture farms, while only the latter was observed in the dataset for monoculture farms only. No protective factor was observed in the dataset for polyculture farms.

This study confirmed the negative effect of sharing water source with other farms and identified several new factors influencing WSSV infection such as feeding live molluscs increases the risk, while feeding with planktons and high mangrove to pond area ratio reduce the risk.

4.1. INTRODUCTION

Diseases cause devastating losses to the shrimp industry world- wide (Wongteerasupaya et al., 1995). In Asia alone, the industry suggests annual losses of about 4 billion USD. Most of these pandemic diseases are of viral origin and one of the two most lethal is the White Spot Syndrome Virus, WSSV (Flegel, 2009).

There is no treatment for WSSV and prevention is the best way to avoid outbreaks (Menasveta, 2002). Several studies have investigated the effect of disinfectants on WSSV (Chang et al., 1998; Maeda et al., 1998; Balasubramanian et al., 2006). The use of vaccines and immunostimulants to control WSSV has also been explored (Citarasu et al., 2006; Satoh et al., 2008; Sajeevan et al., 2009). Biosecurity measures to exclude the pathogen or reduce its risk have been suggested (Lotz, 1997; Mohan et al., 2004). Measures adopted by the shrimp industry include the stocking of shrimp larvae confirmed WSSV negative with a polymerase chain reaction (PCR), use of disinfectants, closed culture system to reduce water exchange, bird scares, crab fence, foot/tire bath, and limited access to the farm.

Most of the studies done on WSSV risk factors dealt on the carrier organism (Lo et al., 1996; Kanchanaphum et al., 1998; Suppamataya et al., 1998; Otta et al., 1999; Corsin et al., 2001; Hossain et al., 2001; Yan et al., 2007; Liu et al., 2006), transmission (Suppamataya et al., 1998; Corsin et al., 2001; Peng et al., 1998), effect of water physico-chemical parameters (Vidal et al., 2001; Granja et al., 2003; Guan et al., 2003; Rahman et al., 2006; Reyes et al., 2007), and genetic studies (Wongteerasupaya et al., 2003; Dieu et al., 2004; Syed Musthaq et al., 2006). Only a few studies reported risk factors related to pond culture.

WSSV infection has been positively correlated with proximity of the pond to the sea and negatively to ponds closely located within a given cluster (Mohan et al., 2008). Sludge removal, ploughing, liming, complete system dry-out between culture cycles, water filtration through 300 µm mesh screen and phosphorus application through fertilization were reported to reduce risk of WSSV infection (Corsin et al., 2001; Velasco et al., 2002; Mohan et al., 2008). Corsin et al. (2001) found no association between stocking density and WSSV infection at harvest. However, Pienado-Guevara and Lopez-Meyer (2006) reported that removal of 40% and 50% of shrimp population with low level WSSV infection may improve survival. Ingestion of infected shrimp or fresh feed and use of commercial feeds have been associated with WSSV infection (Chou et al.,

1995, Chou et al., 1998; Corsin et al., 2001).

WSSV still continues to plague the shrimp industry, despite the bulk of information available. Most of this information resulted from experimental or "laboratory"-based studies relating to transmission and to the physicochemical properties of the water. Epidemiology is another approach of understanding a contagious disease. Factors related to pond location and management practices seem to affect WSSV epidemiology but these have hardly been investigated. A survey is appropriate to gather information from a population, which is beyond the control of the investigator on an experimental unit. The relationship between a disease and the hypothesized causal factors in a specified population, like between WSSV and farm practices, can be investigated using a questionnaire in a cross-sectional survey.

This study hypothesizes that factors such as pond site and pond management affect the occurrence of WSSV. Using a structured questionnaire, this study aims to identify these risk factors using an epidemiological approach.

4.2. MATERIALS AND METHODS

4.2.1. DEFINITION OF TERMS

WSSV incidence is reported when white spots were observed on the shrimp body, with or without laboratory confirmation. Monoculture farms are those farms that culture *Penaeus monodon* only. Polyculture farms culture *P. monodon* with other species such as milkfish, crab, and tilapia in the same pond. Entire dataset refers to the combined monoculture and polyculture farms.

Season denotes to the wet (June–November) and dry (December–May) seasons while climate refers to the cold (November to March) and warm (April–October) months. Classification of season and climate were based on Tendencia et al. (2010a). Sludge removal is the removal of the topsoil from the pond bottom after shrimp harvest. Crop rotation is the culture of phylogenetically different species alternately within a given area. Greenwater refers to the innovative technique wherein shrimp are cultured in water coming from another pond where tilapia and other fish species are grown. Other culture refers to culture techniques other than crop rotation and green water. Flushing refers to filling in and draining out of water into the pond during pond preparation before shrimp culture. Animal waste refers to the use of chicken/pig

manure or cow dung to fertilize the pond.

Reservoir is the use of a compartment or pond where water is stored/disinfected before use. Settling pond refers to the presence of a compartment or pond where effluent from the culture pond is drained and treated before release into the environment. Fry analysis refers to analysis of fry for abnormalities and diseases like WSSV and luminous bacteria, before stocking. Birdscare are nets or strings installed above the pond to ward off or scare birds. Crab fence are bamboo, nylon screen, or tarpaulin placed on the dike to prevent entry of crabs into the culture pond. Foot and tire baths are containers with disinfectants like chlorine placed in entry points were hands and vehicles can be rinsed or washed before entry to the pond or farm premises. Limited access is the restricted entry to the farm.

4.2.2. STUDY POPULATION AND STUDY SAMPLE

A cross sectional study was conducted from November 2007 to December 2008 in eight provinces of the Philippines with reported WSSV incidence, namely: Bulacan, Antique, Aklan, Negros, Cebu, Bohol, Northern Samar and Leyte. The questions addressed the production cycle that was harvested the same year as the interview. Farms that had two croppings per year were interviewed twice.

A total of 174 shrimp farmers were interviewed, 77 were monoculture farms and 97 were polyculture farms. Of the 77 monoculture farms sampled, 34 were interviewed twice and the remaining 33 interviewed only once. The 77 is a census of all monoculture shrimp farms in the eight provinces while the 97 polyculture shrimp farms represent a 10% randomly selected sample of all polyculture farms in the eight provinces. Staff from the local government unit of the different provinces and from a local cooperative assisted in identifying the farms to be interviewed.

4.2.3. DATA COLLECTION

The data were gathered using a structured questionnaire that addressed seven classes of variables: (1) pond site which included history and description, (2) period of culture, (3) pond preparation techniques, (4) water management, (5) culture methods, (6) feed and other inputs and, (7) biosecurity measures (please refer to columns 1 and 2 of Table 4.1). The selection of these variables was based on the different farming techniques implemented by farmers and on the measures suggested to prevent WSSV

Table 4.1. Spearman's rho correlation coefficients (r) and significance level (P) of all variables with WSSV occurrence. Values in bold were included in the univariate binary logistic analysis.

Class	Factor	Entire dataset (*n*=174)		Monoculture (*n*=77)		Polyculture (*n*=97)	
		r	*P*	*r*	*P*	*r*	*P*
Site Description	Province	-0.04	0.63	**-0.17**	**0.14**	+0.04	0.69
	Yrs in oper	-0.06	0.46	+0.05	0.66	-0.08	0.45
	MPR	**-0.12**	**0.13**	-0.12	0.29	-0.11	0.27
	Adjacent farm	**+0.17**	**0.03**	**+0.18**	**0.11**	-0.05	0.60
	Prev mangrove	**-0.02**	**0.04**	-0.01	0.97	-0.02	0.84
	Proximity to sea	+0.08	0.33	+0.12	0.32	-0.02	0.89
	Prox to road	+0.07	0.34	**+0.18**	**0.12**	-0.07	0.51
	Pond size	+0.64	0.63	**+0.28**	**0.01**	-0.02	0.87
Period	Season	-0.03	0.74	+0.07	0.57	-0.07	0.47
	Climate	+0.09	0.24	+0.57	0.76	**+0.17**	**0.04**
	Mo of stocking	+0.03	0.67	+0.09	0.46	+0.12	0 26
	Mo of harvest	-0.06	0.47	-0.02	0.90	-0.05	0.62
Pond preparation	Sludge removal	**+0.13**	**0.10**	+0.03	0.79	**+0.35**	**0.00**
	Ploughing	-0.10	0.21	-0.02	0.86	-0.12	0.25
	Flushing	+0.09	0.23	+0.03	0.83	**-0.17**	**0.10**
	Drying	-0.06	0.47	-0.10	0.38	-0.06	0.58
	Lime appli	+0.05	0.50	-0.02	0.88	**+0.15**	**0.14**
	Teaseed	-0.00	1.00	-0.03	0.83	+0.03	0.78
	Animal waste	-0.06	0.43	+0.06	0.62	-0.03	0.80
	Inorganic fert	+ 0.05	0.51	+0.10	0.69	+0.00	0.97
Water mgt	Drain to mang	+0.05	0.54	+0.03	0.75	+0.05	0.65
	Share water	**+0.19**	**0.01**	**+0.33**	**0.00**	-0.05	0.63
	Same Rec/sourc	**+0.17**	**0.02**	**+0.25**	**0.03**	+0.03	0.80
	Reservoir	-0.02	0.87	+0.04	0.72	+0.02	0.84
	Settling pond	-0.10	0.20	**-0.18**	**0.12**	+0.08	0.42
	Tidal	**+0.13**	**0.10**	+0.13	0.28	+0.10	0.34
	Water depth	+0.01	0.94	-0.03	0.82	-0.01	0.94
Culture method	Crop rotation	+0.00	0.98	+0.02	0.86	+0.02	0.82
	Greenwater	-0.08	0.30	-0.15	0.18	+0.14	0.16
	Other	-0.02	0.80	**-0.12**	**0.13**	+0.18	0.25
	croppings/yr	-0.08	0.27	+0.01	0.97	-0.09	0.37
	Stocking density	**-0.20**	**0.01**	**-0.30**	**0.01**	-0.03	0.77

Table 4.1. Continuation

Class	Factor	Entire dataset (*n*=174)		Monoculture (n=77)		Polyculture (n=97)	
		r	*P*	*r*	*P*	*r*	*P*
Feed	Comm pellet	+0.07	0.43	-0.04	0.76	**+0.21**	**0.04**
	Live mollusks	**+0.22**	**0.00**	**+0.26**	**0.02**	**+0.20**	**0.05**
	Trash fish	+0.02	0.83	+0.05	0.64	-0.03	0.78
	Cereals	-0.08	0.29	-0.02	0.90	-0.14	0.17
	Planktons	**-0.18**	**0.02**	**-0.21**	**0.06**	**-0.29**	**0.01**
	Other food	-0.04	0.60	-0.02	0.90	-0.08	0.45
other inputs	Probiotics	-0.04	0.56	-0.03	0.83	-0.00	0.99
	Antibiotics	+0.06	0.45	+0.05	0.64	+0.08	0.42
	Other chemicals	+0.09	0.24	+0.14	0.21	+0.08	0.42
Biosecurity measures	Fry analysis	+0.03	0.73	-0.04	0.76	**+0.18**	**0.08**
	Birdscare	-0.01	0.94	+0.03	0.85	-0.00	0.99
	Crab fence	-0.04	0.64	-0.04	0.73	+0.03	0.74
	Foot bath	-0.03	0.73	+0.02	0.89	-0.03	0.77
	Tire bath	-0.02	0.79	+0.02	0.85	-0.30	0.77
	Limited access	-0.04	0.59	-0.08	0.52	-0.34	0.74

occurrence as mentioned in the introduction.

The questionnaire was developed in English, and administered in the local language to ensure that farmers would understand all the questions. It was pre-tested or validated with 10 farmers outside the study sample and questions were refined according to feedback from the farmers. The final questionnaire was administered by the main author with the help of the staff from the provincial government unit or from the local cooperative. It was emphasized that answers would be highly confidential, and that correctness of the answers was necessary for the proper analysis of the results. Interviewers were allowed to enter the farm premises in most cases. This allowed the validation of the gathered information. Staffs of the local government unit and of the local cooperative were knowledgeable of the farms' operation, and thus also assisted in the validation.

The respondents were persons involved in farm management: farm owners, managers, technicians and caretakers. If farm owners were interviewed, they referred to the managers or technicians for questions they were unable to answer.

4.2.4. STATISTICAL ANALYSIS

A total of 47 variables (please refer to column 2 of Table 4.1) were used for the analysis. Data gathered were mostly binary (yes/no) except for months of stocking or harvest, mangrove to pond area ratio (MPR), proximity to the sea or road (km), pond size (ha), water level inside the pond (cm), and number of croppings/year. No scaling was used in the case of the non-binary data. Data analysis was done in two steps: variable reduction to increase model stability and binary logistic regression to identify the protective and risk factors. WSSV incidence was used as the dependent variable that needs to be explained. The procedure was applied to the entire dataset and to two sub-sets of the data: farms into monoculture, and farms into polyculture. All the statistical analyses were done with SPSS V. 17.

4.2.4.1. VARIABLE REDUCTION

Variable reduction was done to increase model stability. The correlation of all variables to WSSV was determined using Spearman's rho correlation analysis. Variables with rho correlation significance level $P<0.1$ were selected. Univariate binary logistic regression analysis was applied on the selected variables. Correlation analysis was used for variable reduction to prevent collinearity of variables included in the final model. Variables with univariate slope parameter that is non-significant with a P-value greater than 0.1 were eliminated. The remaining variables are the reduced variables.

4.2.4.2. BINARY LOGISTIC REGRESSION

Binary logistic regression's backward stepwise method with a likelihood ratio test was applied on the reduced variables to identify the predictors that will best explain WSSV occurrence.

The backward stepwise logistic regression eliminates at each consecutive step the variable that contributes the least to explain the model until the smallest possible log-likelihood ratio, indicator for the statistical fit of the model, is attained. The software lists all steps and for each step it gives indications for the model fit. The model giving the best combination of low negative log-likelihood value, R^2 value close to one, and high classification ratio (CR), in predicting WSSV incidence for each group was retained. The negative log-likelihood ratio is the measure of the relationship between the dependent and independent variables; while the R2 value is the indication of the strength of the

relationship between the two. The CR is the percentage correctly classified cases, either 0 or 1, by the model. The multicollinearity diagnostics from the linear regression analysis was used to assess if the model parameters were biased. In case of multicollinearity, the procedure was repeated replacing a specific factor with a correlated independent factor of the same class of equal importance to check the contribution to the explanation of the variability.

4.3. RESULTS

4.3.1. VARIABLE REDUCTION

Table 4.1 shows the correlation coefficient, including significance level, of the different factors or variables correlated with WSSV incidence using Spearman correlation analysis. A positive correlation coefficient indicates that an increase in the corresponding variable will increase the risk of WSSV incidence. On the contrary, a negative correlation coefficient implies that an increase in the level of that variable will reduce the risk of WSSV occurrence.

Factors correlated ($P<0.1$) with WSSV incidence using the entire dataset were high MPR, presence of adjacent farms, sharing of water source with other farms, having the same receiving and water source, water change dependent on tide (tidal), sludge removal, stocking density, and feeding the shrimp with live molluscs and planktons. High MPR, presence of adjacent farms, sharing of water source with other farms, having the same receiving and water source, pond size, stocking density, and feeding with live molluscs and planktons were correlated to WSSV incidence in monoculture farms. Climate, sludge removal, pond flushing, lime application, fry analysis, and feeding commercial pellets, or molluscs and planktons to the shrimp were correlated to WSSV in polyculture farms.

Table 4.2 shows the unstandardized coefficient of the different variables including their significance levels. The unstandardized coefficients explain the effects of the variables on the odds of WSSV incidence. A positive coefficient indicates that an increase in the corresponding variable will increase the risk of WSSV incidence. On the contrary, a negative coefficient implies that an increase in the level of that variable will

Table 4.2. The unstandardized coefficients (ß) with significance values (P) of the different variables following univariate binary logistic regression (BLR). Values in bold were used in the final BLR.

Class	Factor	Entire dataset (*n*=174)		Monoculture (*n*=77)		Polyculture (*n*=97)	
		ß	*P*	ß	*P*	ß	*P*
Site description	Ratio	**-0.31**	**0.13**	**-0.57**	**0.07**		
	With adjacent farm	**+0.84**	**0.03**	**+0.78**	**0.11**		
	Previously mangrove	-0.06	0.85				
	Proximity to the road			+0.04	0.56		
	Climate					**+0.76**	**0.1**
	Pond size			**+1.43**	**0.09**		
	Province			-0.07	0.58		
Pond Preparation	Sludge removal	**+0.51**	**0.10**			**+2.04**	**0.00**
	Flushing					**+0.78**	**0.10**
	Lime application					+0.64	0.18
Water management	Share water	**+0.81**	**0.02**	**+0.40**	**0.00**		
	Same receiving/water source	**+0.76**	**0.03**	**+1.00**	**0.00**		
	Tidal	**+0.54**	**0.10**				
	Settling pond			- 0.48	0.23		
Culture method	Stocking density	**- 0.04**	**0.01**	**-0.07**	**0.02**		
	Other culture method			- 21.39	1.00		
Feed and other input	Commercial pellet					**+0.90**	**0.04**
	Live molluscs	**+0.92**	**0.00**	**+1.70**	**0.04**	**+0.83**	**0.06**
	Natural food	**-0.81**	**0.02**	**-1.86**	**0.10**	**-1.17**	**0.07**
Biosecurity measures	Fry analysis					**+0.84**	**0.08**

MPR=mangrove to pond area ratio; Climate=cold (November to March) and warm (April–October) months; Sludge removal=removal of the topsoil from the pond bottom after shrimp harvest; Flushing=filling in and draining out of water into the pond; Settling pond=compartment or pond where effluent from the culture pond is drained; Tidal=water change dependent on tide; Fry analysis=health analysis of fry before stocking;

reduce the risk of WSSV occurrence. Factors or variables with unstandardized coefficients significance level of P<0.1 were considered for the final model of WSSV infection risk factors. All variables included in the univariate binary logistic regression analysis had unstandardized coefficients significance level of P<0.1 and thus were all considered for the final model.

4.3.2. BINARY LOGISTIC REGRESSION

The overall CR of the models that will best explain WSSV infection was comparable for all subsets. Overall CR for the entire dataset was 63%; for monoculture 73% and for poly-culture 66% (Table 4.3). No collinearities were observed between variables in all models in the analysis for the entire dataset and the two subsets, monoculture and polyculture farms. The variance loadings are onto different dimensions. Tolerance remains largely above 0.1 (0.57 and higher) and variance inflation factor far below 10 (1.76 and lower), which are the frontier values according to Menard (1995) and Myers (1990), both cited by Field (2005). The models produced using binary logistic regressions are valid.

Table 4.3. Characteristics of the binary logistic regression of retained WSSV risk factors on the complete dataset and on the subsets of monoculture and polyculture farms (for variables considered in the model see Table 4).

	-2LL	R^2	Classification Ratio		
Subsets			0	1	0verall
Entire	219	0.13	38	83	63
Monoculture	86	0.31	64	81	73
Polyculture	112	0.18	28	92	66

-2LL=-2 log likelihood; R2 according to Nagelkerke ;

0 = without WSSV incidence; 1=with WSSV incidence.

The factor common in the models for the entire dataset and monoculture was feeding with planktons as a protective factor. Feeding molluscs and plankton to the shrimp was a common factor for the entire dataset and polyculture. Factors included in the model for WSSV as well as the direction of their association with WSSV infection are presented in Table 4.4.

