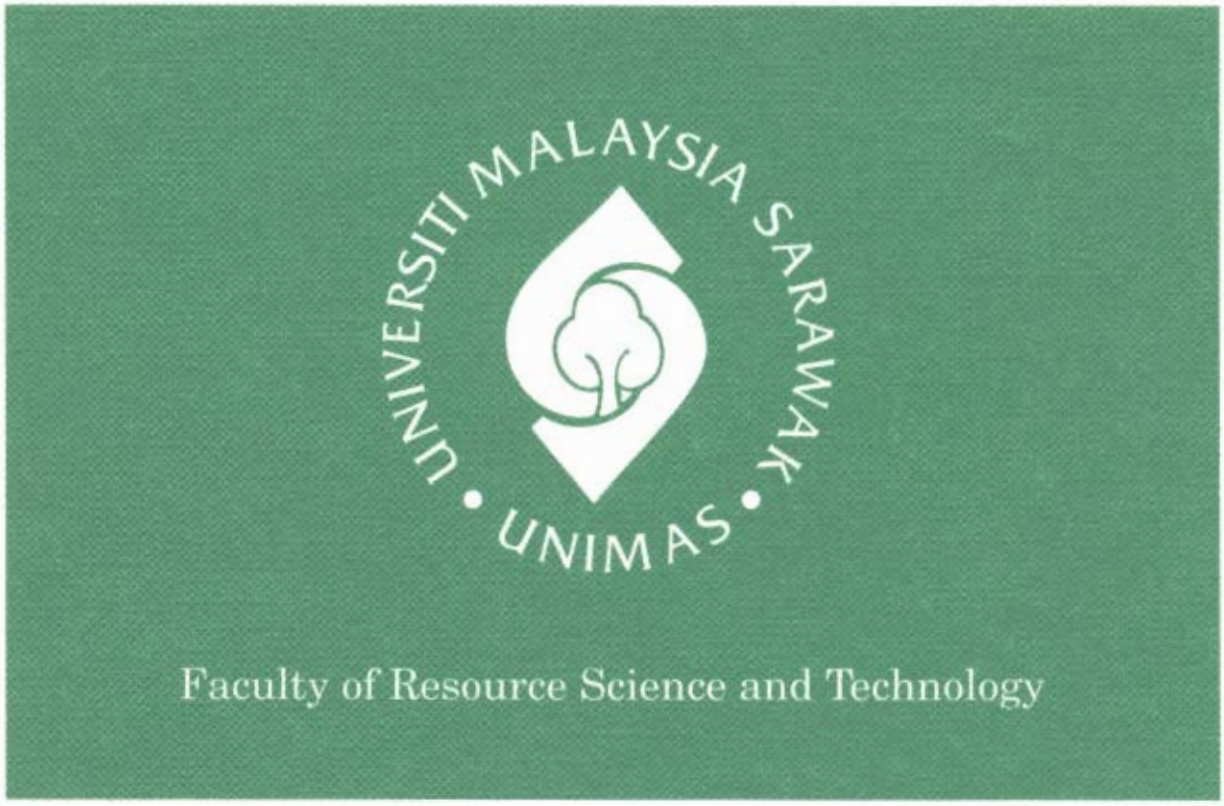

PREVALENCE OF *Vibrio spp.* IN SHRIMP CULTURE SYSTEM: ENUMERATION AND APPLICATION OF THE MULTIPLEX PCR FOR SPECIES-SPECIFIC SCREENING

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Bachelor of Science with Honours
(Resource Biotechnology)
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Prevalence of *Vibrio spp.* in shrimp culture system: Enumeration and application of the Multiplex PCR for species-specific screening.

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Prevalence of *Vibrio spp.* in shrimp culture system: Enumeration and application of the Multiplex PCR for species-specific screening.

