Luminous Bacteria Associated with Shrimp Mortality

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ABSTRACT : Luminous disease caused by luminous bacterial infection is currently a significant problem among the Asian countries where marine shrimp is the main aquaculture product. Luminous bacteria isolated from the shrimp farm environment include *Vibrio fischeri*, *V. harveyi*, *V. cholerae* biotype *albensis*, and *Photobacterium leiognathi*. Of these, *V. harveyi* is claimed to be the causative agent associated with shrimp mortality. In hatcheries, its pathogenicity is correlated with the developmental stage of the shrimp larvae. However, successful prophylactic measures have been established. In grow-out ponds, luminous disease frequently causes mortality with 2-3 month old stock of *Penaeus monodon* and a bacteroiphage of *V. harveyi* has been implicated in mortality of such shrimp exhibiting tea-brown gill syndrome (TBGS). Chemotherapeutics are more or less effective for controlling the disease but are not effective for curing it. Recent *in vitro* studies have demonstrated growth inhibition of this bacteria using biological control and the information gained paves the way for further investigations.

Key words : Luminous bacteria, Shrimp mortality, *Vibrio harveyi*

INTRODUCTION

Widespread interest in shrimp culture in Thailand has developed only in the past 15 years. During that period, intensive farming systems have been introduced and the areas of shrimp farms number of farms has rapidly expanded. In order to reduce the introduction of high organic matter into the coastal environment and thus avoid severe damage to the production system (Nygaard et al. 1992), either closed pond systems or recycle systems are necessary. These closed and recycle systems increase the problems associated with bacterial pathogens. It is recognized that damage to shrimp stocks is frequently associated with bacterial diseases that are mostly caused by luminous bacteria (Ruangpan 1987, Songserm et al. 1990, Ruangpan et al. 1997). Luminous bacterial diseases have also been reported to cause economic losses to the shrimp industry in the Philippines (Fernandez & Mayo 1994), Vietnam (Nguyen & Le Trong 1994), India (Raju 1994), and Indonesia (Sulasmi et al. 1994, Taslihan & Wijayati 1994). The problem seems to be common among the Asian countries where shrimp farming is the main aquaculture activity. This presentation describes the species composition (incidence and intensity) of luminous bacteria in shrimp farms and the coastal environment in Thailand, their pathogenicity and recent studies on the chemotherapy and biological control. It is hoped that the information will contribute towards further investigations leading to successful resolution of the luminous disease problem.

SPECIES COMPOSITION

Preliminary studies on luminous bacterial species in Thailand have been conducted using numerical taxonomic analysis (Ruangpan et al. 1995). Media used for isolation included thiosulfate citrate bile salts sucrose (TCBS, Difco) agar and modified sea water complete (MSWC) media. A total of 180 luminous and nonluminous vibrios were isolated from diseased *P. monodon* cultivated in several areas of Thailand. Each strain was tested for 98 characters. Luminescence was observed on MSWC medium 18-24 h after isolation using the technique of Furniss et al. (1978). Based on unweighed average linkage analysis at 80% similarity, 15 phenotypic clusters were obtained. Among those, luminous vibrios represented 4 clusters, according to their similarity in phenotypic characters, in guanine-cytosine (G + C) contents and to reference cultures. Members in the first cluster comprised 21 strains identified as *V. harveyi*. The second cluster containing 2 strains identified as *V. fischeri*. The last 2 clusters containing 7 strains which could not be identified as any known species. However, their characters and G+C contents were very close to either *V. harveyi* or *V. cholerae* non-01 reference cultures ATCC 14126 and NCTC 8021, respectively (Fig. 1).

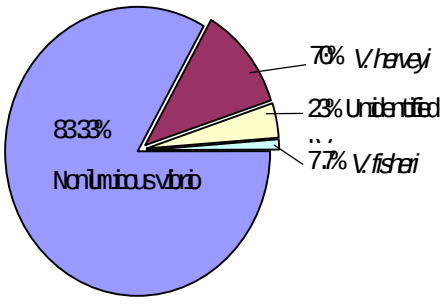

Figure 1. Species composition of luminous and nonluminous vibrios from cultured black tiger shrimp. The exploded portion of the pie (16.7% of total strains) are subdivided into groups by percentage of isolates.