Table 4.4. Variables included in the final binary logistic regression (BLR) model that best describe WSSV risk factors associated to shrimp farming practices in 8 provinces of the Philippines. Variables with values are included in the final model. The mentioned values are the unstandardized coefficients (ß) of the constant with the significance values (P).

Class	Factor	Entire (*n*=174)	Monoculture (*n*=77)	Polyculture (*n*=97)
		ß (P)	ß (*P*)	ß (*P*)
Site description	Ratio	-0.28 (0.18)		
	Pond size		+1.16 (0.23)	
Period	climate			+0.85 (0.09)
Pond Preparation	Sludge removal			+1.99 (0.00)
Water management	Share water		+0.80 (0.15)	
	Same receiving and source		+0.82 (0.12)	
Culture method	Stocking density		-0.06 (0.07)	
Feed and other input	Live molluscs	+0.98 (0.00)		+0.90 (0.06)
	Natural food	-0.93 (0.01)	-2.46 (0.06)	

MPR=mangrove to pond area ratio; Climate=cold (November to March) and warm (April–October) months; Sludge removal=removal of the topsoil from the pond bottom after shrimp harvest.

4.3.3. WSSV RISK/PROTECTIVE FACTORS

Eight variables that describe the farm site were analysed in relation to WSSV infection. Only five were correlated to WSSV and two were included in the final model. Pond size was identified as a risk factor for monoculture farms: the larger the pond the greater the odd of WSSV infection. On the other hand, MPR was retained in the model for the entire dataset as protective factor. The higher the MPR the lesser is the odd of WSSV incidence.

The possible interaction between WSSV occurrence, province and MPR was investigated in a univariate ANOVA. Within the complete dataset province effected the occurrence of WSSV ($P<0.01$) but neither 2- way nor 3-way interaction occurred between province, farm type and MPR. Neither of the subsets showed interaction between MPR and province; province effected the occurrence of WSSV in the case of monoculture only ($P<0.05$). The representation of farms with a MPR>0 is lower among

the polyculture farms compared to the monoculture farms of which half were interviewed twice (Tendencia et al., unpublished).

Only one factor related to the period of stocking was correlated to WSSV infection: climate: the cooler the climate the greater the odd of WSSV infection. However, climate was identified as a WSSV risk factor in polyculture farms only.

Three variables related to pond preparation were associated with WSSV infection. However, only one was retained in a final model. Sludge removal, i.e. removing the topsoil of the pond bottom after shrimp harvest and its deposition on the dike was identified as a risk factor in the analysis for polyculture farms only.

Sharing water source with other farms, having the same receiving and water source, and water change dependent on tide were associated with WSSV infection during variable reduction. However, only sharing water source with other farms and having the same receiving and water source were retained in the final analysis for monoculture farms. No factor related to water management was retained in the final model for the entire dataset and for polyculture farms.

Only one factor related to culture method was associated to WSSV infection: stocking density was a WSSV risk factor in monoculture farms. The higher the stocking density the greater the odd of an infection. Stocking density has a negative unstandardized coefficient, which implies that an increase in stocking density will reduce risk of WSSV. However, the Spearman correlation between stocking density and WSSV occurrence was negative.

Five variables were identified as important factors for WSSV infection, however one of them was excluded in the final analysis because of its similarity in its effect to WSSV with another variable, which was more strongly correlated. Using commercial pellets was identified as a risk factor in polyculture farms, and feeding live molluscs in the analysis that included either either farm types or monoculture farms only. Feeding planktons to the shrimp was included in the final models for the entire dataset and for monoculture as a protective factor.

Only one factor related to biosecurity measures was correlated with WSSV infection. Fry analysis, i.e. analysis of fry for abnormalities and diseases like WSSV and luminous bacteria, before stocking, was identified as an important factor associated with WSSV infection during variable reduction but was excluded in the final model.

4.4. DISCUSSION

Presence of mangrove can reduce the risk of WSSV infection, the higher the MPR, the less WSSV incidence. Mangroves were reported to have the capacity to process nutrients from pond effluents (Trott and Alongi, 2000). Mangrove is inhabited by diverse bacterial and phytoplankton communities that are responsible for the degradation and recycling of nutrients making them available to other organisms in the food web (Alongi, 1994; McKinnon et al., 2002; Al-Sayed et al., 2005). Another protective factor was feeding planktons to the shrimp. Some planktons are reported to improve water quality and have probiotic effect (Tinh et al., 2006; Sreesai and Pakpain, 2007). However, mangroves harbour WSSV carriers like crabs and some planktons are WSSV carrier (Yan et al., 2004; Esparza-Leal et al., 2009). The low representation of farms with a MPR>0 among those in polyculture might explain the absence of a significant effect on WSSV occurrence in this dataset.

Climate was one of the WSSV risk factors. Climate as a risk factor referred to stocking of shrimp during the cool months. Low atmospheric temperature, which affects water temperature, is an important WSSV risk factor (Tendencia et al., 2010b). Exposure to low water temperature could lead to WSSV infection in pond cultured *P. monodon* (Tendencia et al., 2010a). This has been attributed to the increase in viral replication and the decrease in the immune response of shrimp at low temperature (Vidal et al., 2001; Reyes et al., 2007). Crops grown during the warmer season were less likely to experience WSSV (Corsin et al., 2005). Stocking during the warm months is advisable.

Shrimp cultured in ponds with a larger size have a greater risk of WSSV infection. Larger ponds are more difficult to manage. The inefficiency in farm management may lead to disease occurrence in cultured shrimp due to stress.

Sharing of water source with other farms poses risk, and is strongly correlated with the presence of adjacent farms. These are in agreement with Mohan et al. (2008) who reported that WSSV infection is negatively correlated to ponds closely located within a given cluster. It is possible that water that is allowed to enter the pond is mixed with discharge of other farms or of the farm itself. This water could be contaminated. This argument also holds for having the same receiving and water source as a risk factor. Contamination of water can also come from the sludge removed from the pond bottom. Contrary to Avinemelech and Ritvo (2003) and Mohan et al. (2008), sludge removal is a risk factor of WSSV infection in the models of entire dataset and of polyculture farms.

Sludge is made up of accumulated organic matter such as excess feed and faeces. Organic matter harbours microorganisms that could be pathogenic to shrimp. In most instances, sludge removed from the pond bottom is placed on the dike. This might allow harmful microorganisms present in the sludge to be washed back into the pond. The amount of sludge that accumulates at the pond bottom is affected by stocking density, which is also a risk factor. The higher the stocking density the more organic matter due to faeces and uneaten feed may accumulate at the pond bottom.

Feeding live molluscs to shrimp poses as risk for WSSV infection, which might be due to two ways of transmission. Molluscs being filter feeders can serve as WSSV carriers, and molluscs could ingest WSSV particles from the soil and the water column, which could be transferred to the shrimp when the latter feed on the former. Feeding commercial pellets as risk factor in polyculture farms might be a consequence of sludge accumulation at the pond bottom due to the absence of pond preparation in these farms.

Contrary to Mohan et al. (2008), biosecurity measures did not prevent WSSV occurrence in this study. Biosecurity measures aim to exclude pathogens and carrier organisms from the culture environment; e.g. farmers installed bird scare and crab fence to prevent the entry of birds and crabs. However, these biosecurity measures often do not reach their purpose. Birds still fly over the strings that are supposed to scare them, might defecate above the pond and the faeces contaminated with WSSV particles could infect the cultured shrimp. Crab fence, usually made of nets, is installed on the pond dike to prevent the entry of crabs; however, crabs could still enter the pond by crawling through the net or by making holes through the dike. Farmers should optimize their biosecurity measures.

Limited access to farm, foot baths and tire baths, and farm management strictly implemented hand disinfection. These measures concern humans as carriers. But the question is whether human can really carry WSSV particles that could infect the cultured shrimp? This question is not just anecdotic; no scientific evidence proves that humans can transmit WSSV or particles carrying WSSV, although Corsin et al. (2005) found that sharing of personnel between ponds is associated with WSSV. Stocking of WSSV negative fry could prevent the entry of WSSV into the culture system, but shrimp once inside the pond can be infected in several ways.

The main effects of the protective and risk factors are on water or pond bottom quality and immune response of shrimp. Some WSSV risk factors such as feeding live

molluscs to the shrimp can be avoided. Sludge removed from the pond should be disposed in a place where washing back into the system is prevented. Stocking during the cold months can be avoided. The effect of other risk factors can be mitigated by the implementation of the identified protective factors. Farms sharing water source with other farms could plant mangroves in the water source and in the receiving environment to improve water quality. Growth of planktons can be enhanced to improve water quality and enhance the shrimp's immune response.

This study confirmed the assumed negative effect of sharing water source with other farms but showed that biosecurity measures had no significant effect on WSSV incidence. Several new factors influencing WSSV infection were identified: feeding live molluscs increases the risk, while feeding plankton and high MPR reduce the risk in monoculture farms. Further studies are needed to verify the effect of ecological protective factors such as the presence of mangroves, taking into consideration the mangrove to pond area ratio. Current best management practices in shrimp culture should be improved and properly implemented.

CHAPTER 5

EFFECT OF DIFFERENT MANGROVE TO POND AREA RATIO ON WSSV OCCURRENCE IN PENAEUS MONODON SEMI-INTENSIVE FARMS USING THE GREENWATER TECHNIQUE

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(Including slight modifications regarding the significance of the effect of MPR)

ABSTRACT

White spot syndrome virus (WSSV) has been affecting the shrimp industry worldwide for two decades now. It continues to bring economic losses to affected farms. Despite the many studies on its epidemiology, there is no proven treatment or control measure. Diseases, like the WSSV, results from the interaction of three factors: host, pathogen and environment. The environment plays an important role in disease development and determines the health or the immune capacity of the shrimp. High mangrove-to-pond area ratio (MPR) is reported as a possible protective factor against WSSV. This study investigates if mangroves affect the physicochemical properties of the water and soil as well as the prevalence of infectious agents like the WSSV by monitoring monoculture shrimp farms with different MPR (0:1, 1:1, 4:1).

Results showed no difference in water quality between farms with different MPR. Significantly higher available sulfur was observed in MPR-4; significantly higher percentage green vibrios in the soil in MPR-0. WSSV was detected in farms with MPR-1 and MPR-4 but did not result in an outbreak; the sample size does not allow concluding whether the presence of mangroves could prevent WSSV outbreak.

5.1. INTRODUCTION

White spot syndrome virus (WSSV) has been affecting the shrimp industry worldwide for two decades now (Walker, 2010). It continues to bring economic losses to affected farms. Diseases, like the WSSV, result from the interaction of three factors: the host, the pathogen and the environment (Lightner and Redman, 1998; Kabata, 1985). Micro-organisms are part of the normal flora of the water (Alam et al., 2002), causing disease if the host is stressed (Horowitz and Horowitz, 2001; Alday-Sanz et al., 2002). On-farm stresses may compromise an organism's ability to resist infection (Crane & Hyatt, 2011). Despite the many studies on WSSV epidemiology, there is no proven treatment or control measure. Studies on control measures were on the use of chemicals and immunostimulants (Balasubramanian et al., 2006; Chang et al., 2003; Rosalind et al., 2006; Witteveldt et al., 2004). Reports on ecological means of disease control are lacking.

In shrimp ponds, water conditions deteriorate when the nutrient level increase due to the accumulation of excess feed and fecal matter (Soundarapandian & Sankar, 2010). Exposure of shrimp to stressors increases the risk of WSSV, since stressors could compromise the shrimp's defence system (Takahashi et al., 1995). Consequently, under stressful conditions, WSSV could replicate rapidly and subsequently cause the death of the shrimp (Lo & Kou, 1998). The best way to prevent disease occurrence is to minimize stress by providing the shrimp with good environmental conditions as manifested by the physicochemical properties of the water and soil.

Water quality could be improved by using probiotics, which may also enhance shrimp immunocompetence (Soundarapandian & Sankar, 2010; Ninawe & Selvin, 2009). However, the use of probiotics does not always prevent WSSV outbreaks (Tendencia et al., 2011). The latter authors identified feeding with plankton as a WSSV protective factor and a trend indicating that high moangrove –to-pond area ratio (MPR) might be a protective factor. This is in consonance with the report that lower prevalence of infectious hypodermal and haematopoietic necrosis virus (IHHNV) disease was observed in *P. monodon* in regions with no major aquaculture industry and with intact mangroves; higher IHHNV prevalence was observed in areas with intensive shrimp farms and severely degraded mangroves (Belak et al., 1999). The way mangrove influences disease occurrence could be via its effect on water quality. Several studies have shown the capability of mangroves to remove nutrients from pond effuents (e.g.,

Saenger et al., 1983; Robertson and Philipps, 1995; Primavera et al., 2007; Shimoda et al., 2007). The capacity of the mangrove estuary to assimilate or transform nutrients received periodically from shrimp ponds was attibuted to tidal flushing complemented by biological processes of mineralization and subsequent dissipation by food webs associated with the habitat (Trott et al., 2004). Furthermore, geomorphological setting, forest type, and biogenic structures such as pneumatophores and crab burrows determines nutrient exchange (Mitra et al., 2011; Adame et al., 2010; Penha-Lopes et al., 2010; Adame & Lovelock, 2011) . There is a high rate of bacterial activity and nutrient turnover in mangrove sediments. The mangrove habitat harbours diverse bacterial communities and possesses self-cleaning properties (Al-Sayed et al., 2005). The self-cleaning property of mangrove habitats is attributed to the phytoplankton and microorganisms present in the ecosystem that remove nutrients from shrimp farm effluents and transform them into more bioavailable form (McKinnon et al., 2002; Holguin et al., 2001). These microorganisms are responsible for the degradation and recycling of essential elements such as carbon, nitrogen and phosphorus (Alongi, 1994). Several studies have reported the importance of mangrove to farm area ratio in relation to nutrient removal. Results vary between the studies. According to Saenger et al. (1983) 4 ha of mangroves for every hectare of pond is needed to establish a healthy ecosystem. Robertson and Philipps (1995) stated that 2 to 22 ha of forest are required to filter the nitrogen and phosphorus loads from the effluent produced by a 1 ha pond. A much different figure was presented by Rivera-Monroy (1999) who found that between 0.04 to 0.12 ha of mangrove forest is required to completely remove the dissolved inorganic nitrogen load from effluents produced by a 1-ha pond. In mixed mangrove and pond systems, between 2.1 and 5.2 unit areas of mangroves is required to remove nitrogen remaining in the aquaculture pond (Shimoda et al., 2007); while, 6.2 to 8.9 ha of mangrove area is required to fully process the phosphorus in a 1 ha shrimp pond (Shimoda et al., 2005). In a detailed study, wherein the physicochemical parameters of the water were monitored monthly in creek, reservoir, shrimp pond, and experimental mangrove, Primavera et al. (2007) calculated that 1.8-5.4 ha of impounded mangroves are required to remove the nitrate wastes from 1 ha of shrimp pond. Furthermore, they found that nitrogen was absorbed by the plants as evidenced by the longer nipa palm leaflets.

Based on on-farm data, the present study aimed to assess the effect of different

mangrove to pond area ratios on the quality of the influent water and its effect on the occurrence of WSSV and other diseases in shrimp pond culture; and to compare water and soil quality at different points and stages of production, occurrence of WSSV and other diseases in shrimp pond culture; and to compare water and soil quality at different points and stages of production.

5.2. MATERIALS AND METHODS

5.2.1. FARM CHARACTERISTIC

Six farms into semi-intensive monoculture of *P. monodon* in the Visayas, central Philippines were used in the study (Figure 5.1). Two of the farms (Farms 3 and 4) had 4 ha mangrove in the receiving environment to every hectare of pond (MPR-4), 2 farms (Farms 1 and 2) had 1.2 ha mangrove to every hectare of pond (MPR-1), and 2 (Farms 5 and 6) did not have mangroves in the receiving environment (MPR- 0). The choice of MPR was based on a cross sectional study done on shrimp farms in the Philippines, wherein the highest MPR observed was 4:1 and the lowest was 0:1 (Tendencia et al., 2011); this fits with the MPR recommended by Saenger et al. (1983) for a sustainable aquaculture. Mangrove community structures of the farms were described by the authors in another paper (Tendencia, unpublished).

All 6 farms had a reservoir stocked with tilapia at >2tons/ha biomass. The reservoir was filled with water from the river (Farms 1, 4, 5 and 6) or from deep wells (Farms 2 and 3) and stocked with tilapia a month before stocking of shrimp in the culture pond. Water from the reservoir was used to culture the shrimp. Minimal water change was implemented and only when needed i.e. in case of high luminous bacterial load. Water lost due to evaporation was replenished to maintain a water depth of 1.0m to 1.2m.

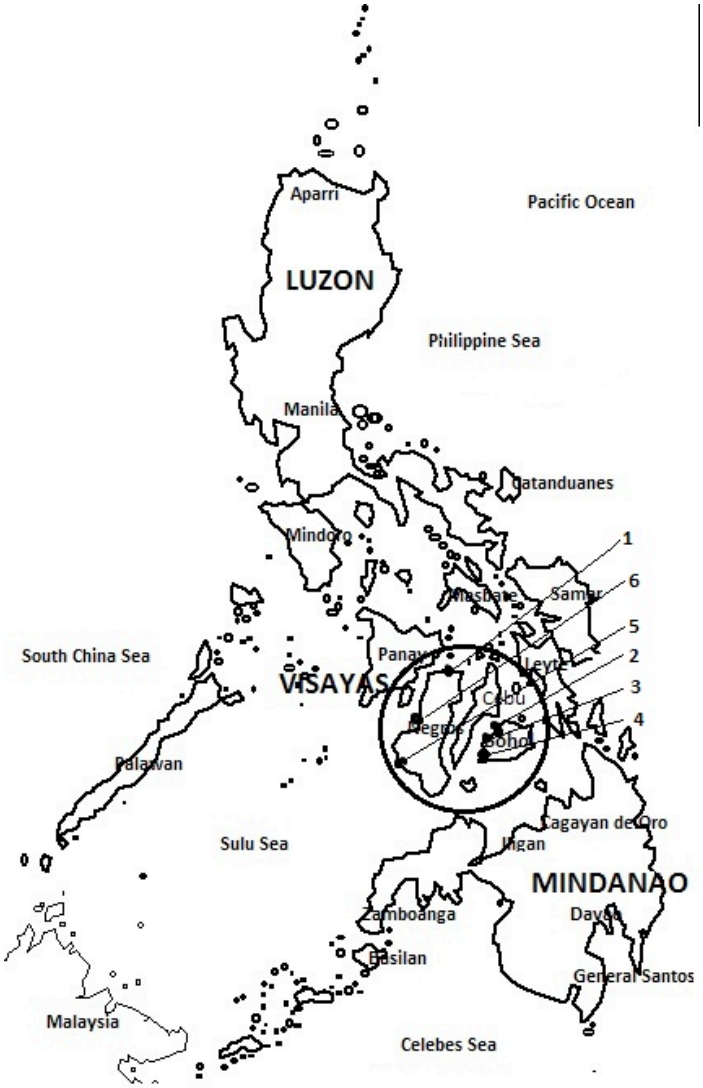
5.2.2. SAMPLING

In the text, unless otherwise specified, the term 'pond' collectively refers to the culture pond, reservoir, intake channel and drainage channel.