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ABSTRACT

In this study, shrimp pond water and shrimp samples were collected monthly from August to November, 2004 from LienHsien shrimp farm, Sadong Jaya, Kuching. Analyses were performed to determine the total population of *Vibrio spp.* and screening for the presence of *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* by using the Multiplex PCR technique. 14 water samples were collected monthly during the four months period and four shrimp samples were collected on November from different pond sources during the cycle of the shrimp growth. TCBS agar and the three tube dilution MPN method was used for estimating the prevalence and dynamics of the *Vibrio spp.* population. Multiplex PCR was then used for the simultaneous detection of four major species of *Vibrios*; *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, in a single tube PCR reaction with four sets of primers designated, *Alg-nspC*, *ompW*, *tl* and *VulCulsl*. The findings of this study showed that the most prevalent of *Vibrio* population peaked in October and this phenomenon is related to the shrimp's life cycle. The Multiplex PCR result indicated that *V. parahaemolyticus* was the dominant species occurring about 100% throughout the sampling period, followed by *V. vulnificus* (48%), *V. alginolyticus* (32%) and *V. cholerae* (5%). This study showed that TCBS agar, MPN method and Multiplex PCR amplification when combined, are useful tools for the studies of *Vibrio spp.* population in shrimp farm environments. Of the three methods, Multiplex PCR was very specific, sensitive, reduce labor and less time consuming to detect the presence of *Vibrio spp.* from shrimp farm samples.

Keywords: *Vibrio spp.*; Multiplex PCR; enumeration; species-specific screening; shrimp culture system.

ABSTRAK

Daripada penyelidikan yang dijalankan ini, sampel udang bersama dengan airnya telah dikumpul secara bulanan daripada bulan Ogos hingga November, 2004 dari ladang udang LienHsien di Sadong Jaya, Kuching. Analisis telah dijalankan demi menentukan populasi bakteria, Vibrio spp. dan penggunaan teknik Multiplex PCR dalam penskrinan kehadiran V. alginolyticus, V. cholerae, V. parahaemolyticus dan *V. vulnificus daripada sampel yang diperolehi. Dalam masa satu kitaran penternakan udang, sebanyak empat belas sampel air telah dikumpulkan setiap bulan dan pada bulan November, tambahan empat sampel udang telah dikumpulkan daripada kolam-kolam berlainan. Agar TCBS dan kaedah MPN dengan pencairan tiga tiub telah digunakan demi menganggarkan jumlah populasi Vibrio spp. populasi. Teknik Multiplex PCR turut digunakan demi mengesan kewujudan empat jenis Vibrio spp.,iaitu V. alginolyticus, V. cholerae, V. parahaemolyticus and V. vulnificus, sekaligus dalam satu tiub PCR yang mengandungi empat rekaan set primer, seperti Alg-nspC, ompW, tl and VulCulsl. Hasil daripada penyelidikan ini, kebanyakan kewujudan Vibrio spp. adalah pada bulan Oktober dan dipengaruhi oleh kitar hidup udang tersebut. Keputusan daripada Multiplex PCR menunjukkan V. parahaemolyticus adalah lebih dominan antara empat Vibrio spp. sepanjang kajian dilakukan, iaitu sebanyak 100%, V.vulnificus pula sebanyak 48%, V.alginolyticus sebanyak 32%, manakala V. cholerae hanya sebanyak 5%. Daripada keputusan yang diperolehi menunjukkan hasil gabungan penggunaan agar TCBS, kaedah MPN dan teknik Multiplex PCR merupakan kaedah amat berguna dalam kajian populasi Vibrio spp. Antara tiga kaedah ini, Multiplex PCR adalah sesuai digunakan atas amplikasinya yang khusus, sensitif, dapat mengurangkan tenaga kerja serta dapat menjimatkan masa dalam pengesahan kehadiran Vibrio spp. daripada sampel ladang udang.*

Kata kunci: *Vibrio spp.; Multiplex PCR; pengiraan; penskrinan khas spesies; sistem pemeliharaan udang.*

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LIST OF ABBREVIATIONS

bp	base pairs
BPW	Buffered Peptone Water
cm	centimeter
cfu	colony forming unit
ddH_2O	double distilled water
dNTPs	deoxynucleotide triphosphates
DNA	Deoxyribonucleic Acid
EDTA	ethylenediamine tetra-acetic acid
g	gram
kbp	kilobase pairs
K-Ac	potassium acetate
LB	Luria Bertani
m	Meter
min	minute(s)
mol	mole
M	Molar or Molarity
mM	miliMolar
MPN	Most Probable Number
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
pmol/ml	picomole per mililiter
ppm	parts per million
ppt	parts per thousand
PCI	phenol-chloroform-isoamyl alcohol
PCR	Polymerase Chain Reaction
rps	revolution per second
rpm	revolution per minute
sdH_2O	sterile distilled water
SDS	Sodium Dodecyl Sulfate
TCBS	Thiosulfate-Citrae-Bile Salts-Sucrose
Taq	*Thermus aquaticus* DNA Polymerase
TBE	Tris-Borate EDTA electrophoresis buffer
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) methylamine
UV	Ultraviolet
V	volts
wt/vol	weight per volume
µm	micrometer
µl	microliter
%	percentage
°C	degree Celsius
>	more than
<	less than