Ruangpan L (1998) Luminous bacteria associated with shrimp mortality. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

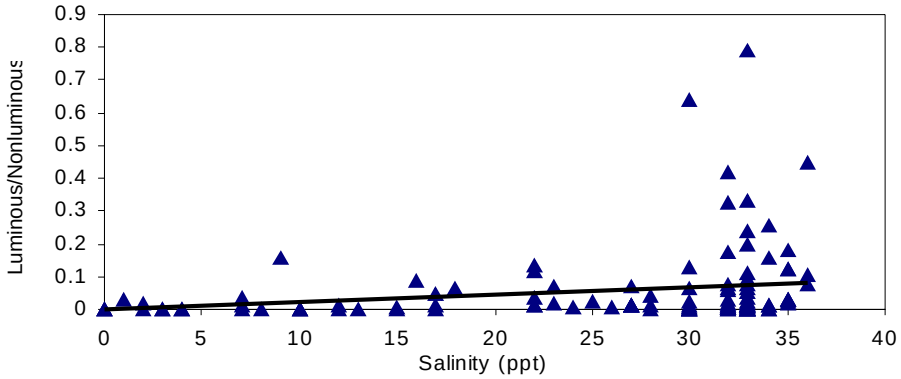

Figure 2. Variation in ratio of liminous/nonluminous bacteria at various salinities.

In 1996, identification of luminous bacteria isolated from the coastal water of Thailand was carried out by Sodthonkong (1996)(Table 1). Water samples were obtained from 128 stations in 21 provinces located along the Andaman and Siam coasts. Based on 49 phenotypic characters, a total of 210 representative isolates were identified to *V. harveyi*, *V. cholerae* biotype *albensis* and *Photo-bacterium leiognathi*.

According to the results most of the *V. harveyi* isolates exhibited green to yellow-green colonies on TCBS. Fifty seven percent of these isolates grew on media supplemented with 6% NaCl. All isolates had the ability to emit light when cultured in media containing 0.5-6% NaCl, although, 10% of them also grew and emitted light in media containing 8% NaCl.

V. cholerae biotype *albensis* exhibited yellow colonies on TCBS. All of its isolates grew on media without added NaCl or with NaCl up to 3%. Forty seven percent grew on media containing 6% NaCl while only 7% grew on media containing 8% NaCl. They gave high light when on media containing 0-6% NaCl but low light with 8% NaCl.

Table 1. The percentage incidence and intensity of luminous bacteria found in the coastal environment (shrimp ponds, pond effluent and hatcheries).

Sources	Incidence (%)	Intensity (cfu/ml)
Water : coastal environment	75	0.2 x 10 - 1.4 x 10
: shrimp ponds	84	9.1 x 10 - 1.2 x 10^2
: effluent from ponds	92	1.5 x 10 - 1.0 x 10^2
: hatcheries	67	0.2 x 10 - 2.9 x 10^3
: effluent from hatcheries	44	1.2 x 10 - 3.8 x 10^2
Sediment : shrimp pond	80	ND

Sources : Sodthongkong 1996, Ruangpan et al. 1997c

P. leiognathi gave green colonies on TCBS. All its isolates grew and emited light in the media containing 0.5-3% NaCl but only 50% grew in 6% NaCl and no growth was found in the media containing 8% NaCl. Ruangpan et al. (1995) indicated that *V. fischeri* exhibited deep green colonies on TCBS, 100% of the isolates grew and emited light on the media containing 1-8% NaCl.