Water and soil samples were taken from 4 locations in the pond: from intake channel before water enters the pond, inside the reservoir, inside the culture pond, and in the drainage channel, 1 m behind the outlet gate. Water was taken midway of the

water column. Soil was taken from the upper 5 cm of the bottom. Samples were taken from the farm <7 days before stocking and every 30±5 days thereafter until 120±5 days of culture (DoC). Three shrimp samples were taken each at DoC 30±5 days and every 30 days thereafter until DoC 120±5 days.

Figure 5.1. Map of the Philippines showing farm sites (1-6).



5.2.2.1. WATER

Temperature, DO, and pH were measured on site. Water samples for NH_3, NH_4,

NO_3, NO_2, and TSS were brought to the laboratory in a chilled container for analysis. Water samples (100ml) for bacteriological analysis were collected using autoclaved (121^{o}C for 15 min) bottles.

Temperature and DO were measured using YSI 55 (YSI instruments); and pH using HI 98108 (Hanna Instruments). The phenate method was used for ammonia analysis (APHA 1995), flow injection analyzer for ammonium and nitrate, colorimetry for nitrite (ISO 13395, 1996), and the ascorbic acid method (APHA 1995) for phosphate. TSS was measured using the filtration drying method (Rainwater and Thatcher, 1960).

Water samples for bacteriological studies were serially diluted ten-fold using sterilized seawater. Representative dilutions were plated in duplicates onto Nutrient Agar (NA; BBL) with 1.5% (weight per volume; w/v) sodium chloride (NaCl) for total and the luminous bacterial counts; thiosulfate citrate bilesalt sucrose agar (TCBS; BBL) for the presumptive Vibrio counts; Pikovskaya Agar (PA; Himedia) for the phosphate solubilizing bacterial counts; Jensen's Agar (JA; Himedia) for the nitrogen-fixing bacterial count; and Czapex Dox Agar (CDA; Himedia) supplemented with 1.5% (w/v) yeast extract (BBL) for the fungal counts. All inoculated media were incubated at room temperature (approx. 30oC). Total bacterial and presumptive *Vibrio* counts were counted after 18–24 h incubation. Luminous bacteria were counted in a dark room to observe luminescence. Phosphate solubilizing bacteria, nitrogen fixing bacteria and fungi were counted after 2-6 days incubation.

5.2.2.2. SOIL

Soil redox and wet pH were measured on-site. Soil samples were air-dried and analyzed for organc matter (OM) and available sulphur (AS). Soil samples for bacteriological study were also collected. Soil redox was measured using ORP meter (Eutech Instruments). Kelway pH meter (Kel Instruments Co., Inc) was used to measure soil wet pH. Soil OM was analyzed using the procedure described by Wackley and Black (1934); and AS using turbidimetry (Fortes & Pahila, 1980).

For the bacteriological study, 0.1 g wet soil was suspended in 1.0 ml sterile seawater, serially diluted, plated, and bacterial growth counted as in water samples.

5.2.2.3. SHRIMP

Shrimp samples were individually processed for bacteriology. Shrimp

hepatopancreas were aseptically taken, weighed in sterile test tube and homogenized in sterile seawater at 1:1 ratio (w/v). Tissue suspension was serially diluted, plated on NA and TCBS, and bacterial growth counted after incubation as in the water samples.

Gill tissues from the 3 shrimp samples were pooled, fixed in 95% ethyl alcohol and stored in the refrigerator at 4oC until WSSV qPCR analysis. For the QSSV quantification, total genomic DNA and associated WSSV viral DNA were extracted from the gill tissue (5 mg) using DNA kit (Quiangen Biotech CO., LTD). The DNA concentration and quality were determined by measuring optical density at OD 260 nm, by using a GeneQuant pro (Spectronic instrument, Inc., New York, USA). A 25 ng/µl DNA was prepared for real time PCR (protocol was based on Durand and Lightner, 2002). A sample of 40 ng total DNA was added to a PCR mixture containing 0.25 µM of each primer and 0.125 µM TaqMan probe in a final reaction volume of 20 µl. The amplification program consisted of 30 s at 95 for initial denaturation followed by 45 cycles of 5 s denaturation and 20 s annealing and extension at 60 s. Thermal cycling was performed on GeneAmp 5700 Sequence Detection System A set of standard dilutions (100 to 106 viral copies/µl) was created from a plasmid containing the target amplicon and run simultaneously with the specimen DNA. All samples were run in duplicate with two negative controls for each run.

5.2.4. STATISTICAL ANALYSIS

Results were analyzed using ANOVA in the repeated measures designs in SPSS V.19. Repeated measure reduces the unsystematic variability in the design and thus provides greater power to detect effects. Mauchley's test was used to test sphericity or the hypothesis that the variances of the differences between conditions are equal. If Mauchley's test is not statistically significant (P>0.05), then there is homogeneity in the variances and the condition of sphericity was met and ANOVA using repeated measures is valid. If data violated the assumption of sphericity, corrected and valid F-ratios are produced by using the estimates of sphericity recommended by Greenhouse and Geisser (1959) and Huynh and Feldt (1976) as described by Field (2004). When sphericity estimates are greater than 0.75 then the Huyn-Feldt correction was used; otherwise, the Greenhouse-Geisser was used. Differences between DoCs and sampling sites were analyzed using Tukey's post hoc test.

The repeated measures were location and DoC; the independent variable was

MPR. Bacterial and fungal counts and viral copies were log 10 transformed before analyses. Averages of the observed physico-chemical water parameters (4% of the whole data set) belonging to the same treatment were computed to fill in missing values. Variables with more than 4 missing values at a given day were not analyzed.

5.3. RESULTS

Abbreviations used for the different parameters and other terminologies found in this paper are presented in Table 5.1. The estimated marginal means for the intake channel are presented in Table 5.2, for the sampling sites in Table 5.3, and for the different MPRs in Table 5.4. The standard deviation of observed measurements of most parameters is large. ANOVA was used to explain the differences between subjects (Table 5.5).

WSSV was detected in one each of the farms in MPR 1 (18.5 WSSV/mg gills at DoC 60) and one in MPR 4 (17.8 WSSV/mg gills at DoC 120).

Mauchley's test was significant in the repeated measures analysis for NH_4, TAN and AS. Estimated sphericity for the 3 parameters was less than 0.75, thus the corrected Greenhouse-Geisser was used to test sphericity. Greenhouse and Geisser test for the three parameters was not significant, thus sphericity was not violated. Significant differences were observed in the AS (P=0.018) and sPGV (P=0.047). The sLB in farms with different MPR is near to significant (P=0.07).

Data analysis showed no significant difference in the water and soil quality of samples taken from the intake channel with different MPR except for NO_2 (P=0.03)(Table 5.2). The trend is decreasing NO_2 as MPR increased.

No significant difference in the sPGV and sLB between DoCs, and locations was observed. The interactions were non-significant thus the significant differences in the sPGV and sLB between farms with different MPR (P=0.047) can be attributed to the presence of mangroves. The AS (P=0.018) was significantly different for both MPR (P=0.05) and site (P=0.033)(Table 5.5).

Increasing levels of NH_3, NH_4, TAN, NO_2, wTBC and wGV were observed as the days of culture increased; significantly higher levels were observed at DoC 120 except for wTBC which was highest at DoC 90 (data not shown). Although not significantly different, increasing levels with DoC were observed for NO_3, PO_4, wYV, wPVC, and wPSB.

Table 5.1. Abbreviations used for the different parameters and terminologies that can be found in the paper.

Parameter	Abbreviation	Unit
Mangrove-to-pond area ratio= 0:1	MPR-0	na
Mangrove-to-pond area ratio=1.2:1	MPR-1	na
Mangrove-to-pond area ratio= 4:1	MPR-4	na
Days of culture	DoC	na
Water		
Ammonia	NH_3	ppm
Ammonium	NH_4	ppm
Total ammonia nitrogen	TAN	ppm
Nitrite	NO_2	ppm
Nitrate	NO_3	ppm
Phosphate	PO_4	ppm
Total suspended solids	TSS	ppm
Temperature	^oT	^oT
Dissolved oxygen	DO	ppm
Salinity	sal	ppt
Water pH	wpH	na
Luminous bacteria	wLB	cfu/ml
Total bacterial count	wTBC	cfu/ml
Yellow vibrios	wYV	cfu/ml
Green vibrios	wGV	cfu/ml
Black colonies on TCBS	wBC	cfu/ml
Presumptive *Vibrio* count	wPVC	cfu/ml
Percentage green *Vibrio*	wPGV	%
Phosphate solubilizing bacteria	wPSB	cfu/ml
Nitrogen fixing bacteria	wNFB	cfu/ml
Filamentous fungi	wF	cfu/ml
Soil		
pH	spH	na
Organic matter	OM	%
Available sulfur	AS	%
Luminous bacteria	sLB	cfu/g
Total bacterial count	sTBC	cfu/g
Yellow *Vibrio*	sYV	cfu/g
Green *Vibrio*	sGV	cfu/g
Black colonies on TCBS	sBC	cfu/g
Presumptive *Vibrio* count	sPVC	cfu/g
Percentage green *Vibrio*	sPGV	%
Phosphate solubilizing bacteria	sPSB	cfu/g
Nitrogen fixing bacteria	sNFB	cfu/g
Filamentous fungi	sF	cfu/g

TCBS=thiosulfate citrate bilesalt sucrose agar, a Vibrio-selectitive medium; na=not applicable; ppm=parts per million; ppt=parts per thousand; cfu/ml= colony forming units per millilitre; cfu/g=colony forming units per gram

Table 5.2. Estimated marginal means of the different biophysicochemical parameters in the intake channel in relation to MPR, including P values using repeated measures.

Parameter	P	0:1	1:1	4:1
Water				
NH_3	0.78	0.2± 0.12	0.18± 0.18	0.08±0.18
NH_4*	0.56	0.36±0.21	0.07±0.21	0.04±0.21
NO_2	**0.03**	0.05±0.01	0.01±0.01	0.003±0.01
NO_3	0.40	0.05±0.02	0.004±0.021	0.03±0.02
PO_4	0.15	0.28±0.05	0.25±0.52	0.09±0.05
TSS	0.53	1.97±0.24	1.83±0.24	2.25±0.24
^oT**		29.63	29.58	31.08
DO**		5.84	3.93	4.16
wpH**		8.33	7.74	7.18
Sala		4.14	13.75	12.13
wLB	0.47	0.0±0.12	0.21±0.12	0.0±0.12
wTBC	0.28	4.6±0.76	2.79±0.76	2.67±0.76
wYV	0.95	1.08±0.59	1.31±0.59	1.32±0.59
wGV	0.98	1.04±0.67	1.07±0.67	0.88±0.70
wBC	0.22	0.18±0.11	0.0±0.11	0.36±0.11
wPVC	0.99	2.3±1.25	2.38±1.25	2.56±1.25
wPGV	0.95	30.33±19.12	29.70±19	22.39±19
wPSB	0.46	3.67±0.89	2.4±0.89	1.95±0.89
wNFB	0.57	3.09±0.92	2.22±0.92	1.59±0.92
wF	0.5	1.03±0.18	0.86±0.18	0.69±0.18
Soil				
Redox	0.50	2.68±0.04	2.62±0.04	2.61±0.04
pH**		4.46	6.17	6.05
OM	0.21	1.59±0.57	3.39±0.57	2.85±0.57
AS*	0.42	2.42±0.25	2.84±0.25	2.94±0.25
sLB	0.18	0.88±0.19	0.77±0.19	0.51±0.19
sTBC	0.57	5.51±0.22	5.16±0.22	5.42±0.22
sYV	0.66	1.84±0.8	2.26±0.8	2.93±0.8
sGV	0.87	2.55±0.57	2.12±0.57	2.34±0.57
sBC	0.07	0.25±0.28	0.12±0.28	0.39±0.28
sPVC	0.85	4.64±1.55	4.5±1.55	5.68±1.55
sPVG	0.16	49.96±3.8	35.76±3.80	40.32±3.8
sPSB	0.26	5.86±0.43	4.72±0.43	4.78±0.43
sNFB	0.65	4.74±0.53	4.12±0.53	4.08±0.53
sF	0.23	1.82±0.26	1.03±0.26	1.31±0.26

*=sphericity was tested using Greenhouse-Geisser; **= not analysed using repeated measures due to >4 missing values; Significant P values are in bold; Values in the same row with different superscripts are significantly different (P<0.05; Tukey's)

Table 5.3. Estimated marginal means of the different parameters for sampling sites across MPR, including P values with DoC as the repeated measures.

Parameter	P	Sampling site			
		Intake channel	reservoir	Culture pond	Drainage channel
Water					
NH_3	**0.01**	0.05 ± 0.00^{ab}	0.10 ± 0.04^{a}	0.26 ± 0.09^{ab}	0.46 ± 0.11^{b}
NH_4*	0.15	0.05 ± 0.03	0.01 ± 0.00	0.06 ± 0.01	0.90 ± 0.56
NO_2	0.15	0.01 ± 0.01	0.02 ± 0.00	0.06 ± 0.02	0.11 ± 0.06
NO_3	0.26	0.06 ± 0.03	0.0 0.00	0.05 ± 0.03	0.00 ± 0.00
PO_4	0.08	0.15 ± 0.04	0.41 ± 0.08	0.44 ± 0.17	0.59 ± 0.22
TSS	0.30	2.02 ± 0.14	2.28 ± 0.12	2.21 ± 0.11	2.20 ± 0.13
^{o}T**		30.89	31.51	29.55	30.97
DO**		4.45	6.20	6.09	4.74
wpH**		8.13	7.8	7.95	7.88
Sal**		10.42	9.92	8.71	11.33
wLBC	0.76	0.34 ± 0.14^{a}	0.16 ± 0.11^{ab}	0.27 ± 0.17^{b}	0.39 ± 0.29^{ab}
wTBC	0.33	3.84 ± 0.35^{a}	3.44 ± 0.40^{ab}	4.45 ± 0.25^{ab}	3.51 ± 0.50^{b}
wYV	0.63	1.49 ± 0.34^{a}	1.88 ± 0.28^{ab}	2.04 ± 0.20^{ab}	1.81 ± 0.28^{b}
wGV	0.058	0.99 ± 0.39^{a}	1.75 ± 0.28^{b}	1.90 ± 0.35^{b}	1.97 ± 0.23^{b}
wBC	0.59	0.03 ± 0.03	0.47 ± 0.27	0.22 ± 0.18	0.44 ± 0.30
wPVC	0.23	2.62 ± 0.50^{a}	3.87 ± 0.50^{b}	4.19 ± 0.62^{b}	4.30 ± 0.77^{b}
wPGV	0.08	33.82 ± 3.00^{a}	33.85 ± 5.76^{ab}	42.67 ± 5.30^{ab}	44.00 ± 5.55^{b}
wPSB	0.73	2.74 ± 0.18	2.91 ± 0.45	3.12 ± 0.24	2.67 ± 0.46
wNFB	0.75	2.38 ± 0.12	2.07 ± 0.55	2.33 ± 0.36	2.47 ± 0.30
wF	0.11	0.33 ± 0.19	0.47 ± 0.19	0.68 ± 0.20	1.14 ± 0.24
Soil					
redox	0.69	2.55 ± 0.03	2.56±0.06	2.59±0.01	2.57±0.01
pH**		5.52	6.49	6.07	4.93
OM	0.08	2.47 ± 0.38	3.40±0.58	2.34±0.28	2.70±0.61
AS*	**0.001**	2.85 ± 0.01	2.21±0.12	2.79±0.01	2.77±0.02
sLB	0.20	0.24 ± 0.16	0.17±0.17	0.97±0.22	0.50±0.35
sTBC	0.44	5.70 ± 0.30	4.77±0.20	5.56±0.56	5.20±0.34
sYV	0.17	2.71 ± 0.22	2.45±0.20	2.80±0.23	3.15±0.35
sGV	0.41	2.79 ± 0.61	2.12±0.20	2.97±0.31	3.04±0.25
sBC	0.77	0.31 ± 0.22	0.57±0.14	0.68±0.41	0.62±0.22
sPVC	0.32	5.81 ± 0.97	5.13±0.50	6.45±0.42	6.81±0.47
sPGV	0.45	41.42 ± 5.77	36.25±4.89	46.98±4.18	45.28±1.65
sPSB	0.76	5.09 ± 0.37	4.63±0.46	4.94±0.24	5.10±0.43
sNFB	0.53	4.41 ± 0.09	3.90±0.56	4.10±0.24	4.47±0.44
sF	**0.04**	4.47 ± 0.44^{ab}	3.90±0.56^{a}	4.10±0.24^{ab}	4.47±0.44^{b}

*=sphericity was tested using Greenhouse-Geisser; **= not analysed using repeated measures due to >4 missing values; Significant P values are in bold; Values in the same row with different superscripts are significantly different (P<0.05; Tukey's)

Table 5.4. Estimated marginal means of the different parameters across locations and DoC'sdifferent mangrove to pond area ratio including P values using repeated measures.