CHAPTER 1

INTRODUCTION

1.0 Introduction

In the Western and Eastern countries, shrimp is a popular seafood and an appreciated luxury product. Nowadays, to capture wild shrimp is no longer enough to fulfill the demand for human needs. Therefore, the industry of shrimp culture has exploded by providing a great opportunity for many poor farmers in developing countries to increase their income (Blomqvist, 2002).

In Malaysia, the shrimp farming has recently received considerable attention by the introduction of new technologies in farm management, water purification, disease control, hatchery design/production, maturation and nutrition management (Mayra *et al.*, 1994). For example, the shrimp farms at the Sadong Jaya vicinities use intensive culture system. Black Tiger shrimp (*Penaeus monodon*) is the main species which are cultured in these farms. This intensive culture system is characterized by increased inputs such as fertilizers and feed, consequently leading to an increase in the nutrients and organic matter.

Such farming practices are vulnerable to attacks by diseases. According to Andreas (1997), several disease outbreaks of *Penaeus monodon* have led to serious production losses in countries, such as Taiwan (1987-1988), China (1993-1994), Indonesia (1994-1995), India (1994-1996), Ecuador (1993-1996), and Honduras (1994-1996). In many of these cases, *Vibrio spp.* is always implicated in many shrimp diseases. The affected shrimps are lethargic and show abnormal swimming behaviour. In severely affected shrimp, the gill covers appear flared up and eroded, and extensively melanised black

blisters can be seen on the carapace/abdomen (Kavlekar, 1998). Although these diseases are the results of infections by *V. harviyei*, this study focuses on four other *Vibrio spp.* that are similarly important, especially medically and agriculturally. These four kinds of *Vibrio* species that are medically and agriculturally important include *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio alginolyticus* and *Vibrio vulnificus*. In this study, total *Vibrio spp.* will be enumerated to determine the population dynamics of these bacteria in a cycle of the shrimp culture.

For the bacterial enumeration, the total populations of the *Vibrio spp.* were identified through direct plate count and MPN method. In the total plate count, the TCBS agar which is a selective agar for *Vibrio spp.* was used and its population was determine through total colonies (yellow and green) counts. Meanwhile, the MPN method was used to further estimate the total population bacterial in the samples.

In this study, the detection of these four species will utilize the polymerase chain reaction (PCR) technology. In the multiplex PCR reaction which was used in this investigation, the primer length was set between 19 and 24 and the number of primers was exactly twice the number of the target genes. The primers that were used in this research targeted the *V. cholerae* gene for outer membrane protein (*ompW*), *V. parahaemolyticus* thermolabile hemolysin (*tl*) gene, *V. alginolyticus* nspC gene for carboxynorspermidine decarboxylase (*Alg-nspC*) and *V. vulnificus* CMCP6 region (*Vulcls1*) on chromosome II.

1.1 Objective

The aim of this study is to determine the dynamics of the *Vibrio* population by monthly sampling, that includes:

1. To enumerate the sucrose fermenting and non-fermenting *Vibrio spp.* by using TCBS agar.
2. To enumerate total population of *Vibrio spp.* through MPN technique.
3. To determine the presence of four *Vibrio spp.* (*V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*) via multiplex PCR.
4. To determine the prevalence and compare the population patterns *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* in untreated, treated water and on shrimp samples.