INCIDENCE AND INTENSITY

Results from the investigation of luminous and nonluminous bacteria in Thai coastal water (Sodthongkong 1996) revealed that luminous bacteria are normal components of the microbial flora of estuaries and brackish water (Table 1). This finding is supported by farmer reports of luminous bacteria in the water and sediments of shrimp ponds, as well as the inlet and outlet water of mariculture operations which depend totally on water supplied from the coastal environment (Sae-Oui et al. 1987, Songserm et al. 1990, Ruangpan et al. 1995 and 1997c).

The incidence of isolation from environmental water samples was found to differ for species and sampling area. Isolates of *V. harveyi* were detected from 96 sampling stations with 75% incidence. The incidence of *V. cholerae* biotype *albensis* was 43% while that of *P. leiognathi* was 9%. Figure 1 demonstrates the correlation between salinity and luminous bacteria or vibrios. Interestingly, high incidences and intensities of both types were manifested at salinities between 30 and 33 ppt. However, the ratios of these bacteria are shown for estuarine water in the range of 1 ppt (Fig. 2).

Table 2. Incidence of *V. harveyi* infection at different developmental stages of *P. monodon* larvae.

Shrimp stock	% Incidence of infection			
	Zoea	Mysis	Postlarvae	Total
A	3.33	5.33	3.00	10.66
B	4.00	4.66	2.66	11.33
C	2.66	5.33	4.00	11.99
D	5.33	6.00	3.00	13.33
E	5.33	6.66	3.00	13.99
Total	20.65	27.98	15.66	61.30

Source : Songserm et al. 1990

Low intensities of luminous bacteria (0-4.2x10 cfu/ml) were found in the sampling stations located far from aquaculture ponds and communities. The highest intensity ($2.9x10^3$ cfu/ml) was recorded from aquaculture effluent water (Sodthongkong 1996). The incidence of *V. harveyi* isolation from shrimp pond sediment was reported to be 80%, although its intensity was not mentioned (Prapassorn 1995). Very little information is available regarding its incidence and intensity in shrimp. Most studies have focused on virulent isolates. Songserm et al. (1990) reported on the incidence from naupliar to postlarval stages of *P. monodon* (Table 2). Other investigators have reported *Vibrio* bacteria which included luminous and non-luminous isolates. Ruangpan et al. (1995) attempted to investigate the incidence of luminous bacterial species found in diseased shrimp. The

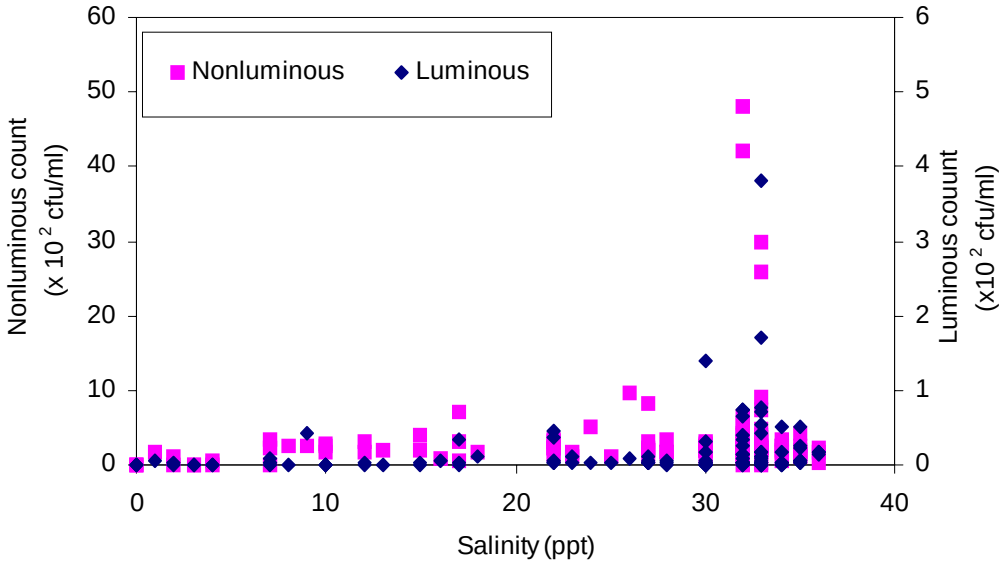

Figure 3. Distribution of luminous and nonluminous vibrio bacteria at various salinities sampled from 21 provinces located along the coast of Thailand.

results indicated that *V. harveyi* was predominant, accounting for 70% of the isolates while *V. fischeri* and other luminous species accounted for 6.7 and 23.3%, respectively (Fig. 3).