		Mangrove to pond area ratio		
Parameter	P	0:1	1:1	4:1
Water				
NH_3	0.47	0.25 ± 0.07	0.14 ± 0.07	0.26 ± 0.07
NH_4*	0.60	0.38 ± 0.23	0.05 ± 0.23	0.34 ± 0.23
NO_2	0.96	0.05 ± 0.03	0.06 ± 0.03	0.05 ± 0.03
NO_3	0.94	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02
PO_4	0.33	0.34 ± 0.20	0.27 ± 0.20	0.58 ± 0.20
TSS	0.89	2.14 ± 0.08	2.14 ± 0.18	2.25 ± 0.18
^oT**		30.3	30.3	31.6
DO**		5.3	5.2	5.6
wpH**		8.3	7.9	7.60
Sal**		4.0	15.3	11.0
wLB	0.79	0.17 ± 0.23	0.39 ± 0.23	0.32 ± 0.23
wTBC	0.18	4.38 ± 0.28	3.55 ± 0.28	3.49 ± 0.28
wYV	0.15	1.38 ± 0.19	2.07 ± 0.19	1.95 ± 0.19
wGV	0.85	1.79 ± 0.41	1.71 ± 0.41	1.46 ± 0.41
wBC	0.19	0.41 ± 0.09	0.11 ± 0.09	0.34 ± 0.09
wPVC	0.93	3.58 ± 0.57	3.89 ± 0.57	3.76 ± 0.57
wPGV	0.67	42.58 ± 7.3	40.13 ± 7.3	33.04 ± 7.3
wPSB	0.61	3.22 ± 0.42	2.76 ± 0.42	2.59 ± 0.42
wNFB	0.77	2.61 ± 0.49	2.22 ± 0.49	2.12 ± 0.49
wF	0.46	0.81 ± 0.15	0.50 ± 0.15	0.66 ± 0.15
Soil				
Redox	0.26	2.66 ± 0.04	2.54 ± 0.04	2.51 ± 0.06
spH**		5.0	6.2	6.01
OM	0.86	2.64 ± 0.73	3.06 ± 0.73	2.49 ± 0.73
AS*	**0.018**	2.26 ± 0.05	2.80 ± 0.04	2.91 ± 0.04
sLB	0.07	0.07 ± 0.13	0.73 ± 0.13	0.61 ± 0.13
sTBC	0.42	5.31 ± 0.17	5.12 ± 0.17	5.49 ± 0.17
sYV	0.40	2.37 ± 0.33	2.87 ± 0.33	3.09 ± 0.33
sGV	0.36	3.04 ± 0.24	2.70 ± 0.24	2.46 ± 0.24
sBV	0.94	0.52 ± 0.24	0.50 ± 0.24	0.62 ± 0.24
sPVC	0.95	5.93 ± 0.53	6.07 ± 0.53	6.16 ± 0.53
sPGV	**0.047**	50.28 ± 2.15	39.29 ± 2.15	37.88 ± 2.15
sPSB	0.54	5.26 ± 0.42	4.54 ± 0.42	5.02 ± 0.42
sNFB	0.77	4.39 ± 0.48	3.93 ± 0.48	4.33 ± 0.48
sF	0.44	1.7 ± 0.34	1.07 ± 0.34	1.27 ± 0.34

*=sphericity was tested using Greenhouse-Geisser; **= not analysed using repeated measures due to >4 missing values; Significant P values are in bold; Values in the same row with different superscripts are significantly different (P<0.05; Tukey's)

Table 5.5. ANOVA table explaining significant difference in sLB, sPGV and AS between different MPR.

	sLBa			sPGV[a]			AS[b]		
Source	df	MS	P	d	MS	P	d	MS	P
MPR	2	0.25	0.07	2	92.23	0.05	2.00	0.15	0.02
Error	3	0.04		3	9.21		2.00	0.00	
DoC	3	0.90	0.72	3	5.59	0.10	1.07	0.66	0.15
DoC*MPR	6	0.95	0.80	6	184.33	0.73	2.14	0.51	0.20
Error (DoC)	9	1.94		9	310.68		2.14	0.13	
Site	3	3.12	0.2	3	544.51	0.45	1.10	4.37	0.03
Site*MPR	6	0.71	0.84	6	399.23	0.66	2.20	4.37	0.09
Error (Site)	9	1.63		9	569.60		2.20	0.19	
DoC*Site	9	0.43	0.63	9	370.37	0.27	1.42	4.31	0.08
DoC*Site*MPR	18	0.90	0.11	18	145.30	0.92	2.83	2.42	0.15
Error (DoC*Site)	27	0.54		27	278.88		2.83	0.60	

a=mauchley's test was used to test sphericity

b=greenhose and geisser testwas used to test sphericity

Significantly lower levels of wLBC, wTBC, wYV, wGV, wPVC, and wPGV were observed in the intake channel. Increasing levels of NH_3, NH_4, TAN, NO_2, PO_4, and sF were observed as the water passed through the different pond compartments or sites. Highest levels were observed in the drainage channel but significantly higher values were observed only for NH_4 and sF. Soil bacteria, generally increased unsignificantly with water movement from one compartment/site to the next; highest counts were observed in the drainage channel.

5.4. DISCUSSION

Decreasing NO_2 concentration with increasing MPR suggests that mangroves may be efficient in the NO_2 removal in the water. Primavera et al. (2007) observed a significant reduction in the NO_2 concentration in the pond effluent, 6 hours after draining into an experimental impounded mangrove. The increasing levels of phosphate and nitrogen species NH_3, NH_4, TAN, NO_2, and NO_3 during the production cycle or days of culture (DoC) and from the reservoir to the drainage channel were expected. Results are

in consonance with Primavera et al., (2007) who reported increasing nutrient levels and TSS in both shrimp pond and impounded mangroves. Feed and fecal matters contribute to nutrient load of the water (Lin and Chen, 2003). Nutrient distribution during the production cycle suggests its accumulation, exceeding the recommended maximum of 0.6 ppm TAN (Funge-Smith and Briggs, 1998), and its disposal in the receiving environment as manifested in the distribution across sampling sites. Contrary to what was expected, lowest nutrient concentrations were observed in MPR-1. Aside from MPR, nutrients are removed from the environment by mangrove tree assimilation, water exchange and microbial processes (Primavera et al., 2007; Prescott et al., 2002; Islam et al., 2004). The latter are affected by forest type, tidal elevation and geomorphology (Adame and Lovelock, 2011; Li et al., 2011; Nickerson and Thibodeau, 1985). It should be noted that mangroves in the present study were found in the receiving area. It is possible that effluent water was purified as it passed through the mangrove habitat before it entered the farm or was integrated in underground water where it found its way into the deep wells. It could be that the filtering capacities of mangroves are seen on the intake channel; nutrient concentrations in the intake channel were generally lowest compared to the other sites. However, this could not be attributed solely to the presence of mangroves, as earlier mentioned, nutrient removal in the water is affected by water exchange, the receiving environment is exposed to tidal fluctuations thus to water exchanges.

AS level was significantly different in farms with different MPR. Highest AS was observed in farms with MPR-4 and lowest in MPR-0. AS is associated with decomposing organic matter, pH and black colonies on TCBS. Vegetated soil is richer in organic matter than non-vegetated soil (Cacador et al., 1996). In the process of decomposition, sulphur is produced from the oxidation of sulphide, which may come from farm effluents and from mangrove habitats (Prescott et al., 2005; Fernandes and D'Sousa, 1999). Higher AS would result in more sulphur available to bind with H20 to produce an acidic condition or lower pH. Moreover, high total sulphur concentration means that pond soil contains iron pyrite, which oxidizes yielding excess acidity. Results of AS in relation to acidity is supported by the lowest wpH observed in MPR-4.

One of the important bacteria in the marine ecosystem are those belonging to the genus *Vibrio* that can either be pathogenic or non-pathogenic. Most of the pathogenic vibrios are non-sucrose fermenters that produce green colonies on TCBS (Amaro and

Biosca, 1996; Fuenzalida et al., 2007). Most of the vibrios reported to have probiotic effects are yellow vibrios that can ferment carbohydrates (Thompson et al., 2010; Austin et al., 1995). A higher percentage of yellow vibrios was identified as a protective factor against WSSV outbreak in a cross sectional study of 90 farms (Tendencia et al., 2010a). In addition, in a case study of six farms, Tendencia and Verreth (2011a) observed consistently more than 50% yellow vibrios in farms with WSSV infection but not resulting in an outbreak. This might explain the absence of disease outbreaks due to WSSV despite its occurrence in one of the farms of MPR-1 and one in MPR-4 at DoC 60 and 120, respectively. Percentage green vibrios in these farms were below 40%; conversely, the percentage yellow vibrios would be 60%. The higher luminous bacterial counts and the presumptive *Vibrio* counts in both the soil and water of farms MPR-1 and MPR-4 compared to MPR-0 can be a factor explaining WSSV incidence in farms with mangroves in the receiving environment. Luminous bacteria and high presumptive *Vibrio* counts are reported as WSSV risk factors (Tendencia & Verreth, 2011a). Total bacterial count in the water was highest in MPR-0 without WSSV incidence. This is again in accordance with Tendencia & Verreth (2011a) who reported higher water total bacterial count in shrimp ponds without WSSV outbreak. Furthermore, they explained that total bacterial count includes all kinds of beneficial as well as harmful bacteria; the beneficial bacteria might have conditioned the pond water, making environmental conditions less stressful to the shrimp and rendering them less susceptible to WSSV infection.

PGV in the soil was lowest in MPR 4 and this was statistically attributed to the MPR persé, since PGV in the different sites and at the different DoCs was not significantly different. This suggests that the presence of mangrove persé has the capacity to control the growth of pathogenic organisms such as the green vibrios. This confirms reports that less disease causing organisms are found in areas with abundant mangroves (Belak et al., 1999). It should be noted however, that the beneficial capability of mangroves depended on the microbial structure and function of the mangrove ecosystem. Some microorganisms important in nutrient transformation in the mangrove ecosystem are the methanogenic, nitrogen fixing, phosphate solubilizing, sulfate reducing and photosynthetic anoxygenic bacteria, and the fungi (Holguin et al., 2001).

Black colonies on TCBS (sBC) were highest in the soil of MPR 4. According to Gram & Huss (1996), the black colonies on TCBS, a selective medium, belong to the

genus Shewanella, a hydrogen sulphide producing organism (Fuenzalida et al., 2007). *Shewanella* is important in the nutrient cycle, i.e. carbon and manganese; and can be used in the remediation of contaminated environments (Prescott et al., 2005; Fredrickson et al., 2008). The probiotic effect of *Shewanella* has also been reported (Zadeh et al., 2010). The higher sBC in MPR-4 might have provided some probiotic effect thus no outbreak was observed despite WSSV occurrence.

The distribution of bacteria across sampling sites was expected. Bacteria get energy from nutrients (Prescott et al., 2005), the increase in the population size follows the nutrient level changes. Bacterial and nutrient levels were lowest in the inlet. In this study, phosphate solubilizing and nitrogen fixing bacteria and fungi in both water and soil were higher in MPR-0. High PSB suggests greater availability of inorganic phosphorus as substrate (Ghosh and Chattopadhyay, 2005). Sengupta and Chaudhuri (1990) reported that nitrogen fixing bacteria do not show specificity for any mangrove species. According to Pereira e Silva et al. (2011), soil type is the main factor influencing N-fixing communities. Phosphate solubilizing bacteria aid in the removal of phosphorus in the water while the nitrogen fixing bacteria are responsible for transforming nitrogenous nutrients into bioavailable form that can be used by organisms lower in the food web present in the ecosystem (Jung and Lovitt, 2011; Makarov et al., 2011).

No disease outbreak was observed despite shrimp exposure to excessive levels of the harmful nitrogenous nutrients (NH_3, NH_4, TAN), OM, wPVC and spH. This is contrary to Alday-Sanz et al. (2002) who reported more pathological changes observed in shrimp challenged with Vibrio with prior exposure to ammonia than those that were not exposed to the stressor; and to Tendencia and Verreth (2011b) who reported that outbreak due to WSSV occurs when the shrimp are exposed to 4 or more stressors simultaneously. These results would suggest that nitrogenous nutrients, OM, spH, and wPVC are not important WSSV risk factors. However, this contention needs to be investigated.

Signifficant difference for NO_2 and trend for NH_4, PO_4, wTBC, wBC, OM, AS, sLB and sPGV in the intake channel showed positive effect of mangrove, but variation was large and sample size small. Results of the present study did not clearly show that that the presence of mangrove improves influent water quality and prevents WSSV infection/ outbreak. This could be attributed to the small number of monitored farms and other factors affecting water quality in a mangrove ecosystem. Moreover, aside from

the MPR, other factors such as the mangrove community structure, geomorphology and tidal elevations affect the efficiency of mangrove habitats. This needs further investigation.

CHAPTER 6

EFFECT OF GREENWATER TECHNOLOGY ON THE OCCURRENCE OF WHITESPOT SYNDROME VIRUS (WSSV) IN POND CULTURED *PENAEUS MONDON*

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Abstract

Whitespot syndrome virus (WSSV) has caused severe production drops in the shrimp industry. Numerous scientific manuscripts deal with WSSV epidemiology, but reports on minimizing disease outbreaks through ecological means are rare. Industry stakeholders resorted to various innovative techniques to recover from heavy economic losses. Some shrimp farmers in the Philippines claimed that "greenwater" (GW) technology could prevent disease outbreak due to WSSV. The efficiency of the GW technology was evaluated by comparing three ponds using the GW culture technique to three ponds not using it. WSSV was detected in the GW ponds but no WSSV disease outbreak occurred. In GW ponds available soil sulphur content was lower, and in water the observed counts of luminous bacteria were lower and of Chlorophyceae higher. Chlorophyceae, i.e. algae, enhance nutrient uptake in effluent streams resulting to an improved in the water quality in *P. monodon* culture ponds. This suggests that the use of the GW technique to culture *P. monodon* might improve water quality.

6.1. INTRODUCTION

A greenwater (GW) culture system is an innovative technique wherein shrimp are cultured in water from a pond in which tilapia or other fish species are grown. Two greenwater culture methods are used by shrimp farmers (Tendencia et al., 2004): 1) shrimp and fish are cultured in separate ponds; water used to culture shrimp comes from the fish pond, 2) shrimp and fish are cultured in the same pond; isolating fish in a net pen inside the shrimp culture pond. Tank based experiments showed that a GW technique wherein shrimp are cultured with different fish species such as tilapias, groupers, seabass, snappers and siganids proved effective in controlling disease due to luminous bacteria (Tendencia and dela Pena 2010). Huervana et al. (2006) reported that GW from broodstock tanks of *Oreochromis mossambicus* inhibited luminous vibriosis for one-week, and that broodstock tanks are a better source of greenwater than juvenile tanks in controlling the disease. Lio-Po et al. (2005) attributed the effectiveness of the greenwater culture to the presence of anti-luminous *Vibrio* factors in the associated bacterial, fungal, and phytoplankton microbiota and the skin mucus of tilapia. The ability of fish mucus and feces collected from a red hybrid tilapia culture and of bacteria isolated from fish mucus and feces to reduce the growth of luminous Vibrio harveyi was also reported by Tendencia and dela Pena (2010).

Fish mucus contains specific and nonspecific antimicrobial compounds including complement (group of proteins in the blood and body fluids that play an important role in humoral immunity), lysozymes, C-reactive protein, immunoglobulins, proteases, lectin-like molecules and glycoproteins (Alexander and Ingram, 1992). Cain, LaPatra, Baldwin, Shewmaker, Jones and Ristow (1996) demonstrated the anti-infectious pancreatic necrosis virus (IPNV) properties of mucus from skin and gastro intestinal tract of rainbow trout. The antiviral activity of gamma inferons of salmonids against IPNV and salmonid alphavirus has also been reported (Sun et al., 2011).

Available literature suggests that the water quality in GW is better resulting in less stress to the shrimp thus enhancing resistance against diseases like whitespot syndrome virus (WSSV) (Cremen et al., 2007). *Vibrio* infection has been reported to increase susceptibility to WSSV (Phuoc et al., 2008), and the potential effect of GW on WSSV outbreaks might pass through the reduction of these or other stress factors. However, a cross sectional study on different farming practices in the Philippines did not identify GW technology as a WSSV protective factor (Tendencia et al., 2011). The lesser

number of GW farms compared to non-greenwater farms that participated in the study could have masked the protective effect of greenwater. In this context, this paper evaluated if the water quality in GW ponds is better than in ponds not applying GW technology. Different bio-physico-chemical properties of the water and soil and WSSV presence in shrimp were monitored during a full production cycle.

6.2. MATERIALS AND METHODS

Abbreviations and terminologies used for the different parameters found in this paper are presented in Table 6.1. In the text, unless otherwise specified, the term 'pond' collectively refers to the culture pond, including the intake channel and the drainage channel.

6.2.1. FARM CHARACTERISTICS

Six semi-intensive monoculture *P.monodon* ponds on the Visayas Island in the Philippines were monitored. The 6 ponds belonged to 6 different farms. Three ponds used the GW technology; three ponds did not (non-GW). Adjacent to the culture ponds, tilapia were raised in reservoir ponds that served as water source for the GW ponds. The water level in reservoir ponds was maintained with nearby river or seawater. Reservoirs were stocked with a salinity tolerant tilapia strain maintained at a biomass >2000kg/ha for minimum one month before water transfer to a GW pond and subsequent post larval stocking. In one of the non-GW ponds, river water was pumped into the reservoir and chlorinated (13ppm) before transfer to GW pond 7 days later. Two non-GW ponds got water directly from deep wells. No water exchange was implemented in any of the shrimp ponds. Water lost due to evaporation and seepage was replenished to maintain a minimum water depth of 1.0 m. Paddle wheels were used from midnight to noon in the shrimp ponds to maintain the dissolved oxygen concentration above 4 ppm. Shrimp were fed commercial pellets (35 % crude protein) daily from stocking until harvest. In one GW pond, ponds received 2.77 sacks/ha/week of mollusc shells starting a month before harvest to improve shell quality. All ponds were successfully harvested at DOC 151±19.

Table 6.1. Abbreviations used for the different parameters and terminologies that can be found in the paper.

Parameter	Abbreviation	Unit
Greenwater pond	GW pond	na
Non-Greenwater Pond	non-GW pond	na
Days of culture	DOC	na
Daily weight gain	DWG	g
Water		
Ammonia	NH_3	ppm
Ammonium	NH_4	ppm
Nitrite	NO_2	ppm
Nitrate	NO_3	ppm
Inorganic nitrogen	IN	ppm
Phosphate	PO_4	ppm
Temperature	T	^oC
Dissolve oxygen	DO	ppm
Water pH	wpH	na
Total plankton count	TPC	cells/ml
Chlorella	CC	cells/ml
Luminous bacteria	wLB	cfu/ml
Total bacterial count	wTBC	cfu/ml
Yellow vibrios	wYV	cfu/ml
Green vibrios	wGV	cfu/ml
Black colonies on TCBS	wBC	cfu/ml
Presumptive *Vibrio* count	wPVC	cfu/ml
Percentage green *Vibrio*	wPGV	%
Phosphate solubilizing bacteria	wPSB	cfu/ml
Nitrogen fixing bacteria	wNFB	cfu/ml
Filamentous fungi	wF	cfu/ml
Soil		
pH	spH	na
Organic matter	OM	%
Available sulphur	AS	%
Luminous bacteria	sLB	cfu/g
Total bacterial count	sTBC	cfu/g
Yellow *Vibrio*	sYV	cfu/g
Green *Vibrio*	sGV	cfu/g
Black colonies on TCBS	sBC	cfu/g
Presumptive *Vibrio* count	sPVC	cfu/g
Percentage green *Vibrio*	sPGV	%
Phosphate solubilizing bacteria	sPSB	cfu/g
Nitrogen fixing bacteria	sNFB	cfu/g
Filamentous fungi	sF	cfu/g
Harvest data		
Feed conversion ratio	FCR	na
Average body weight	ABW	g

na=not applicable

6.2.2. SAMPLING

Per pond studied, water and soil samples were taken from 3 locations: (1) the intake channel, (2) the culture pond, 2 m before the outlet gate and (3) the drainage channel after draining, 1 m behind the outlet gate. On GW ponds, additional samples were taken from the reservoir. Previous research showed no significant difference in the water and soil microbial flora between replicate samples per location (Tendencia and dela Pena, 2002). Therefore, one sample was taken per location. Care was taken to sample at the same spot per location on different sampling dates, by using physical markers such as feeding tray position or distance from the gate. In addition, water samples were taken from each deep water well, and from soil and water samples inside reservoir ponds.

Per location, water, soil and shrimp were sampled. Water samples were collected 0.5m below the water surface by divers. Water samples were stored in sterile 100-ml bottles for bacteriological analyses. Water samples for physicochemical and plankton counts were stored in 250 and 1000 ml bottles, respectively. Soil samples were taken from the top 5 cm of the sediment. Shrimp were collected using a cast net thrown once from the dike. Of the 50-100 shrimp trapped, 3 shrimp that appeared to be weakest or showed disease related abnormalities (i.e. reddish, soft-shelled of spotted outside skeleton) were stored for WSSV quantification.

Samples were taken from the pond -7 to 0 days before stocking (except for shrimp) and every 30±5 days after stocking until 120±5 days of culture (DoC). Samples were taken between 08:00 and 10:00 a.m.

6.2.3. DATA COLLECTION/SAMPLE ANALYSES

Shrimp. Shrimp average body weight (ABW), day of culture (DoC), feed conversion ratio (FCR) and survival were recorded at harvest. Shrimp daily weight gain (DWG) was computed by dividing ABW by DoC.