CHAPTER 2

LITERATURE REVIEW

2.0 The genus *Vibrio*

Various kinds of Gram-negative, polarly flagellated rod-shaped bacteria were previously classified as the genus *Vibrio* in the family *Spirillaceae* (Sakazaki and Albert, 1981). They are capable of both fermentative and respiratory metabolism and also do not form endospores or microcysts. Members of this group are widespread in aquatic habitats of various salinities. They are very common in marine animals and estuarine environments and on the surfaces of marine plants and animals (Baumann *et al.*, 1984). They also occur naturally in the intestinal content of marine and appear to be influenced by the physicochemical features of the environment (Elena *et al.*, 1999).

The genus *Vibrio* includes more than 30 species, and 12 of these are human pathogens or have been isolated from human clinical specimens. 8 of the 12 human-associated *Vibrio* species have been isolated from extraintestinal clinical specimens (Lee *et al.*, 1998). Four kinds of *Vibrio* species that are widely studied include *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio alginolyticus* and *Vibrio vulnificus.*

Table 10 shows the biochemical characteristic and the differential characteristic of the *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. alginolyticus*, respectively.

2.0.1 *Vibrio cholerae*

Vibrio cholerae (Figure 1) is a gram-negative, facultative anaerobe bacterium that occupies a variety of aquatic niches. *V. cholerae* is rod-shaped (0.5-0.8 μm x 1.4-2.6 μm) and can be curved or straight. They are mesophiles in that their optimum growth temperatures are at 20°C to 48°C. They achieve motility through the use of a single polar flagellum. Usually, the consumption of contaminated food or water which contains pathogenic *V. cholerae* will cause diarrheal episodes. The bacteria will colonized the human intestinal tract and multiply within it (Afsar *et al.*, 2000).

V. cholerae was first described as the cause of cholera by Pacini in 1854. Pathogenic *V. cholerae* produces a heat-sensitive enterotoxin that causes the characteristic cholera symptoms, namely the "rice water stool" (Elliot *et al.*, 1998).

Since the 19th century, practically all the continents have reported outbreaks by the classical or Asiatic cholera pandemics that took millions of lives. In spite of the fact that Asiatic cholera appeared in 1817 and consequently induced six successive pandemics (1817– 1926), we now dispose of the data that only the fifth and sixth pandemics were caused by *Vibrio cholerae* of the classical biotype (Smirnova *et al.*, 2003).

Today there are more than 193 serogroups of *V. cholerae* that are classified on the basis of biochemical tests and DNA homology studies. These are further subdivided into serogroups based on the antigenicity of surface polysaccharides (Jutta *et al.*, 2002). Among the 193 currently recognized O serogroups of *V. cholerae*, only serogroups O1 and O139 are capable of causing the life-threatening epidemic disease cholera. These strains

have acquired a large pathogenicity island, TCP-AC or VPI (*Vibrio* pathogenicity island), which has recently been reported to be the genome of a filamentous phage, VPIΦ (Gabriela and Karen, 2000). The other serogroups of *V. cholerae*, collectively referred to as non-O1 and non-O139 serogroups, have not been associated with epidemics but can cause sporadic diarrhea and are ubiquitously distributed in the aquatic environment (Soumen *et al.*, 2000).

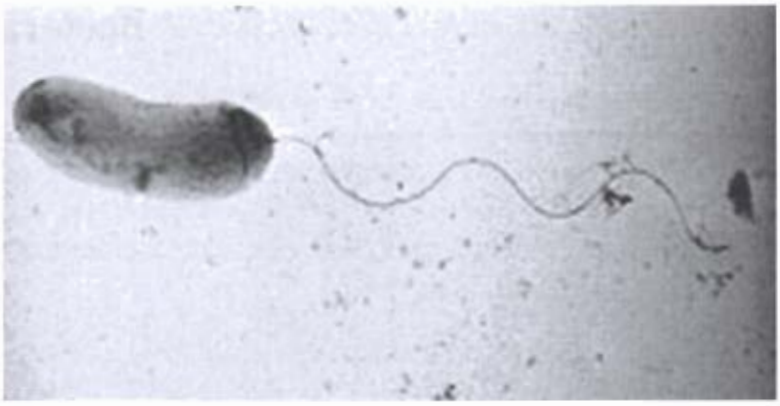

Figure 1: A typical *Vibrio cholerae* cell as seen under an electron microscope. (Prestero *et al.*, 2003)

2.0.2 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus (Figure 2) is a halophilic bacterium found naturally in estuarine waters and animals. It has been isolated from many species of fish, shellfish and crustaceans. Its densities in the environment and seafoods vary greatly by season, location and sample type (Depaola *et al.*, 2003). Some *V. parahaemolyticus* strains cause gastroenteritis in humans, usually through the consumption of raw or undercooked seafood and seafood recontaminated with the bacterium after cooking.