PATHOGENESIS AND HOST SUSCEPTIBILITY

Among the luminous bacterial species reported from shrimp ponds, hatcheries and moribund shrimp, only *V. harveyi* has been confirmed to cause mortality of shrimp. The disease is widely known as "luminous disease" or "Kung ruangsang" in Thai. Epizootics of this disease occur several times a year and are expanding throughout shrimp farming areas along the coast. A number of shrimp farmers have suffered severely from damage of long duration caused by this disease. Because of similar problems throughout Asia, scientists from many countries including the Philippines, Indonesia, Malaysia, India, Thailand and Taiwan are becoming increasingly interested in this disease.

The first report on shrimp luminous disease in Thailand was published in 1987 by Sae-oui and collaegues. The report indicated an outbreak of the disease in shrimp hatcheries in the central part of the country, which used to be the largest productive area for marine shrimp. The bacterial pathogenesis resulted in mortalities up to 100% for naupliar to zoeal stages of *P. merguiensis*. Living and dead shrimp larvae and even the sea water in disease outbreak areas were luminescent in dim light. Other gross features of the diseased shrimp were milky white bodies, weakness, swimming disorders and loss of appetite, eventually leading to death. Using luminous media (LM) (Baumann & Baumann 1981), luminescent bacterial colonies could be isolated from the diseased specimens as well as from hatchery water. Based on 47 phenotypic characters, the causative agents were identified to *V. harveyi*. Experimental trials for pathogenicity gave 100 and 95% mortality for naupliar and zoeal stages, respectively (Table 3), but no mortality for the larvae that reached the mysis stage.

Table 3. Pathogenicity of *V. harveyi* CHA 86021 to *P. merguiensis* larvae infected by bath exposure for 24 h.

	% Mortality		
	control	Treatment (cfu/ml)	
Shrimp stages		10^7	10^8
Nauplius	70	100	100
Zoea	60	ND	95

Source : Sae-Oui et al. 1987

Table 4. Mortality of different developmental stages of *P. monodon* exposed to V. harveyi at various concentrations.

	% Mortality				
	Control	Treatment/(cfu/ml)			
Shrimp stages		10^8	10^6	10^5	10^4
Nauplius	35	100	100	ND	100
Zoea	25	100	100	75	100
Mysis	25	75	70	ND	53
Postlarva	20	50	50	45	35

Source : Limsuwan 1989, Songserm et al., 1990

In *P. monodon*, luminous disease was also reported initially from larvae at naupliar to mysis stages (Ruangpan 1987). Since then, a rising incidence of luminous disease that can cause 80-100% mortality to the larval stocks has occurred in hatchery areas all over the country (unpublished results). In 1990, Songserm et al. reported that luminous disease in *P. monodon* larvae was also caused by *V. harveyi*. Results from experimental bath infections confirmed that zoea and mysis stages were more susceptible than postlarvae (PL). Prayinto & Latchford (1995) reported similar results with experimental infections of *P. monodon* larvae using suspensions of luminous bacteria related to *Photobacterium* and

Vibrio. Mortalities of zoea and postlarvae, after 48 h exposure, were 75 and 45%, respectively. Furthermore, Limsuwan (1989) has found that larvae beyond PL3 and 4 developmental stages resisted luminous bacterial infection (Table 4). Subsequently, various reports have indicated that luminous bacteria are also associated with shrimp mortality in grow-out ponds. In a survey of rearing water from numerous shrimp farms in southern Thailand from April to August 1996, Ruangpan et al. (1997a) found large numbers of the luminous bacteria. They also noted that massive mortality occurred in some farms within 3 to 4 days after the first appearance of moribund shrimp exhibiting tea-brown colored gills or tea-brown gill syndrome (TBGS). Isolations of bacteria from the moribund shrimp revealed that *V. harveyi* was dominant. Based on results from electron microscopy and injection trials with naturally and experimentally infected TBGS shrimp, it was suggested that a bacteriophage might be implicated in shrimp mortality by mediating toxigenesis of *V. harveyi*.