Water. Water samples (100ml) were collected using autoclaved (121^{o}C for 15 min) bottles for bacteriological study using the serial dilution technique (Prescott et al., 2005). Briefly, 0.2 ml of the sample was diluted to 2.0 ml and serially diluted ten-fold using sterilized seawater. Representative dilutions were plated onto 5 media in duplicates: Nutrient Agar (NA; BBL by Becton Dickinson) with 1.5% (w/v) sodium

chloride (NaCl) for the total and luminous bacterial counts; Thiosulfate Citrate Bilesalt Sucrose Agar (TCBS; BBL by Becton Dickinson) for the presumptive *Vibrio* (green and yellow colonies) and *Shewanella* counts (black colonies); Pikovskaya Agar (PA; Himedia) for the phosphate solubilizing bacterial counts; Jensen's Agar (JA; Himedia) for the nitrogen-fixing bacterial count; and Czapex Dox Agar (CDA; Himedia) supplemented with 1.5% (w/v) yeast extract (BBL by Becton Dickinson) for the fungal count. All media were incubated at 30^{o}C. Total bacterial and presumptive *Vibrio* counts were done after 18–24 h of incubation. Luminous bacteria were counted in a dark room to observe luminescence. Phosphate solubilizing bacteria, nitrogen fixing bacteria and fungus were counted after 2-6 days of incubation. Incubation periods were based on manufacturer's instruction. The following parameters were measured in situ: T and DO (YSI 1000; YSI instruments) and pH (HI 98108; Hanna Instruments). Water samples (0.5L) were brought to the laboratory in a chilled container and analysed for NH_3-(phenate method; APHA 1995), NH_4 and NO_3 (flow injection analyser), NO_2 (colorimetry; ISO 13395, 1996), PO_4 (ascorbic acid method; APHA 1995) and TSS (filtration drying method; Rainwater and Thatcher, 1960). Water sample (1L) for TPC was filtered using plankton net fixed in 5% formalin and counted using a haemocytometer.

Soil. The following parameters were measured in situ: Soil redox (ORP meter; Eutech Instruments) and wet pH (Kelway pH meter; Kel Instruments Co., Inc). Collected soil samples (0.5kg) were air-dried, powdered and passed through a sieve (2mm mesh size) before analysis for organic matter (OM; Wackley & Black; 1934) and available sulphur (AS; turbidimetry; Walkley & Black, 1934). Soil samples for bacteriological study were processed for bacteriology within 2h of collection. Soil (0.1 g) was suspended in 1.0ml sterile seawater, serially diluted, plated on the same media as in water sample. Bacterial growth was counted was similarly counted.

WSSV quantification. Gill tissues from the 3 shrimp samples were pooled, fixed in 95% ethyl alcohol and stored in the refrigerator (4^{o}C) until WSSV real time PCR analysis. Total genomic DNA and associated WSSV viral DNA were extracted from the gill tissue (5 mg) using DNA kit (Quiangen Biotech CO., LTD). The DNA concentration and quality were determined by measuring optical density at OD 260 nm, by using a GeneQuant pro (Spectronic instrument, Inc., New York, USA). A 25 ng/µl DNA was prepared for real time PCR. Realtime PCR protocol was based on Durand and Lightner (2002). A sample of 40 ng total DNA was added to a PCR mixture containing 0.25 µM of each primer and

0.125 µM TaqMan probe in a final reaction volume of 20 µl. The amplification program consisted of 30 sec at 95°C for initial denaturation followed by 45 cycles of 5 sec denaturation and 20 sec annealing and extension at 60 sec. Thermal cycling was performed on GeneAmp 5700 Sequence Detection System. A set of standard dilutions (10^0 to 10^6 viral copies/µl) was created from a plasmid containing the target amplicon and run simultaneously with the specimen DNA. All samples were run in duplicate with two negative controls for each run.

6.2.4. STATISTICAL ANALYSIS

Bacterial counts, plankton counts and available sulphur levels were log 10 transformed before analyses. Harvest data i.e. FCR, SR, DoC, ABW, DWG, were analyzed using one-way ANOVA.

Samples taken at monthly intervals were considered repeated measures. Mauchley's sphericity test was used to test if the variances of the differences between levels of repeated measures factor were equal. If the criterion of sphericity was not reached (P>0.05), the Greenhouse & Geisser (1959) correction as described by Field (2004) was applied.

6.2.4.1. WATER AND SOIL QUALITY BETWEEN GW AND NON-GW, DIFFERENT DOC'S AND LOCATION

A 3-way repeated measures ANOVA with 'farm type' as main factor, 'sample location' as sub-factor and 'DoC' as sub-sub-factor was applied (SPSS V.19).

6.2.4.2. Water quality between different water intakes

Intake channel of non-GW, from intake channel of GW-reservoir and intake channel to GW culture ponds were analyzed using a 2-way repeated measures ANOVA with 'water intake' as main factor, and 'DoC' as a sub-factor(SPSS V.19).

6.2.4.3. WATER AND SOIL PARAMETERS IN THE CULTURE PONDS OF GW AND NON-GW

Intake channel of non-GW, from intake channel of GW-reservoir and intake channel to GW culture ponds were analyzed using a 2-way repeated measures ANOVA with 'water intake' as main factor, and 'DoC' as a sub-factor (SPSS V.19).

6.3. RESULTS

6.3.1. WATER AND SOIL QUALITY BETWEEN GW AND NON-GW, DIFFERENT DOC'S AND LOCATIONS

Mauchley's test was significant for NO_3, wLB and sNFB. Sphericity was not violated in all other cases. Results are given in Tables 6.2 through 6.5. The estimated main effect means and standard deviations of the different parameters across days of culture are presented in Table 6.2, across sampling sites in Table 6.3 and between greenwater and non-greenwater in Table 6.4. ANOVA tables were used to explain the differences in wYV, sLB, and sPGV (Table 6.5); in sBC and redox (Table 6.6) between GW and non-GW ponds..

6.3.1.2. Water parameters

In all cases, measured concentrations of nitrogenous wastes were above the safe level for shrimp culture which are 0.1 ppm for NH_3, 0.001 ppm for NH_4, 3.7 ppm for TAN and 0.1 ppm for IN (Chen and Lei, 2007; Funge-Smith and Briggs. 1994; Crab et al., 2007); the concentration increased with DoC ($P < 0.05$) (Table 6.2). Sampling location was also significant ($P < 0.05$); highest concentration was observed in the culture pond and lowest in the reservoirs (Table 6. 3). Total suspended solid (TSS) was above the Best Aquaculture Practices (BAP) standard, established by the Aquaculture Certification Council, Inc., of <100 mg/L for all cases, though no significant difference was observed between GW and non-GW ponds, between DoC's and between locations. Higher TSS was observed in non-GW ponds (Table 6.4) and in the drainage channel (Table 6.3). Water pH was significantly higher in the culture pond (Table 6.3). There was a trend that salinity of non-GW ponds was significantly higher ($P=0.06$) than GW ponds (Table 6.4). Highest salinity was reached at the end of the sampling period ($P=0.04$; Table 6.2). Total plankton count (TPC) was not significantly different between GW and non-GW ponds. Chlorophyceae count (CC) was significantly higher in GW ponds compared to non-GW ponds ($P=0.003$; Table 6.4). The use or non-use of GW per se explained for the difference in CC. Significant difference in location ($P=0.02$; Table 6.3) and DoC ($P=0.005$; Table 6.2) was observed for IN. Significantly higher IN was observed in the drainage channel at DoC 120 of non-GW ponds ($P=0.039$). Significantly higher water yellow *Vibrio* (wYV)

Table 6.2. Estimated main effect means and standard deviation of the different parameters across days of culture including and p values from ANOVA of repeated measures.*

		Days of culture				
Parameter	P	0	30	60	90	120
Water						
NH_3	**0.002**	0.07±0.04	0.04±0.02	0.13±0.03	0.31±0.07	0.53±0.15
NH_4	**0.046**	0.009±0.006	0.16±0.15	0.02±0.02	0.23±0.11	1.18±0.58
NO_2	0.33	0.05±0.04	0.17±0.10	0.03±0.02	0.12±0.06	0.16±0.05
NO_3	0.53	0.04±0.02	0.01±0.01	0.04±0.002	0.08±0.03	0.15±0.13
PO_4	**0.02**	0.10±0.04	0.18±0.08	0.25±0.11	0.66±0.24	0.74±0.32
IN	**0.005**	0.17±0.08	0.33±0.24	0.22±0.05	0.74±0.23	2.01±0.65
TSS	0.20	114±26	142±19	216±71	190±17	222±26
T	0.86	30.23±0.34	29.68±0.56	29.43±0.83	29.78±0.34	29.60±0.61
DO	0.70	5.13±0.87	4.27±0.48	4.62±0.69	4.33±0.46	4.06±0.23
pH	0.71	7.89±0.14	7.98±0.24	7.97±0.09	7.79±0.09	7.45±0.06
Sal	**0.04**	11.56±2.05	11.78±1.64	11.28±2.09	10.83±1.69	15.88±2.11
TPC**	0.056	na	4.13±3.48	4.61±4.2	5.03±4.65	5.04±4.58
CC**	0.618	na	3.94±0.14	4.36±0.17	4.27±0.096	4.09±0.08
wLB**	0.54	0.33±0.33	0.18±0.06	0.65±0.49	0.25±0.25	0.00±0.00
wTBC**	**0.04**	2.88±0.17	3.44±0.22	4.18±0.46	3.90±0.37	3.36±0.23
wYV**	**0.01**	1.28±0.29	1.07±0.19	2.04±0.35	2.14±0.30	2.00±0.13
wGV**	0.13	1.02±0.27	1.22±0.2	1.64±0.47	1.88±0.55	2.04±0.41
wBC**	0.58	0.19±0.14	0.33±0.10	0.48±0.29	0.82±0.38	0.54±0.27
wPVC**	**0.05**	2.49±0.52	2.61±0.41	4.16±0.93	4.84±1.18	4.58±0.54
wPGV**	0.69	28.16±11.23	39.4±5.73	29.89±7.64	37.15±6.31	38.58±2.8
wPSB**	0.18	3.17±0.37	2.91±0.04	3.13±0.23	3.26±0.37	2.55±0.43
wNFB**	0.72	2.91±0.59	2.55±0.17	2.28±0.37	2.58±0.53	2.47±0.72
wF**	0.33	0.30±0.15	0.44±0.17	0.44±0.20	1.01±0.52	1.24±0.69[c]
Soil						
Redox	0.87	216±60	170±15	217±40	173±47	176±20
pH	0.97	7.58±3.17	6.02±0.26	9.75±6.77	9.63±6.54	6.76±2.39
OM	0.26	1.39±0.91	1.87±0.46	2.46±1.0	1.38±1.03	1.31±0.60
AS	0.96	2.53±0.12	2.46±0.55	2.55±0.43	2.52±0.12	2.75±0.17
sLB**	**0.001**	0.22±0.43	0.06±0.11	0.00±0.00	1.91±0.17	0.00±0.00
sTBC**	**0.04**	4.37±0.29	5.86±0.55	5.97±0.15	4.49±0.41	4.84±0.04
sYV**	0.11	2.45±0.51	2.78±0.25	3.92±0.47	3.19±0.24	2.75±0.28
sGV**	0.08	1.91±0.46	3.63±0.48	4.11±0.79	2.84±0.26	2.84±0.39
sBC**	0.25	1.21±0.00	1.29±0.36	0.94±0.34	1.18±0.53	0.5±0.22
sPVC**	0.10	5.56±0.97	7.69±0.69	8.98±1.57	7.21±0.32	6.09±0.09
sPGV**	0.16	27.78±9.52	50.15±2.57	46.87±2.44	40.85±5.33	39.54±3.27
sPSB**	**0.004**	3.69±0.37	5.20±0.24	5.85±0.19	5.82±0.32	4.87±0.17
sNFB**	0.06	3.14±0.53	4.37±0.17	4.38±0.20	4.36±0.41	4.72±0.12
sF**	**0.01**	0.32±0.22	1.11±0.64	1.89±0.86	3.33±0.35	4.20±0.69

Significant P values are in bold; Na=not analyzed due to 2 missing data in GW ponds; *= please refere to Table 1 for the units; **= values are in log 10 of the counts

Table 6.3. Main effect means and standard deviation of the different parameters across sampling locations including and P values from ANOVA of repeated measures.*

		Sampling Location		
Parameter	P	Intake channel	Culture Pond	Drain Channel
Water				
NH_3	**0.002**	0.08±0.04	0.29±0.04	0.28±0.05
NH_4	**0.05**	0.13±0.10	0.51±0.17	0.31±0.11
NO_2	0.11	0.009±0.005	0.18±0.10	0.95±0.04
NO_3	0.92	0.05±0.02	0.07±0.06	0.07±0.04
IN	**0.022**	0.28±0.14	1.06±0.28	0.75±0.15
PO_4	0.13	0.19±0.06	0.56±0.25	0.41±0.11
TSS	0.29	154±28	151±18	225±49
T	0.57	29.97±0.38	29.61±0.42	29.65±0.28
DO	**0.07**	4.37±0.42	4.93±0.36	4.16±0.26
pH	**0.02**	7.77±0.1	8.00±0.04	7.86±0.06
Sal	0.35	14.43±2.38	10.61±0.90	11.75±2.81
TPC**	0.9	na	4.84±4.44	4.82±4.27
CC**	0.946	na	4.16±0.06	4.17±0.057
wLB**	0.62	0.41±0.30	0.16±0.14	0.24±0.17
wTBC**	0.21	3.3±0.33	3.62±0.11	3.74±0.18
wYV**	**0.01**	1.06±0.32	2.10±0.18	1.96±0.17
wGV**	**0.02**	1.05±0.43	1.60±0.33	2.03±0.24
wBC**	0.28	0.31±0.10	0.57±0.14	0.53±0.13
wPVC**	**0.007**	2.42±0.78	4.28±0.53	4.51±0.46
wPGV**	**0.02**	22.47±7.81	33.28±5.07	48.15±1.61
wPSB**	0.09	2.66±0.42	3.09±0.21	3.27±0.18
wNFB**	**0.002**	2.17±0.48	2.28±0.34	3.23±0.50
wF**	0.54	0.62±0.27	0.66±0.22	0.78±0.27
Soil				
Redox	0.59	183±14	197±12	190±8
pH	0.78	5.97±0.14	7.98±3.55	9.88±4.30
OM	0.51	1.5±0.58	1.83±0.88	1.71±0.83
AS	0.54	2.55±0.3	2.43±0.12	2.70±0.29
sLB**	**0.03**	0.03±0.07	0.72±0.12	0.56±0.13
sTBC**	0.87	5.07±0.12	5.08±0.17	5.16±0.07
sYV**	0.46	3.28±0.33	3.05±0.30	2.72±0.31
sGV**	0.84	3.0±0.18	3.20±0.44	3.0±0.38
sBC**	**0.04**	0.95±0.06	0.60±0.20	1.52±0.26
sPVC**	0.82	7.24±0.42	6.84±0.64	7.24±0.94
sPGV**	0.71	37.53±1.91	43.26±5.86	42.34±4.14
sPSB**	0.24	5.37±0.09	5.03±0.26	4.86±0.1
sNFB**	**0.03**	3.86±0.16	4.57±0.24	4.16±0.14
sF**	**0.02**	1.7±0.16	2.66±0.22	2.16±0.30

Significant P values are in bold; ; na=not analyzed due to missing data in the inlet of two GW ponds; *= please refer to Table 1 for the units; **= values are in log 10 of the counts

Table 6.4. Main effect means and standard deviation of the different parameters between greenwater and non-greenwater intake channel, ponds, and drainage channel including and P values from ANOVA of repeated measures.*

		Classification	
Parameter	P	Non-GW ponds	GW ponds
Water			
NH_3	0.38	0.25±0.05	0.19±0.05
NH_4	0.42	0.42±0.15	0.22±0.15
NO_2	0.4	0.14±0.06	0.05±0.06
NO_3	0.18	0.11±0.04	0.2±0.04
IN	0.22	0.91-0.21	0.47±0.21
PO_4	0.42	0.51±0.19	0.27±0.17
TSS	0.38	196±27	158±27
^oT	0.29	30.11±0.43	29.38±0.43
DO	0.62	4.31±0.44	4.65±0.44
wpH	0.26	7.80±0.08	7.95±0.08
Sal	**0.06**	**16.4±2.3**	**8.13±2.3**
TPC**	0.972	4.84±4.45	4.83±4.45
CC**	**0.003**	**4.33±0.022**	**4.0±0.027**
wLB**	0.98	0.28±0.22	0.27±0.22
wTBC**	0.09	3.15±0.25	3.95±0.25
wYV**	**0.04**	**2.22±0.24**	**1.19±0.24**
wGV**	0.83	1.49±0.43	1.63±0.43
wBC**	0.43	0.54±0.11	0.40±0.11
wPVC**	0.38	4.26±0.75	3.22±0.75
wPGV**	0.09	26.06±5.39	43.21±5.38
wPSB**	0.99	3.00±0.36	3.00±0.36
wNFB**	0.77	2.43±0.60	2.70±0.60
wF**	0.76	0.77±0.34	0.61±0.34
Soil			
Redox	**0.04**	**235±16**	**146±9**
pH	0.23	6.41±1.58	9.48±0.91
OM	0.61	1.22±1.32	2.14±0.76
AS	0.37	2.81±0.37	2.32±0.21
sLB**	**0.04**	**0.73±0.10**	**0.14±0.06**
sTBC**	0.44	5.04±0.11	5.16±0.06
sYV**	0.06	3.76±0.34	2.28±0.20
sGV**	0.27	3.48±0.48	2.65±0.28
sBC**	**0.05**	**1.59±0.23**	**0.45±0.13**
sPVC**	0.09	8.83±0.98	5.38±0.57
sPGV**	**0.04**	**36±2**	**46±1**
sPSB**	0.09	5.34±0.14	4.83±0.08
sNFB**	0.59	4.3±0.28	4.09±0.16
sF**	0.08	2.89±0.37	1.45±0.21

Significant P values are in bold; *= please refere to Table 1 for the units; **= values are in log 10 of the counts

(P=0.04) was observed in non-GW ponds (Table 6.4) and lowest in the intake channel (P=0.01; Table 6.3). Repeated measures showed significant difference in wYV (P=0.01) between DoC's (Table 6.2). Water green *Vibrio* (wGV; P=0.02) was highest in the drainage channel (Table 6.3). Significantly higher presumptive *Vibrio* count (wPVC) was observed at DoC 90 (P=0.05; Table 6.2) and in the drainage channel (P=0.007; Table 6.3). Water nitrogen fixing bacteria (wNFB; P=0.002) and wPGV (P= 0.02) were significantly higher in the drainage channel.