In fact, *V. parahaemolyticus* is an important food-poisoning pathogen in coastal countries, especially in Japan and Taiwan. In Taiwan, more than half of bacterial food-

poisoning outbreaks are associated with this pathogen. However, only a few reports regarding this pathogen in fish and shellfish have been published (Lee *et al.*, 2003). *V. parahaemolyticus* was first described as the cause of gastroenteritis in Japan and was first found in the United States by Baross and Liston (1968) in the estuarine waters of Puget Sound. Ever since then, *V. parahaemolyticus* has been implicated in numerous outbreaks of seafood-borne gastroenteritis in the United States. Between 1971 and 1978, crab, oyster, shrimp and lobster were implicated in 14 outbreaks, which may have resulted from the consumption of raw or insufficiently heated seafood or properly cooked seafood contaminated after cooking (Elliot *et al.*, 1998). Furthermore, 650 cases of four multistate *V. parahaemolyticus* illness outbreaks had been reported with the consumption of raw shellfish since 1997 (Gooch *et al.*, 2001).

Like all other *Vibrio* species, the growth of the *V. parahaemolyticus* is stimulated by sodium ions, where it grows best in the condition with the presence of 2-3% of sodium chloride (NaCl). *V. parahaemolyticus* may be serotyped on the basis of three major antigens, such as thermostable (TS) somatic O antigen, thermolabile (TL) capsular K and flagellar H, of which a serological grouping scheme has been developed based on the O and K antigens (Baumann *et al.*, 1984).

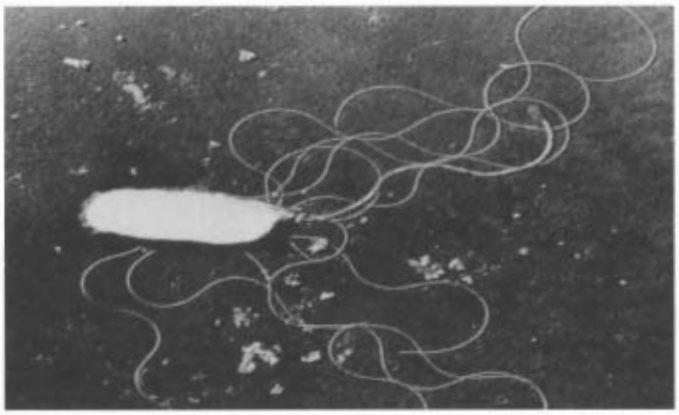

Figure 2: A typical *Vibrio parahaemolyticus* cell as seen under an electron microscope. (Kogure, 2003)

2.0.3 *Vibrio vulnificus*

As a member of the genus *Vibrio*, *Vibrio vulnificus* (Figure 3) also is a halophilic bacterium found in the estuarine environment. It is phenotypically similar to *Vibrio parahaemolyticus* (Elliot *et al.*, 1998). The species was first described as "lactose-positive" because most strains ferment lactose and are *o*-nitrophenyl-β-D-galactosidase (ONPG) positive. The organism was first reported as a cause of human illness in 1979 (Maitra, 2004) and is one of the most invasive and lethal pathogens known today. It exhibits seasonal occurrence and is sensitive to cold temperatures. *V. vulnificus* accounts for approximately 95% of all deaths associated with seafood consumption in the United States.

V. vulnificus affects immonce-compromised individuals with hepatic diseases, heavy alcohol drinking habit, diabetes mellitus, hemochromatosis, and immunosuppression from corticosteroid therapy, AIDS, or malignancy. The wound and/or soft tissue infections it causes and fatal primary septicemia progresses robustly and results in high mortality, usually more than 50% within a day or two (Lee *et al.*, 1998, Marion *et al.*, 2002).

According to Pfeffer *et al.* (2003), individuals who are predisposed to infection by increased serum iron levels, or who are immunocompromised, will show the symptom of primary blood poison (septicemia) after consumptions of *V. vulnificus* contaminated shellfish, especially oyster. However, the symptom of the wound infections have been shown on healthy individuals who come in contact with this bacterium through the infection of a previously inflicted wound or one incurred in an estuarine environment.