Disease induced by *V. harveyi* has been investigated by experimental infection of various crustacean species under stress conditions. It has the ability to induce disease and cause mortality in *P. monodon*, *P. indicus*, and *Artemia* nauplii (Prayinto & Latchford 1995) and *P. merguiensis* (Sae-Oui et al. 1987). No significant mortality occurred in *Macrobrachium* species (Kasornchan 1987, Prayinto & Latchford 1995) and banacles (Prayinto & Latchford 1995).

PREVENTION AND CONTROL

Prevention and control of luminous disease in shrimp are generally achieved by chemical treatment. Chemotherapeutants may be applied in the feed or in the water. Usually antibiotics are applied in the feed while disinfectants (and sometimes antibiotics as well) are applied in the water (Ruangpan 1987, Ruangpan et al. 1997c).

To prevent the luminous disease in hatchery operations, water should be filtered through a biofilter system prior to chemical treatment with 60% active chlorine at 10-30 g/ton or commercial grade formalin at 50-100 ml/ton. During treatment aeration is necessary. In addition, *in vitro* trials have been conducted on the inhibition of luminous bacterial growth using several disinfectants. Table 7 shows the effective doses for growth inhibition of *V. harveyi* treated with benzalkonium chloride, formalin and povidone iodine. Antibiotics treatment via the water is a common practice during rearing the early larval stages (nauplius to postlarvae 3-4). Application of antibiotics at 3-5 g/ton into the rearing water, especially during periods of metamorphosis has been found to greatly reduce luminous bacteria and stock mortality (Chayarat 1997). Oxytetracycline (OTC) was the main drug used for several years in all areas of the country. The effectiveness of OTC gradually declined after repeated application for long periods due to the development of bacterial resistance (Ruangpan et al. 1997c). Recently, various drugs have been tested against luminous and nonluminous vibrios, including resistant strains (Tables 5 and 6). Therefore, alternative drugs can be recommended based on sensitivity tests. After harvesting, treatment of the larval rearing facilities by flushing or vigorous spraying with freshwater has been suggested as a routine practice.

The major disinfectants used to reduce bacterial numbers in grow-out ponds are formalin and chlorine. When the *Vibrio* count in a pond rises to 10^3 to 10^4 cfu/ml, addition of 5 to 20 liters of disinfectant (per 1 ha pond) has been recommended. Benzalkonium chloride and povidone iodine have also been found to be effective chemicals to inhibit growth of *V. harveyi* (Table 7). At present, these chemicals are seldom used due to their high cost.

Antibiotics are usually applied in grow-out pond by addition to pelleted feed at approximately 3-5 g/kg administered to the shrimp for 7-10 days. Oxytetracycline and oxolinic acid were commonly used in the past. Recently they appear to be of limited value due to the development of bac-

Table 5. Minimal inhibitory concentration (MIC) ranges and inhibitory concentration (IC) of 6 drugs against 60 strains of luminous bacteria.

Drugs	MIC ranges (ug/ml)	IC s (ug/ml) 50% *1	90% *2
Oxytetracycline	6.25 - 100	15.00	69.20
Oxolinic acid	12.5 - 100	28.20	56.70
Norfloxacin	0.8 - 100	65.50	100
Chloramphenicol	3.13 - 50	8.75	45.20
Trimethoprim	3.13 - 25	9.37	15.00
Kanamycin	100 -> 100	>100	>100

*1 and *2 : Concentration inhibiting 50% and 90% of the strains, respectively. Source: Ruangpan et al. 1997c

Table 6. Drug resistant strains of luminous and nonluminous vibrios isolated from cultured shrimp *P. monodon* and the farm environment.