Table 6.5. ANOVA table explaining significant difference in wYV, sLB and sPGV between GW and Non-GW ponds.

	wYV			sLB			sPGV		
Source	df	MS	P	df	MS	P	df	MS	P
GW	1	1.609	0.038	1	0.26	0.039	1	80.008	0.042
Error (GW)	4	0.172		2	0.011		2	3.623	
Location	2	9.556	.010	2	1.916	0.025	2	141.853	0.714
Location * GW	2	2.612	.156	2	1.215	0.053	2	1157.060	0.16
Error(location)	8	1.105		4	0.182		4	386.292	
DOC	4	4.410	.010	4	6.178	0.001	4	665.389	0.163
Error(DOC)	16	.922		8	0.473		8	306.560	
DOC * GW	4	1.477	.222	4	5.977	0.002	4	141.226	0.763
Location * DOC	8	1.009	.386	8	1.712	0.000	8	176.393	0.619
Location * DOC * GW	8	.690	.644	8	1.451	0.00	8	150.950	0.706
Error(Location*DOC)	32	.914		16	0.151		16	223.398	

6.3.1.2. Soil Parameters

Soil redox was significantly higher in non-GW ponds than in GW ponds (Table 6.6). Significantly higher soil luminous bacteria (sLB) were observed in non-GW ponds (P=0.04), at DoC 90 (P=0.001) and inside culture ponds (P=0.03). All 2- and 3- way interactions are significant (Table 6.5). Significantly higher sBC in the drainage channel was observed. The 3-way interaction of site with DoC and GW was significant for sBC (Table 6.6). Higher concentrations of sBC were observed inside culture ponds (P=0.001), in the intake channel (P=0.005) and in the drainage channel (P=0.001). A significantly higher percentage of soil green *Vibrio* (sPGV) was observed in GW ponds

(Table 6.5). Significantly higher soil nitrogen fixing bacteria (sNFB; P=0.03) and soil filamentous fungi (sF; P=0.02) were observed inside culture ponds (Table 6.3). Significant differences were also observed in the sPSB and sF between different DoC's; and sNFB increased with DoC (P=0.06) (Table 6.2).

Table 6.6. ANOVA table explaining significant difference in sBC and redox between GW and Non-GW ponds.

	sBC			Redox		
Source	df	MS	P	df	F	P
GW	1	0.978	0.051	1	23.664	0.04
Error (GW)	2	0.054		2		
Location	2	3.255	0.044	2	0.595	0.594
Location * GW	2	1.260	0.165	2	1.492	0.328
Error(location)	4	0.432		4		
DOC	4	0.914	0.538	4	0.293	0.874
Error(DOC)	8	1.089		8		
DOC * GW	4	1.959	0.222	4	0.467	0.759
Location * DOC	8	3.416	0.000	8	0.997	0.474
Location * DOC * GW	8	2.865	0.0000	8	1.344	0.292
Error(Location*DOC)	16	0.323		16		

6.3.2. WATER QUALITY BETWEEN DIFFERENT WATER INTAKES

The criterion of sphericity was met except for NO_3, TSS, pH, wTBC, wYV wPSB, and wNFB. Except for NO_2 (P=0.02), there were no significant differences between water parameters measured in intake channel of non-GW ponds, intake channel to the GW reservoir and intake water to GW culture ponds (Table 6.7). In GW farms, nutrient levels were lower in the intake channel to the culture pond than in the intake channel to the GW reservoir. No trend was observed in the water microflora. TPC was highest in the intake channel of non-GW and lowest in the intake channel to GW culture ponds. CC was highest in the intake channel to GW reservoir and lowest in the intake channel to GW culture ponds.

Table 6.7. Main effect means and standard error of the different water parameters observed in the different water intakes across DoC's including and P values from ANOVA of repeated measures.*

		Sampling Location		
Parameter	P	Intake channel of NGW	Intake channel of GW reservoir	Intake channel to GW culture pond
NH_3	0.56	0.08±0.06	0.16±0.06	0.08±0.06
NH_4	0.825	0.07±0.16	0.19±0.16	0.20±0.16
NO_2	0.02	0.002±0.01	0.06±0.01	0.02±0.01
PO_4	0.67	0.19±0.06	0.26±0.06	0.19±0.06
TSS	0.29	178±37	100±37	129±37
pH	0.57	7.62±0.36	7.43±0.36	7.98±0.36
TPC	0.317	4.86±4.35	4.83±4.35	4.39±4.35
CC	0.673	4.27±3.8	4.40±3.8	4.20±3.8
wLB	0.44	0.65±0.34	0.00±0.34	0.18±0.34
wTBC	0.07	2.68±0.38	4.21±0.38	3.91±0.38
wYV	0.73	1.28±0.41	0.88±0.41	0.84±0.41
wGV	0.80	0.77±0.60	0.97±0.60	1.34±0.60
wBC	0.75	0.34±0.13	0.21±0.13	0.28±0.13
wPSB	0.29	2.52±0.5	3.69±0.5	2.8±0.5
wNFB	0..40	2.04±0.55	3.14±0.55	2.29±0.55
wF	0.84	0.74±0.32	0.75±0.32	0.50±0.32

6.3.3. WATER AND SOIL PARAMETERS IN THE CULTURE PONDS OF GW AND NON-GW

The criterion for sphericity was met for NH_3, NH_4, TAN, NO_2, temperature and OM. Salinity (P=0.02), AS (P=0.003), wYV (P=0.01), sLBC (P=0.006), and sYV (P=0.03) were significantly lower in greenwater ponds (Table 8). The effect of DoC and the 2-way interaction between DoC and GW were significant (P<0.001) in sYV. Though again much higher, the count of sBC in non-GW (1.27±0.27) was not significantly different from the GW (0.36±0.27).

Table 6.8. Main effect means and standard deviation of the different parameters between greenwater and non-greenwater culture ponds including and P values from ANOVA of repeated measures.*

		Classification	
Parameter	P	Non-GW	GW
Water			
NH_3	0.32	0.33±0.05	0.25±0.05
NH_4	0.30	0.72±0.25	0.31±0.24
NO_2	0.37	0.28±0.14	0.095±0.14
NO_3	0.30	0.14+0.08	0.007+0.08
PO_4	0.33	0.85±0.36	0.28±0.36
TSS**	0.13	0.21±0.09	1.98±0.09
^oT	0.80	29.75±0.60	29.51±0.60
DO	0.48	5.23±0.51	4.67±0.51
wpH	0.72	8.01±0.06	7.98±0.06
Sal	**0.02**	**13.73±1.26**	**7.40±1.26**
TPC**	0.57	4.58±0.15	4.45±0.15
CC**	0.52	4.06±0.15	4.21±0.15
wLB**	0.46	0.05±0.20	0.28±0.20
wTBC**	0.19	3.45±0.16	3.79±0.16
wYV**	**0.01**	**2.91±0.25**	**1.3±0.25**
wGV**	0.96	1.62±0.46	1.59±0.46
wBC**	0.77	0.53±0.20	0.62±0.2
wPGV**	0.36	28.02±7.17	38.54±7.12
wPSB**	0.80	3.14±0.29	3.03±0.29
wNFB**	0.88	2.22±0.48	2.33±0.48
wF**	0.4	0.87±0.32	0.45±0.32
Soil			
Redox**	0.07	2.29±0.04	2.26±0.04
pH	0.5	6.3±2.66	9.1±2.66
OM	0.76	1.6±0.63	1.89±0.63
AS	**0.003**	**2.81±0.09**	**2.004±0.09**
sLB**	**0.006**	**1.06±0.11**	**0.23±0.11**
sTBC**	0.52	4.83±0.20	5.03±0.2
sYV**	**0.03**	**3.65±0.3**	**2.18±0.30**
sGV**	0.42	3.08±0.44	2.52±0.44
sBC**	0.78	1.27±0.27	0.36±0.27
sPGV**	0.36	28.02±7.12	38.02±7.12
sPSB**	0.64	5.05±0.4	4.77±0.4
sNFB**	0.99	4.2±0.53	4.21±0.53
sF**	0.28	2.76±0.4	2.60±0.4

Significant P values are in bold; *= see Table 1 for the units;
**= values are in log 10 of the counts

6.3.3. HARVEST DATA

FCR, ABW, DoC, DWG, and survival of shrimp at harvest, including WSSV detection are presented in Table 6.9. FCR was significantly higher (P<0.001) in GW ponds (1.84) than non-GW ponds (1.68). WSSV was detected in one of the greenwater ponds at DoC 120 (63.5 WSSV/mg gills) and not in any other sample. Survival and shrimp DWG was higher in GW ponds.

Table 6.9. Average body weight (ABW; g) of shrimp, days of culture (DOC) and survival (SR; %) at harvest; daily weight gain (DWG; g) including WSSV detection in shrimp cultured using (GW) and not using (non-GW) technology.

	Harvest data				WSSV detection		
	DOC	ABW	SR	FCR	Quantity WSSV/mg gills	DOC	DWG
GW pond 1	178	39	100	2.1	63.5	120	0.22
GW pond 2	165	33	90	1.9	nd	na	0.2
GW pond 3	138	43	90	1.6	nd	na	0.31
Non-GW pond1	143	25	100	1.7	nd	na	0.17
Non-GW pond 2	125	20	30	1.68	nd	na	0.16
Non-GW ponds 3	132	29	77	1.65	nd	na	0.22
GW pond Average	160	38[a]	93	1.84±0.21[b]			0.24
Non-GW pond Average	133	25[a]	69	1.78±0.03[b]			0.19

a=significant difference observed between GW and non-GW ponds (P=0.025)

b= significant difference observed between GW and non-GW ponds (P<0.001)

6.4. DISCUSSION

The levels of nitrogenous wastes were higher than the safe level but this was expected. Furthermore, feed and faecal matter load increase during the production cycle.

In the present study, the aerators used in the culture ponds explain for the higher DO observed in this location. Highest DO was observed at DoC 0. Shrimp farmers using the greenwater technology prefer a lower salinity level to culture the shrimp because they believe that shrimp are not easily infected by WSSV at low salinity. This explains for the significantly lower salinity observed in GW ponds. The same trend was observed in the analysis for the 'sample location' nested in 'farm type' with 'DoC' as the repeated measure and in the analysis for culture ponds only. In both analyses, parameters in the culture pond of GW farms were better than for non-GW farms.

Though not statistically different, comparison of the three water intakes showed a trend that the intake channel of non-GW had lower nutrient load than the reservoir of GW, which is lower than the intake channel of GW. This implies that comparison of non-GW with GW was not fair because from the start the non-GW had better water quality. In addition, subsequent observations indicated that passing the river water through a tilapia reservoir pond before transfer to the shrimp pond lowers NH_3, NO_2, and PO_4 concentrations.

The soil redox was positive in GW and non-GW systems. This might be attributed to the use of paddle wheels. Paddlewheel induced aeration and water mixing could have loosened the sediments while providing more oxygen to the bottom layer. Masuda & Boyd (1994) reported that the upper 0-5 cm layer of the sediment in shrimp pond is more reactive with the water column. Lower redox potential in the GW system could be due to bioturbation caused by the tilapias stocked in the reservoir exposing more organic matter to oxygen. Joyni et al. (2011) suggested that bioturbation associated with the swimming activity of tilapias is effective down to 7cm of the sediment.

Luminous bacteria were lower in the soil of GW culture ponds. Luminous bacteria belong to the green vibrios, wherein most pathogenic variants of vibrios belong (Travers et al., 2009; Junpeng et al., 2007; Diggles et al., 2000). Interestingly, the percentage green *Vibrio* in the soil (sPGV) was higher in GW pond. This implies that luminous bacteria were not the only green vibrios present in the GW ponds.

Yellow *Vibrio* count was significantly higher in the water of non-GW ponds. This could explain for the non-occurrence of WSSV infection in non-GW ponds in addition to the significantly higher salinity (16 ppt). Dominance of the yellow *Vibrio* has been reported as a WSSV protective factor, while frequent exposure to salinity below 15 ppt as a risk factor (Tendencia and Verreth, 2011a). Furthermore, higher black colonies on TCBS (sBC) were recovered in non-GW ponds. The black colonies on TCBS belong to the genus *Shewanella*, (Gram and Huss, 1996; Fuenzalida et al., 2007). *Shewanella* are important in the nutrient cycle, and have been reported to have probiotic effect against *Vibrio parahaemolyticus* (Prescott et al., 2005; Zadeh et al., 2010). The probiotic effect of the black colonies on TCBS warrants further study.

The higher CC in GW ponds is in accordance with Cremen et al. (2007). Higher CC suggested that the use of the GW technique to culture *P. monodon* improves water quality. *Chlorella, a Chlorophyceae,* has been reported to improve pond water quality (Sivarikumar et al., 2011; Hernandez et al., 2009). Aside on its effect on water quality, the abundance of Chlorophyceae in GW ponds might have improved the shrimp's immune competence to resist disease thus resulting in higher survival; however, this needs further investigation. It has been reported that hydrolyzed *Chlorella vulgaris* promotes the release of inferons (IFN) in mice (Kim et al., 2010). Furthermore, IFN's have protective immunomodulatory function against pneumonia virus of mice resulting to high survival (Rigaux et al., 2012).

There was one observation of WSSV infection in a GW pond but no outbreak occurred. The low salinity (8 ppm) used to culture the shrimp in GW ponds could have predisposed the shrimp to WSSV infection. Vargas-Albores et al. (1998), Cheng and Chen (2000), Yu et al. (2003), Lu-Qing et al. (2005) and Liu et al. (2006), correlated low salinity with infection The present study did not provide a clear explanation for the absence of a disease outbreak. However, three mechanisms might be involved. One is the possible decrease in viral load. Tendencia et al. (2012) showed a decrease in WSSV viral load in shrimp cultured in a simulated greenwater environment although mortality was highest in this treatment. Another mechanism is the higher CC in GW ponds. Tendencia et al. (2012) observed higher shrimp survival despite increased WSSV viral load in shrimp cultured in tanks with *Chlorella* added. The use of natural food i.e phytoplankton is identified as a WSSV protective factor (Tendencia et al., 2011). The absence of other

predisposing factors like the Vibrios, could be another mechanism. The lower sLB in GW ponds did not act as additional stressor to the *P. monodon*, which are bottom dwellers.

Results of the present study suggest that the water quality in GW might improve as it passes through the reservoir; and that some soil and water qualities of the GW were better than the non-GW ponds. However, these results did not clearly showed the protective role of GW compared to non-GW against WSSV infection, as most differences were statistically insignificant. These could be attributed to two limitations of the study. One was the small number of monitored ponds due to the absence of commercial farms that fit the criteria. Another limitation is the uncontrolled experimental environment on the commercial *P. monodon* farms where quality of input water differs and where farm managers of both systems implemented measures to prevent WSSV infection and/or outbreak. These preventive measures could have affected the pond ecosystem thus further masking the real differences between GW and non-GW. To clearly show the differences between GW and non-GW, it is suggested that a longitudinal study be done on several small experimental ponds that will allow more repetitions and controlled farm management techniques.

CHAPTER 7

GENERAL DISCUSSION

The shrimp industry expanded over the years and diseases accompany this expansion. Some diseases have limited impact but others linger for decades like the ones caused by viruses. The most lethal of the shrimp viruses is the white spot syndrome virus (WSSV) first reported in 1992 in Asia. Since then it has spread over most shrimp producing countries of the world (Flegel, 2012; Lightner, 2011; Stentiford and Lightner, 2011). After 2 decades of existence, WSSV continues to bring havoc to the shrimp industry without proven preventive or control measure and this results often in bankruptcy of many shrimp farmers, especially those with small capital support. Several tank–based studies identified temperature, salinity and pH as WSSV risk factors in crustaceans other than *P. monodon* (Gao et al., 2011; Rahman et al., 2007; Du et al., 2006; Jiravanichpaisal et al., 2006; Carbajal-Sanchez et al., 2008; Liu et al., 2006). The pharmaceutical industry brought specific products on the market like probiotics, disinfectants, and immunostimulants that claim to prevent or control WSSV. Also farming practice plays an important role in the occurrence of the WSSV disease (Joffre and Bosma, 2009). Farms implemented biosecurity measures, including the import of pathogen free fry for which production a specific industry emerged. Several shrimp culture techniques emerged like aquasilviculture, biofloc technology, crop rotation, greenwater technology, low salinity, and zero discharge-recirculating system among others (Avnimelech 1999; Tendencia and Lavilla-Pitogo, 2005). However, most of these practices failed to reduce the impact of WSSV, except the use of the greenwater technique. Most shrimp farmers observed that the use of greenwater renders some temporary if not permanent protection against WSSV.

In recognition of the foregoing gaps, this thesis aimed to study the epidemiology of WSSV in on-farm situations, to have a better understanding of the factors that trigger disease infections or outbreaks, and to identify factors that render protection against infection or outbreak. We hypothesized that these factors would provide management strategies to prevent WSSV infection or to control WSSV infection from resulting in an outbreak.

In this final chapter, results of the research study are integrated and discussed in relation to the hypothesis, and limitations of the study and research gaps are identified. The discussion is grouped into the following issues: 1) The role of water/sediment quality; 2) Possible ecological means of disease prevention/control; 3) Implication on aquaculture management; and 4) Limitations and research gaps.

7.1. THE ROLE OF WATER/SEDIMENT QUALITY

At the start of our study we hypothesized that WSSV outbreak is affected by the water and sediment quality. The thesis identified on-farm WSSV risk and protective factors related to water quality, which will be divided into the physico-chemical parameters, the microflora, and farm practices. The physico-chemical parameters are the abiotic factors, while the microflora is a biotic factor.

7.1.1. PHYSICO-CHEMICAL

At the start of the study, results from several tank based experiments on WSSV risk factors related to temperature were available but they were done on crustaceans other than *P. monodon*. It was reported that viral replication and load increased at constant low water temperature (22-27^{o}C) (Rahman et al., 2006; Reyes et al., 2007), while the shrimp' immunocompetence is compromised (Vidal et al., 2001) thereby making them more susceptible to WSSV infection which increased in viral load. Although low temperature observed during shrimp culture is a WSSV risk factor, extremely low temperature is a protective factor as far as viral replication and load is concerned; Du et al. (2008) and Jiravanichpaisal et al. (2004) reported reduced WSSV replication at 4-15^{o}C. On the otherhand, at constant high temperature (>28^{o}C) viral replication is reduced or completely inhibited, while shrimp immune response is strengthened thereby making them less susceptible to disease agents (Reyes et al., 2007; Rahman et al., 2006; Guan et al., 2003; Vidal et al., 2001). However, Rahman et al. (2007) demonstrated that when temperature fluctuates between 27-33^{o}C, viral load in shrimp increased resulting to an increased mortality.

In this thesis, exposure to low temperature (26-27^{o}C), temperature fluctuation of 3-4^{o}C in 10h and pH fluctuation of 1 point in 1h were identified as important WSSV risk factors in *P. monodon* in ponds, which will result in an infection but not necessarily in an outbreak (Chapters 2 & 3; Tendencia and Verreth, 2011b). Contrary to low temperature, high temperature reduces the risk of WSSV infection (Chapter 2).

Prior to this thesis, only a few studies were done on salinity as a WSSV risk factor with contradictory results and mostly in species other than *P. monodon*. Joseph and Philip (2007) reported a reduced immunocompetence in *P. monodon* challenged with WSSV after exposure to salinity fluctuations from 15 to 0 ppt and from 15 to 35 ppt for

7h. Liu et al. (2006) reported increased viral load in *Fenneropeneaus chinensis* exposed to salinity fluctuation of 8ppt (from 22 to 14 ppt) in 1h but not in a lesser fluctuation of 4 ppt (from 22 to 18 ppt). Carbajal-Sanchez et al. (2008) reported more severe WSSV infection in *Littopenaeus vannamei* cultured at 15 ppt than those at 2, 5, 25 and 35 ppt. Gao et al. (2011), on the other hand, reported that best viral replication in *F. chinensis* is at 35ppt among different investigated salinities (15, 20, 25, 30 ppt).