	% of drug resistant strains/year					
Drugs	1989	1990	1992	1994	1995	1996
Trimethoprim	0	23.4	25.5	ND	ND	20
Chloramphenicol	0	2.92	5.1	14.8	47.1	20
Oxolinic acid	90	0	0	11	20.5	45
Oxytetracycline	ND	32.2	36.4	45.9	71.4	0.4
Norfloxacin	ND	22.9	7.3	41.5	87.6	90
Kanamycin	0	ND	14.5	100	100	100
Sulfamonomethoxine	0	61.95	100	ND	ND	ND

Sources : Ruangpan and Kitao 1992, Sangrungruang et al. 1993, Prapassorn 1995, Sudthongkong 1996, Ruangpan et al. 1997

Table 7. Summary of *in vitro* trials for control of *V. harveyi*.

Agents **Biological control**	**Application**	**Dosage**	**Effectiveness**	**Reference**
Bacillus S11	Probiotic/feeding	ND	74 % RPS	Phianphak et al. 1997
Lactobacillus casei *L. acidophilus*	Probiotic/feeding	ND	Growth-inhibition	Jiravanichpaisal et al. 1997
Vibrio alginolyticus	Concomitant culture	1 : 10 cfu	Growth inhibition 0 to 100%	Ruangpan et al. (in press)
Chlorella sp.	Concomitant culture	1000 : 1 cell/efu	Growth inhibition	Direkbusarakom et al. 1997
Skeletonema costatum	Concomitant culture	500:1 cell/cfu	Growth inhibition 35 to 100 %	Panichsuke et al. 1997
Guava leaf extract	Feeding	MIC 1250 µg/ml	Growth inhibition	Direkbusarakom et al. 1997
Fresh water	Spray or bathe containers and equipment	Several times	Growth inhibition	Unpublished data
Chemical control				
Benzalkonium chloride	Water treatment	40% MIC 64 ppm MBC 64 ppm	Growth inhibition	Lin and Nash 1996
Formalin	Water treatment	40-50 ppm	Growth inhibition	Lin and Nash 1996
Providone iodine	Water treatment	1-3 ppm MBC 64 ppm MIC 1024ppm	Growth inhibition	Areechon 1990

ND = No data; MIC = Minimal inhibitory concentration; RPS = Relative percent of survival
MBC = Minimal bactericidal concentration

terial resistance (Ruangpan et al. 1997c). Several drugs claimed to be effective against luminous bacteria are supplied in commercial grade although many show very low bacterial growth inhibition (Ruangpan et al. 1997b).

Governmental guidelines to restrict the use of antibiotics in shrimp farms have been issued. To prevent problems regarding drug residues, the guidelines recommend that antibiotics should not be used beyond 3 months in shrimp grow-out ponds.

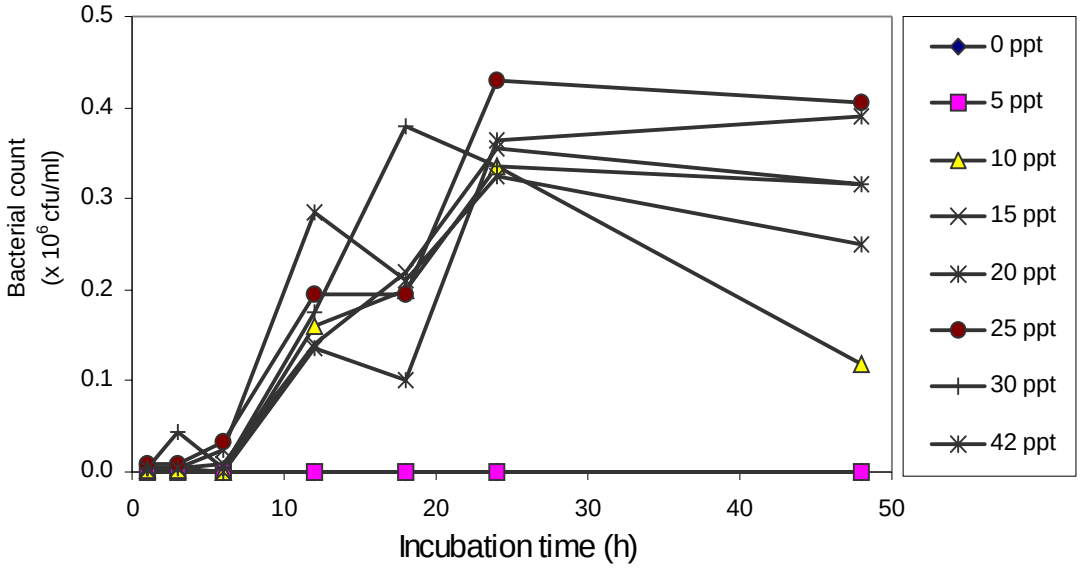

Figure 4. Number of luminous vibrios cultured at various salinity levels.

Experiments on preventing and controlling luminous disease using alternative methods such as probiotics or biological control have been attempted (Table 7). Phianpark et al. (1997) successfully used *Bacillus* sp. as probiotic feed in laboratory scale tests to achieve 74% relative survival after challenge with *V. harveyi*. *Lactobacillus casei* and *L. acidophilus* have also been claimed to inhibit the growth of *V. harveyi* when used as a probiotic feed (Jiravanichpaisal et al. 1997). Beneficial microorganisms found to be effective for growth inhibition of *V. harveyi* on the laboratory scale include *V. alginolyticus* (Ruangpan et al. 1998) *Chlorella* sp. (Direkbusarakom et al. 1997) and *Skeletonema costatum* (Panichsuke et al. 1997). Sodthongkong (unpublished data) studied the multiplication of *V. harveyi* at various salinity levels and found that the number of bacteria was reduced from $7.5x10^3$ to $8.9x10^2$, $4.0x10^2$, 1.3x10 and 0 cfu/ml in 1, 6, 12 and 18 h, respectively, after transfer from 25 ppt to 5 ppt. By contrast, bacteria transferred to 10 ppt decreased to $2.0x10^3$ and $32.x10^2$ cfu/ml after 1 and 6 h, respectively, but then increased to $1.6x10^5$ and $3.4x10^5$ after 12 and 24 h, respectively. At salinities between 15 and 42 ppt, numbers of *V. harveyi* increased gradually from 1-48 h of cultivation (Fig. 4). The results suggested that shrimp culture in seasons of low water salinity may experience less risk of luminous disease. Further studies are needed to ascertain the efficacy of using various microorganisms to control luminous bacteria in shrimp operations. They may prove a better alternative than antibiotics in overcoming problems.

CONCLUSIONS

Thailand is now one of the top of shrimp producing countries and highly developed culture systems have been established and expanded in many shrimp farming areas. However, more attention should be focused on how to develop shrimp culture as a sustainable industry. Disease problems still exist and are associated with serious crop losses. The survey data on luminous bacteria has shown that they are members of the natural estuarine and brackishwater environment. The studies also suggest that they induce disease more frequently in dry season than the rainy season and that their is related to salinity levels and effluent from farming operations. Only *V. harveyi* has been confirmed to be a pathogenic agent associated with shrimp mortality. In hatcheries, luminous disease can be controlled by chemical treatment with disinfectants and antibiotics, while in grow-out ponds, effective treatment and control methods are still uncertain. Some experiments indicate effective growth inhibition of *V. harveyi* by probiotics and biological treatments. However, effectiveness has not yet been proven in field trials. Although luminous pathogens exist naturally in the water, they certainly need carbon and introgen (C+N) for growth and multiplication. Thus, the first action to take in reducing their numbers is to be careful not to overfeed the shrimp and thus supply plentiful nutrients in the pond and the adjacent environment.

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