This thesis confirmed the role of salinity in WSSV epidemiology in *P. monodon*. In healthy shrimp, Ye et al. (2009) reported significantly low survival in shrimp cultured at 5ppt compared to those maintained at 10-35ppt. Salinity fluctuations are important WSSV risk factors but not at constantly low salinity. Constant salinity of 10 ppt, which is below optimum for shrimp culture, is not a WSSV risk factor (Tendencia and Verreth, 2011b). The effect of low salinity could be on the general well-being of the shrimp and not on viral replication. Salinity fluctuation in WSSV infected shrimp is an important factor for an outbreak of the disease (Chapter 3). The effect of salinity on WSSV is opposite to that of temperature. High viral load is observed at low temperature and low viral load at high temperature. Whereas, at low salinity, low viral load or replication is observed and high viral load or replication are observed at high salinity. Salinity and temperature fluctuations could be related to rainfall, which has been identified as another WSSV risk factor (Tendencia et al., 2010b). Furthermore, Tendencia et al. (2010b) explained that continuous raindays will result in lower water temperature and salinity.

Among the physicochemical properties of the water related to WSSV epidemiology, ammonia and pH are the least studied. This thesis identified that pond water with a pH below 8 is a WSSV risk factor (Tendencia and Verreth, 2011b), however, as mentioned earlier, this may be related to pH fluctuation, which is also identified as a risk factor (Chapters 2 & 3). Furthermore, the role of pH in WSSV epidemiology may be indirect through the toxicity of substances in the system like NH_3, which affects the shrimp's immune response and viral virulence. An increase in pH would increase the NH_3 concentration in the water, which could reach toxic levels. Jiang et al. (2004) reported that 5mg/L of NH_3 could reduce the immunocompetence of *P. japonicus* but suggested that it lowers WSSV virulence. The effect of pH on WSSV epidemiology is not as important as temperature since the effect on the shrimp and virus are indirect through the effect of NH_3, which is increased with increased pH. Furthermore, the

parallel effect of NH_3 concentration on the shrimp and WSSV decreased its effect on shrimp mortality; high ammonia concentration decreases shrimp immune response but likewise decreases WSSV virulence, thus shrimp mortality is not as pronounced as in the effect of temperature. This parallel effect of NH_3 on shrimp and WSSV virulence could explain for the pH<8 as a risk factor. At pH>8, NH_3 is high thereby decreasing the shrimp's immunocompetence but WSSV virulence is also low; when pH drops to below 8, WSSV virulence is increased at a time when the shrimp's immune system is compromised, consequently, the infection results in an outbreak. Furthermore, Gao et al. (2011) reported best WSSV proliferation at pH 8 and worst at pH 9, suggesting that WSSV proliferates better at low pH. The direct role of pH on WSSV epidemiology is through the possible apoptosis or death of the cells of the shrimp's lymphoid organ during exposure to extreme pH. Wang et al. (2007) reported apoptosis of the lymphoid organ cells after exposure to pH 7 or 9. The lymphoid organ plays an important role in the immune response of shrimp (Kondo et al., 1994, Martin et al., 1996). Apoptosis of the lymphoid organ cells after exposure to high pH might have rendered the shrimp more susceptible to WSSV at the same time that WSSV proliferation increased due to the subsequent decrease in pH.

This thesis found that factors that caused outbreaks seemed to be related to fluctuations of the parameters; either from low to high or high to low could result in an outbreak. Results of this thesis could assist shrimp farmers on the proper time to culture *P. monodon*.

7.1.2. MICROFLORA

Water microflora in this thesis are divided into the phytoplanktons and the bacteria. The role of water microflora in WSSV epidemiology might be due to a modified water quality and enhanced shrimp immune response. The latter is not addressed in this thesis.

7.1.2.1. PHYTOPLANKTON

At the start of the study, no WSSV protective factor was associated with the water microflora. This thesis identified abundant phytoplankton acting as a WSSV protective factor (Chapter 4), which is supported by the protective effect of water transparency (Chapter 2). Water transparency, measured using a secchi disk, is used to monitor the

abundance of phytoplankton, in the absence of a laboratory. Nitrogen concentrations are lower in ponds with higher phytoplankton count (Chapter 6; Sivarikumar et al., 2011). Phytoplanktons use the nitrogen present in the water thereby lowering nitrogenous wastes to non-toxic concentrations; water quality becomes suitable for shrimp culture (Hernandez et al., 2009; Glibert and McCarthy, 1983). The role of phytoplankton on shrimp immunity is unclear, it is possible that phytoplankton uptake by the shrimp stimulates the production of interferon which are produced by animals in the presence of pathogens such as WSSV. Ponprateep et al. (2011) suggested the binding of WSSV particles by a proteinase important in the innate immune response of shrimp which give the organism protection against WSSV. Substances like proteases and lectins are important in the innate immune response of shrimp (Luo et al., 2011; Chen et al., 2011; Ponprateep et al., 2011). Furthermore, Khimmakthong et al. (2011) demonstrated that the immune response of shrimp can be stimulated; the ability of *Chlorella vulgaris* hydrolysate to trigger the immune response in mice has been reported earlier (Kim et al., 2010). Therefore, it is possible that the protective effect of phytoplankton is by triggering the production of the shrimp's innate anti-viral substances.

7.1.2.2. BACTERIA

Among the bacterial flora, dominance of the yellow vibrios over the green ones or percentage green vibrios lower than 50% is identified as a WSSV protective factor (Chapters 2 & 3). The yellow vibrios referred to in this thesis, are the yellow colonies on thiosulphate citrate bilesalt sucrose agar, a *Vibrio* selective medium. Yellow vibrios belong to the heterotrophic bacteria reported to use nitrogen in the water for cell growth and reproduction (Zher and Ward, 2002). Removal of nitrogen in the water could result in lower NH_3 level, which affects the shrimps' immune response, and WSSV virulence as explained in section 1.1. Also, most vibrios reported to have probiotic effects seem to belong to the yellow ones. Krupesha Sharma et al. (2010) demonstrated WSSV removal from *P. monodon* hemolymph after feeding with biofilm of *V. alginolyticus*, a yellow *Vibrio*. It is possible that the yellow vibrios present in the ponds also have this probiotic effect against WSSV.

Results of this thesis identified that abundant growth of phytoplanktons such as *Chlorella* and the proliferation of the yellow vibrios are ecological means of preventing WSSV infection from resulting in outbreaks. Farm practices that will encourage the

abundant growth of phytoplanktons and the proliferation of the yellow vibrios are management tools that shrimp farmers can adopt to prevent disease outbreaks without resorting to the use of chemicals or other synthetic products which can be harmful to the environment.

7.1.3. FARM PRACTICES

At the start of our study we hypothesized that farm management affects water and soil quality. Prior to and during the conduct of the thesis several studies have been done in relation to farm practices and WSSV occurrence. Complete system dry-out between culture cycles, water filtration, fertilization with phosphorus, and use of commercial feeds, were reported to contribute to excellent shrimp survival in spite of the presence of WSSV (Velasco et al., 2002; Corsin et al., 2001). Contradictory effects of stocking density on WSSV infection were reported. Corsin et al. (2001) did not observe an association between stocking density and WSSV infection at harvest. Pienado-Guevara and Lopez-Meyer (2006) reported that removal of 40 and 50% of the shrimp population at a low level WSSV infection improved shrimp survival, suggesting that at lower density there is less disease prevalence.

Looking into farm practices, this thesis identified more WSSV risk factors related to water and sediment quality. Most of them were related to risks for contamination such as sharing of water source with other farms, using the same water body for intake and outlet, removing sludge and depositing it on the dikes, and feeding with molluscs (Chapter 4). WSSV may persist in the water for 20 months (Quang et al., 2009). WSSV infection via recycled water in extensively managed pond and from neighbouring ponds in semi-intensive farms have been demonstrated (Walker et al., 2011; Hoa et al., 2011; Esparza-Leal et al., 2010). Based on molecular studies, Hoa et al. (2012) reported that spread of WSSV in improved-extensive system occurs within the pond; while transmission via pond to pond occurs more in the semi-intensive systems. Contaminated effluents from farms that had WSSV outbreak may be taken in by adjacent farms or even by the farm itself during water change. Consequently, the WSSV-contaminated water may infect the cultured shrimp. Sludge removed from the pond bottom may harbour WSSV that can persist in the substrate for more than 10 months (Natividad et al., 2008). The WSSV-contaminated sludge deposited on the dike may find its way back to the pond

during rainy days or through crabs that can crawl on and in the dike covered with sludge, get contaminated with WSSV and carry the virus into the pond and infect the cultured shrimp. Ingestion of molluscs can infect shrimp. Molluscs are filter feeders that can ingest WSSV particles from the water column or those from the soil, which can then be ingested by the shrimp when the shrimp feed on the molluscs. Chang et al. (2011) and Vasquez-Boucard et al. (2010) demonstrated the ingestion of WSSV particles by the molluscan bivalves, *Meretrix lusoria* and *Crassostrea gigas*. Furthermore, the former reported that though WSSV may accumulate in the bivalve and infect *Litopennaeus vannamei* through feeding, it couldn't replicate inside the molluscs.

Other factors pertain to management strategies such as larger pond size, higher stocking density and stocking during the cold months (Chapter 4). Karim et al. (2011) found that larger ponds and higher stocking density are WSSV risk factors. Large pond size and high stocking density could lead to difficulty in management in terms of feeding, monitoring shrimp health status, efficient aeration, proper pond preparation and maintaining good water quality. Underfeeding could compromise the shrimp's immune defence system resulting in increased susceptibility to infectious agents, which are not readily detected due to limited time to oversee all activities. Pond preparation in large ponds are usually shortened thus resulting in poor water quality during shrimp culture which consequently lead to increased susceptibility to diseases like WSSV. Farmers would like to do more crops a year to make more profit. Stocking during the cold months can be avoided but some farmers still take the risk for the same financial reason and shorten the pond preparation period.

This thesis reiterated the importance of farm practices such as rest period, ploughing, sludge removal, pond drying, lower stocking density, and stocking during the warm months; site selection i.e, presence of other farms in the area, water source; and pond size for monoculture ponds only, in relation to WSSV occurrence. Results of this thesis can be used by legislators in the promulgations of laws on good management practices that could prevent disease outbreaks.

7.2. ECOLOGICAL MEANS OF DISEASE CONTROL

It was hypothesized that ponds with a higher mangrove to pond area ratio (MPR) and that greenwater (GW) ponds will have better water quality as far as it relates to factors with potential disease incidence.

7.2.1. MANGROVE TO POND AREA RATIO

Aside from a survey done by Belak et al. (1999), no other paper was published on the relationship of mangroves with disease occurrence. We were not able to prove our hypothesis but identified high mangrove to pond area ratio (Chapter 4) as a WSSV protective factor. Farms with mangroves in the receiving environment had WSSV infection not resulting in an outbreak (Chapter 5). Unfortunately, the thesis cannot provide an explanation for this. Measured water quality parameters are not significantly different between farms with different MPR (0:1, 1:1, 4:1). This may be because the MPR in the investigated farms were below those reported to be efficient, although it fits the MPR of 4 : 1 as suggested by Saenger et al. (1983) for a healthy ecosystem. In separate systems, as in the farms in this thesis, where mangroves are outside the culture pond, between 2 and 8 hectares of forest are required to filter the nitrogen from effluent produced by a 1 ha pond (Primavera et al., 2007; Robertson and Philipps, 1995). A relatively low MPR (0.04 to 0.12 ha of mangrove forest to 1-ha pond) is required to completely remove the dissolved inorganic nitrogen load from effluents (Rivera-Monroy, 1999), but a high MPR of up to 22:1 is required to process phosphate (Primavera et al., 2007; Robertson and Philipps, 1995). In mixed mangrove and pond systems, a generally lower MPR is required to remove nutrient levels from the pond water: 2.1-5.2 : 1 to remove nitrogen (Shimoda et al., 2007) and 6.2-8.9 : 1 to fully process the phosphorus (Shimoda et al., 2005).

The fact that WSSV infection does not result in a disease outbreak with mangroves in the receiving environment (Chapter 5), suggests that the mangrove habitat itself has anti-viral property or might have stimulated the shrimp's innate immune response leading to the production of anti-viral substances. Investigation of this hypothesis was beyond the scope of the present thesis. Nga et al. (2006) found higher weight gain and survival in shrimp fed with *Rhizophora apiculata* leaves than those fed with commercial pellets. Although the authors did not give an explanation for this

observation, the high survival could be due to the antimicrobial properties of the mangrove leaves, which give the shrimp protection against diseases. Sudheer et al. (2011) demonstrated that WSSV-infected *P. monodon* fed with pellets coated with extracts from the leaves of *Ceriops tanggal* got rid of all WSSV virions; the same effect was observed in a challenge experiment using pure *C. tanggal* extract on WSSV. In addition to *C. tanggal*, leaves extracts of *Rhizophora mucronata* and *Sonneratia* sp were also found to have anti-WSSV activities. Farms with mangroves have lower green *Vibrio* counts (Chapter 5) that are supposed to be the pathogenic ones. This again suggests that the mangrove habitat might have antimicrobial activity. However, this contention needs to be investigated.

The present thesis did not provide clear evidence that the presence of mangrove improves soil and water quality, or provides protection against WSSV infection or outbreak. However, it suggested that a high MPR may provide some protection against WSSV.

7.2.2. GREENWATER TECHNOLOGY

The thesis did not provide clear evidence that GW (greenwater) provides a better water quality than non-GW and that the first suffer less from WSSV infection and disease outbreaks. Chlorophyceae are more abundant in GW ponds (Chapter 6). As explained in section 1.2, phytoplanktons play important roles in the nitrogen removal from the water. Furthermore, the microflora may have probiotic effects that might stimulate the shrimp's immune response; proving this is beyond the scope of the thesis. The efficiency of the GW technology, using different fish species such as tilapia, seabass, snapper, siganid, and grouper against luminous bacterial disease of *P. monodon* has been demonstrated; tilapia was found to be the most efficient at a biomass not lower than 300 g/m^3 to shrimp biomass of 80 g/m^3 (Tendencia et al., 2004; Tendencia et al., 2005; Tendencia et al., 2006b). Furthermore, the efficiency of tilapia at a biomass a higher biomass of 500 g/m^3 is reduced if the shrimp biomass is greater than 80 g/m^3 (Tendencia et al., 2006). The anti-luminous bacterial property of the greenwater technology was attributed to the associated bacterial, fungal, and phytoplankton micro biota and the skin mucus and faeces of tilapia; and bacteria associated with the skin mucus and faeces of tilapia (Lio-Po et al., 2006; Tendencia et al., 2010). *Vibrio* infection was reported to enhance susceptibility to WSSV (Phuoc et al., 2008), and the potential effect

of GW on WSSV outbreaks might pass through the reduction of these or other factors stressing shrimp.

Results of the present thesis did not provide clear evidence that the use of the greenwater culture system can improve water and soil quality. Water and soil parameters between greenwater and non-greenwater farms were mostly not statistically different but those in the former were better. This suggest that the use of the greenwater culture system might improve water quality, but this was not apparent in this thesis, which may be due to the limited number of ponds monitored.

7.3. IMPLICATION ON AQUACULTURE MANAGEMENT

Identified WSSV risk factors related to the physico-chemical parameters of the water may be avoided by not stocking during the cold months when temperatures are low or during the rainy season when temperature, salinity and pH fluctuations could be very frequent. Even larger farms in the Philippines, one being integrated in a vertical market chain of a global supermarket company, avoid the rainy season by stocking the ponds only once a year and use the GW technology thus not filling all available water surface with shrimp.

Science-based recommendations on disease (including WSSV) prevention are tank- and biotechnology-based. These kind of studies have mostly a reductionist approach and do not consider affordability, field applicability and effect on the ecosystem of the newly tested substance or method. Organisms have innate substances that protect them from disease. The innate immune system could be altered through the introduction of other objects in the system. In the case of aquatic organisms, any addition into the water, like aquatic products, may affect water quality, which may affect the shrimp immune response and consequently the organism's response to diseases. Aside from their negative impact on the environment, these aquaculture products affect shrimp health in two ways: 1) indirectly by modifying the environment making it stressful to the shrimp, compromising shrimp's immune defence system and consequently resulting in disease and mortality; and, 2) directly by affecting the shrimps resistance to disease or the shrimp's response to treatments. The negative effect of aquaculture products can be avoided by considering the principles of aquatic ecology in

pond management which is environmentally and financially more sustainable, and affordable to small-scale shrimp farmers.

In this thesis we could not find undisputable evidence, but our results suggested that the use of the greenwater technology and the association of mangroves in the receiving environment (associated greenwater-mangrove system) might prevent WSSV infection to result in an outbreak. We were not able to demonstrate that water quality is better in greenwater than in non-greenwater ponds, which would have provided basis to explain our observation of a mitigating effect of greenwater. Phytoplankton and heterotrophic bacteria are abundant in ponds of farms using the greenwater technology (Chapter 6). Cultured shrimp may feed on both microflora, which might enhance their innate response against pathogenic agents (Kim et al., 2010). Integration of mangroves in the receiving environment diminishes the prevalence of harmful bacteria thus reducing the risk due to primary bacterial infection. Based on literature, a mangrove to pond area ratio higher than 4 : 1 is suggested.

Use of the greenwater technology with mangroves in the receiving environment might mitigate the risk due to sharing water source with other farms and using the same water body for intake and outlet (Chapter 4). Water may undergo purification and decontamination and suspended solids settle in the reservoir, which serve as the intake water for the shrimp culture pond. Effluents from the shrimp culture pond are also purified and might be decontaminated in the mangrove habitat where they are drained. This system might reduce the risk due to poor water quality and contamination from other farms or ponds but this thesis was unable to identify the responsible factor (s).

The greenwater culture system and mangroves in the receiving environment could also provide additional sources of livelihood to the shrimp farmers (Bosma et. al., 2012; Warren-Rhodes et al., 2012; Karim et al., 2011). A study in South Vietnam revealed that households that integrated mangroves in the shrimp culture system had (on the long term) a higher income compared to those that did not (Tran et al., 2012). Fully-grown trees can be cut and sold as firewood or timber for houses. In greenwater, bigger finfish in the reservoir can be harvested and sold, while the smaller ones are left to grow and provide the greenwater effect.

Based on literature, the use of mangroves mitigates effects of climate change that are WSSV risk factors such as extremely high temperature and high salinity. Temperature could be lower in mangroves areas due to the shades the leaves provide.

The rooting network of mangroves introduces oxygen into the sediment and focuses evapo-transpiration and salt exclusion in the area (Comeaux, 2012) resulting to lower salinity. Mangroves assist in carbon sequestration and are a better carbon pool than all pools combined in upland systems (Donato et al., 2012). Some of the other positive implications of mangroves in relation to climate change are: prediction of temperature and sea-level rise (Aral et al., 2012), dissipation of wave energy that in turn reduce wave run-up (Villanoy et al., 2012) thus preventing erosion of pond dikes.

Although this thesis did not identify the factors responsable for the interest of farmers in the GW system, nor of the factors reducing disease risks by the association of mangrove, both systems have advantages. The farming systems may be adopted in both extensive or semi-intensive *P. monodon* culture.

7.4. LIMITATIONS AND RESEARCH GAPS

The mechanisms behind the identified on-farm WSSV risk and protective factors were beyond the scope of this thesis. Although the effect of physical parameters such as temperature, salinity and pH on the immune response of shrimp and on viral replication has been reported, the effect of yellow vibrios and phytoplanktons on shrimp immune response and on viral replication needs further investigation.

In vitro challenge tests of the different components of the greenwater technology such as fish mucus and faeces, phytoplanktons and bacterial flora, and the different components of a mangrove habitat such as microflora and leaf litters of various mangrove species, against WSSV need to be done to elucidate their role in WSSV epidemiology. If indeed, the various components of the greenwater technology and mangrove habitat have anti-WSSV activity the next step is to investigate their possible use as shrimp feed or for improving water quality.

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SUMMARY

SUMMARY

White spot syndrome virus (WSSV) has brought economic devastation to all kinds of shrimp farming systems. Most studies done on WSSV are tank-based and mostly on species other than *Penaeus monodon*. This thesis aimed to study WSSV epidemiology in on-farm situation and find ecological means of WSSV prevention or control measures. Case, cross sectional and longitudinal studies were done to achieve these goals. The thesis is divided into 7 chapters.

The Introduction in **Chapter 1** gives a short background on factors associated to WSSV occurrence. The rationale behind the consideration of the presence of mangroves and the greenwater technology as ecological means of disease prevention was presented. The research problem, hypotheses and questions the thesis tried to answer were identified in this chapter; the approaches used to address them were summarized.

Chapter 2 identifies WSSV risk factors related to the physico-chemical parameters of the water by analyzing the water physico-chemical properties and microflora of 91 production cycles of 8 semi-intensive shrimp farms. Low water temperature, low salinity, low transparency and high water level, and fluctuations in temperature, salinity and pH were identified as important risk factors that will result in an infection but not necessarily in an outbreak. However, further studies are suggested to clarify the effects of water depth, water transparency, and various bacterial counts which could be individual or interactive. Exposure to high salinity and high temperature are important factors for an infection to result in an outbreak. The risk of an infection is reduced when the water temperature is high, salinity fluctuations are small, and percentage of yellow *Vibrio* colonies is greater than the green ones. The contradictory effect of high temperature on WSSV infection and outbreak might be related to temperature fluctuation.

A case study that compared bio-physico-chemical properties of three ponds of a farm having experienced a WSSV outbreak in 2006 but not in 2005 is presented in **Chapter 3.** This chapter mentioned that luminous bacterial count (10^3 cfu/ml) and percentage green vibrios (>50%) were higher in ponds with infection resulting in an outbreak. The synergistic effect of temperature fluctuation of 3-4°C within a 10 h period, frequent salinity fluctuation to lower than 15ppt and high presumptive *Vibrio* count (10^4 cfu/ml) were identified as risk factor for an infection to result in an outbreak.

Risk factors associated with two different farming systems: polyculture with crab or finfish like milkfish and tilapia, and semi-intensive monoculture of *Penaeus monodon* are discussed in **Chapter 4**. Data were gathered from a total of 174 shrimp farmers in eight provinces of the Philippines using a structured questionnaire. In the analysis of the dataset aggregating monoculture and polyculture farms, feeding live molluscs was identified as important WSSV risk factor. In addition to feeding live molluscs, sharing of water source with other farms, having the same receiving and intake water, larger pond size, and higher stocking density were identified as important WSSV risk factors in monoculture farms. Climate, i.e. stocking during the cold months and sludge removal and its deposition on the dikes were identified as WSSV risk factors in polyculture farms. Analysis of the dataset aggregating polyculture and monoculture farms identified feeding with phytoplanktons as a protective factor. Only high mangrove to pond area ratio was confirmed as a protective factor in monoculture farms. No protective factor was identified in the dataset of only polyculture farms.

Chapter 5 determines if mangroves affect the physicochemical properties of the water and soil as well as the prevalence of infectious agents like the WSSV by monitoring farms with different mangrove to pond area ratio (MPR; 0:1, 1:1, 4:1). WSSV infection not resulting in an outbreak was observed in the four farms with mangroves in the receiving environment; two of these farms used deep well as intake channel. Results of the investigation did not provide explanation for the infection not resulting in an outbreak. There was no difference in water quality between farms with different MPR. However, soil analysis showed significantly higher available sulphur in MPR 4:1, and significantly higher percentage green vibrios in the soil in MPR 0:1. Results suggest that the presence of mangroves could prevent prevalence of harmful vibrios but no statistical evidence related to an effect on WSSV infection or outbreak was identified.

Some shrimp farmers in the Philippines claimed that the greenwater (GW) technology could prevent disease outbreak due to WSSV. **Chapter 6** evaluated the efficiency of the GW technology by comparing three farms using the GW culture technique to three farms not using it. Lower total suspended solids and nutrient levels despite higher feed conversion ratio; higher plankton counts in GW suggest that the technology improves water quality. Furthermore, greater shrimp daily weight gain and

higher shrimp survival despite WSSV infection in GW farms were observed, suggesting that the use of the GW technology grow healthier shrimp compared to non-GW farms.

The general discussion, **Chapter 7**, integrated the results of the research study and discussed these in relation to the hypothesis. The thesis could not detect statistical significant evidence for a protective effect against WSSV by the use of GW and the presence of mangroves. Yet, when these pond management strategies were used, disease outbreaks due to WSSV were not observed. Therefore, it is postulated that these measurements need further attention.

Some of the knowledge gaps needing research are the anti-viral activity and immunomodulatory effect of the GW technology, the mangrove habitat and the components of these ecosystems such as the phytoplanktons, the yellow vibrios, tilapia mucus and faeces, and leaf litters including mangrove species.

SAMENVATTING

SAMENVATTING

Het witte-vlek-syndroom-virus (WSSV) heeft zowel kleine als grote telers van garnalen aan de financiële afgrond gebracht. De meeste onderzoeken aan WSSV zijn uitgevoerd in tanks en veelal met andere garnalensoorten dan *Penaeus monodon*, de meest geteelde soort. Het doel van mijn PhD-promotieproject was het bestuderen van de WSSV-epidemiologie op bedrijven en van ecologische methoden om WSSV te voorkomen of te controleren. Daartoe zijn, naast enkele casussen, een enquête en twee observatie studies gedaan, waarvan de resultaten in zeven hoofdstukken zijn gepresenteerd.

De introductie, **Hoofdstuk 1**, beschrijft de factoren die verband houden met sterfte van garnalen ten gevolge van WSSV, en de redenen voor het beschouwen van de aanwezigheid van mangrovebossen en de 'greenwater'-technologie als ecologische middelen van ziekte-preventie. Tot slot bevat dit hoofdstuk de op dit probleem gebaseerde onderzoekshypotheses en onderzoeksvragen, evenals een samenvatting van de gebruikte methoden om deze te beantwoorden.

Het onderzoek in **Hoofdstuk 2** identificeertde WSSV-risicofactoren van het kweekwater door een analysevan de fysisch-chemische eigenschappen en de microflora in vijvers voor 91 productiecycli van 8 semi-intensieve garnalenbedrijven. Lage water temperatuur, laag zoutgehalte, lage doorzichtigheid, een hoog waterniveau en schommelingen in temperatuur, zoutgehalte en pH zijn belangrijke risicofactoren die tot een WSSV besmetting kunnen leiden, maar niet noodzakelijkerwijs tot een ziekte-uitbraak. Blootstelling aan een te hoog zoutgehalt een te hoge temperatuur zijn factoren die een besmetting kunnen doen omslaan in een uitbraak. Het risico van besmetting is verlaagd bij hoge watertemperatuur, bij geen of slechts kleine schommelingen van het zoutgehalte en wanneer het percentage gele *Vibrio*-kolonies groter is dan die van de groene (geel, groen, en hieronder lichtgevend hebben betrekking op de eigenschappen van de bacterie kolonie na groei op een laboratorium medium). Het tegengestelde effect van hoge temperatuur op WSSV-besmettingen-uitbraak kan samenhangen met temperatuurschommelingen. Verdere studies zijn nodig om te bepalen of de effecten van

waterdiepte en helderheid, en van allerlei bacteriesoorten, enkelvoudig zijn of onderling afhankelijk.

De studie in **Hoofdstuk 3** vergelijkt de chemisch een biofysische eigenschappen van drie vijvers van een bedrijf waar in 2006 een WSSV-uitbraak was, maar in 2005 niet. In de vijvers waar zowel het aantal lichtgeven de bacteriën (>10^3cfu/ml) als het percentage groene *Vibrio's* (>50%) hoger was, resulteerde besmetting in een uitbraak. Deze studie gaf aan dat het synergetische effect van temperatuurschommelingen van 3 tot 4°C binnen 10 uur, regelmatige schommelingen van het zoutgehalte tot onder de 15gr/kg en hoge aantallen *Vibrio*(10^4cfu/ml), hoge risicofactoren waren voor het omslaan van besmetting naar uitbraak.

Met gegevens uit een enquête analyseert **Hoofdstuk 4** de risicofactoren van twee verschillende bedrijfssystemen: polycultuur met krabben of vin vissen als melk visen Tilapia, en van semi-intensieve monocultuur van *Penaeus monodon.* Met behulp van een gestructureerde vragenlijst zijn gegevens van 174 garnalenbedrijven in acht provincies van de Filippijnen verzameld. De analyse van de geaggregeer de gegevens van de monocultuur en polycultuurbedrijven, gaf aan dat het voeren van levende weekdieren een WSSV-risicofactor was. Daarnaast waren het delen van de waterbron met andere bedrijven, het gebruiken van dezelfde bron voor waterverversing en -lozing, grotere vijverafmetingen en hogere garnalendichtheden WSSV-risicofactoren voor monocultuurbedrijven. Het starten van een cyclus in de koude maanden en het verwijderen en opslaan op de dijk van modder waren de bijkomende WSSV risicofactoren op de polycultuurbedrijven. De geaggregeerde gegevens van polycultuur- en monocultuurbedrijven gaven aan dat het voeren met fytoplankton beschermend werkte. Alhoewel statistisch niet bewezen, geven de gegevens van de monocultuurbedrijven aanwijzingen voor een mogelijk beschermend effect van een hoge verhouding tussen het oppervlak van mangroves en van vijvers (MPR), terwijl die van de bedrijven met polycultuur geen aanwijzing gaven voor bijkomende beschermende factoren.

Door het observeren van bedrijven met een MPR van 0:1, 1:1 of 4:1, onderzocht **hoofdstuk 5** het effect van mangroves op de fysisch-chemische eigenschappen van het water en de bodem alsmede op de aanwezigheid van ziekmakende organismen zoals

WSSV. Er was geen verschil in waterkwaliteit tussen bedrijven met de verschillende MPR's, maar de bodemmonsters van MPR 4:1 bevatten significant hogere hoeveelheden beschikbaar zwavel en van MPR 0:1 significant hogere percentages groene *Vibrio*'s. De resultaten suggereren dat mangroves de aanwezigheid van schadelijke *Vibrios* kunnen voorkomen.

Sommige garnalenbedrijven op de Filippijnen beweren dat de 'greenwater' (GW)-technologie ziekte-uitbraken ten gevolge van WSSV-besmetting kan voorkomen. **Hoofdstuk 6** evalueert de effectiviteit van de GW-technologie door drie bedrijven die deze gebruiken te vergelijken met drie bedrijven die deze niet toepassen. Lagere gehaltes aan totaal zwevende vaste stoffen en voedingsstoffen, ondanks de hogere (=slechtere) voerconversie, en hogere aantallen plankton bij GW-bedrijven suggereren dat de technologie de waterkwaliteit verbetert. Ook waren de dagelijkse groei van de garnalenen de overleving, ondanks besmetting met WSSV, hoger op de bedrijven die de GW-technologie toepasten, hetgeen suggereert dat deze bedrijven gezondere garnalen produceren dan bedrijven die GW niet gebruikten.

De algemene discussie in **Hoofdstuk 7** integreert en bediscussieert de resultaten van deze studie in relatie tot de gestelde onderzoekshypothesen. Het onderzoek kon geen statistisch significant beschermend effect van de GW-technologie en de aanwezigheid van mangrovebossen tegen ziekte uitbraken als gevolg van WSSV infecties aantonen. Toch werd in vijvers waar deze management strategieën toegepast werden, geen ziekte uitbraken geconstateerd. Daarom wordt geconcludeeerd dat deze management strategieën zeker nader onderzoek vragen.

De studie beveelt nader onderzoek aan naar de antivirale activiteit en het immuno modulaire effect van de'greenwater'-technologie, van de mangrove habitat en van de componenten van het ecosysteem zoals fytoplankton, de gele *Vibrio*'s, het slijm en de ontlasting van Tilapia, en van de bladeren van onder andere mangrove soorten.

ABOUT THE AUTHOR

SHORT BIOGRAPHY

Eleonor V. Alapide Tendencia is a researcher at The Fish Health Section of the Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDEC AQD) based in Tigbauan, Iloilo, Philippines. She has 6 children: Kristine (February 1985), Korina Marion (December 1985), Kempee (July 1989), Karen (November 1990), Kevin (February 1992) and Karlo (July 1996).

Gigi as friends and relatives fondly calls her, is the only daughter and first of the three children of Rosalia Cruz Villanueva and Lorenzo Soliveres Alapide Jr. She was born on March 3, 1962 in Manila, Philippines. She attended her elementary school at St. Paul College, Bocaue, Bulacan and graduated in 1974. She finished her secondary school in March 1978 from the same school. She then enrolled at the University of the Philippines, Diliman, Quezon City and obtained her BS Fisheries major in Aquaculture degree in 1983.

She joined SEAFDEC in 1983 as a research technician under the mollusk project. In 1984, she started working as a research assistant at SEAFDEC's Fish Health Section. As such, she assisted in various research studies exposing her to different aspects of fish diseases including diagnosis. Her work as a research assistant included bacterial isolation and identification, parasite detection, fungal isolation, cell culture and viral assays. Aside from research activities, she was also tasked to process or analyze organisms submitted to the Fish Health Section for disease diagnosis and recommended control and preventive measures.

In 1987, she again enrolled at the University of the Philippines in pursuit of higher education. She balanced her role as wife, mother, research assistant and student; and finally got an MSc degree in 1994. Her MSc thesis on the red disease of *Penaeus mondon* was published in the journal Aquaculture in 1997. Her very first research study after her MSc thesis was approved in 1998. Since then she had other research projects on fish diseases, antibiotic resistance, disinfection and on the control of luminous bacterial disease in shrimp. In 2006, she was awarded a Sandwich PhD Fellowship by the Wageningen University.

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AWARDS

Elvira O. Tan Memorial Award for Best Published Paper in Aquaculture/Inland Fisheries given in 2008 by the Philippine Council for Aquatic and Marine Research Development of the Department of Science and Technology Republic of the Philippines for the paper entitled Polyculture of green mussels, brown mussels and oysters with shrimp control luminous bacterial disease in a simulated culture system which appeared in Aquaculture 272: 188- 191

AFMA R&D Paper award given in 2006 by the Bureau of Agricultural Research Department of Agriculture Republic of the Philippines for the paper entitled Evaluation of the Anti-*Vibrio harveyi* activity of *Kappaphycus striatum* Schnitz and *Gracilaria heteroclada* Zhang ad Xia in a simulated shrimp culture system.

National R&D Paper award given in 2005 by Bureau of Agricultural Research, Department of Agriculture Republic of the Philippines for the paper entitled Presence of seabass, snapper, siganid inhibit the growth of luminous bacteria in a simulated shrimp culture system.

DA's Secretary award given in 2003 by Bureau of Agricultural Research, Department of Agriculture Republic of the Philippines for the paper entitled Level and Percentage recovery of resistance to oxytetracycline and oxolinic acid of bacteria from shrimp ponds.

DA's Secretary award given in 2003 by Bureau of Agricultural Research, Department of Agriculture Republic of the Philippines for the paper entitled Investigation of some components of the greenwater system, which makes it effective in the initial control of luminous bacteria

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To all who has been part of my journey.....a heartfelt gratitude!

TRAINING AND SUPERVISION PLAN

TRAINING AND SUPERVISION PLAN	Graduate School WIAS
Name PhD student	Eleonor A. Tendencia
Project title	The relation between fsrming practices, Ecosystem, and White Spot Syndrome Virus (WSSV) disease outbreaks in *Penaeus monodon* farms in the Philippines

EDUCATION AND TRAINING	year	credits *
The Basic Package		**3**
WIAS Introduction Course	2007	
Course on philosophy of science and/or ethics	2009	
Scientific Exposure		**15**
International conferences		
Asia Pacific Aquaculture 2007 (APA2007); Hanoi, Vietnam	2007	
International Workshop on Emerging Fish and Shellfish Diseases (IWEFSD); Bangkok, Thailand	2007	
Diseases in Asian Aquaculture VII (DAA7); Taipei, Taiwan	2008	
9th Asian Fisheries and Aquaculture Forum (AFAF 9); Shanghai, China	2011	
Diseases in Asian Aquaculture VIII (DAA8); Mangalore, India	2011	
Seminars and workshops		
WIAS Science Day; WU, The Netherlands	2007	
RESCOPAR Seminar Series; WUR, The Netherlands	2007	
RESCOPAR First Scientific Meeting; WUR, The Netherlands	2007	
Symposium on "Microbial Diversity in Mangrove Ecosystems; Philippines	2007	
2-day seminar on Shrimp diseases; Taipei, Taiwan	2008	
RESCOPAR Second Scientific Meeting: Samarinda, Indonesia	2009	
WOTRO Research Day; Utrecht, The Netherlands	2009	
Presentations		
Prevalence of white spot sydrome virus in shrimp *Penaeus monodon* in Negros Island, Philippines: 2000- 2006 (Poster; APA 2007)	2007	
Exposure to multiple stressors increases risk of WSSV outbreak in pond cultured *Penaeus monodon* (Oral; IWEFSD)	2007	
Modified greenwater culture system prevents WSSV outbreak in pond cultured *Penaeus monodon* (Oral; DAA7)	2008	
Comparison of the biophysical properties of the water and soil of environments with and without mangroves, receiving *P. monodon* farm effluents (Oral; AFAF9)	2011	
Shrimp farming practices for livelihood and ecosystem resilience: the Philippine Scenario (Oral; AFAF9)	2011	

Effect of different temperatures on the mortality and viral load of experimentally infected *Penaeus monodon* with WSSV (Oral; DAA8)	2011	
In-Depth Studies		**32**
Disciplinary and interdisciplinary courses		
Fish Immunology workshop	2007	
Training on Mangrove Ecology, Taxonomy and Community Structure	2008	
Training on WSSV challenge test	2009	
Advanced statistics courses		
WIAS Advance statistics course: experimental design	2007	
WIAS Course Statistics for the Life Sciences	2007	
MSc level courses		
General and Environmental Microbiology (MIB 10806)	2007	
Nutrion, Welfare and Reproduction in Aquaculture (AFI 32306)	2007	
Practical Aquatic Ecology and Water Quality (AEW 20706)	2007	
Aquaculture Production Systems (AFI 31806)	2007	
Professional Skills Support Courses		**4**
Techniques for Writing and Presenting a Scientific Paper	2007	
Information Literacy, including introduction to endnote	2007	
Competencies for Integrated Agricultural Research	2007	
Training on improving presentation skills	2009	
Managing with Kindness, A seminar on how to be tough and demanding in your people and still be liked by them	2011	
Stress Management Assertiveness Responsibility workshp	2011	
Research Skills Training		**6**
Preparing own PhD research proposal	2007	
Education and Training Total		**60**

* one ECTS credit equals a study load of approximately 28 hours