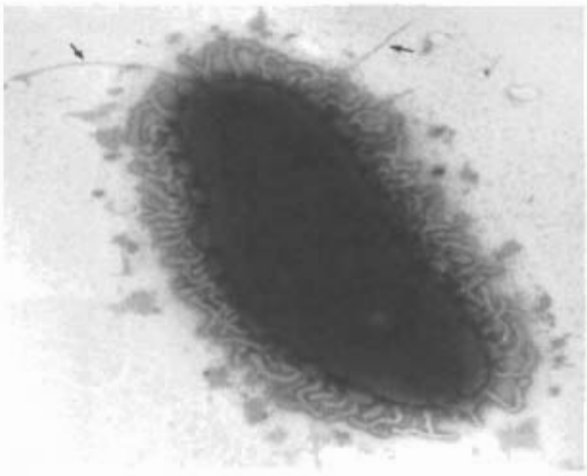

Figure 3: A typical *Vibrio vulnificus* cell as seen under an electron microscope. (Mark, 2002)

2.0.4 *Vibrio alginolyticus*

Vibrio alginolyticus (Figure 4) is another important member of the genus *Vibrio*. *V. alginolyticus* is part of the normal marine flora especially during warmer periods. According to Ripabelli *et al.* (2003), this species is the most common *Vibrio* isolated from mussels and seawater. This bacterium can reach concentrations in the shellfish sufficient enough to cause disease in humans. Recent studies reported several clinical infections caused by *Vibrio alginolyticus* (Stefania *et al.*, 1999), such as wound infections, otitis

media, and otitis externa. *V. alginolyticus* has also been reported as associated with gastrointestinal infection however its exact role as an enteric pathogen is unclear.

V. alginolyticus and *V. parahaemolyticus*, which are closely related, have similar flagellar systems. They have two types of flagella, namely the polar flagella (*Pof*) and the lateral flagella (*Laf*), in one cell (Ikuro *et al.*, 1995). The lateral flagella, which have proton-driven motors, are expressed when cells are transferred to high-viscosity environments. The polar flagella have sodium-driven motors and work better for swimming in low-viscosity environments. The rotation of polar flagella is very fast, about 1,700 rps in 300 mM NaCl at 35°C (Yukako *et al.*, 1999).

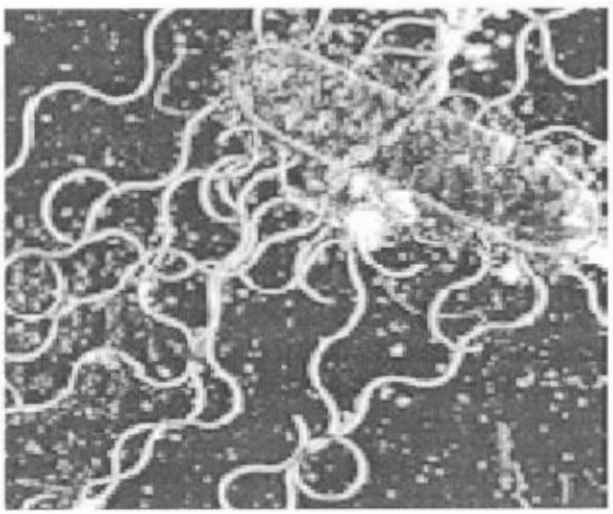

Figure 4: A typical *Vibrio alginolyticus* cell as seen under an electron microscope. (Ken, 2003)

2.1 Shrimp culture system

The shrimp industry is an important industry in many parts of Southeast Asia, including Malaysia, which supplies the shrimp for local diet. These shrimp industries provide both job opportunities and foreign currency for the countries involved. Of the various shrimp species which found naturally in Southeast Asia, the Black Tiger shrimp (*Penaeus monodon*) is the most popular for culture, as catches from natural sources declined through the 1970s (CSN & Associates, 2001). Therefore the shrimp culture industry has exploded. It is a great opportunity for quick wealth for many poor farmers in developing countries. People use the land they have and the standard they can afford, which is not much, and therefore the shrimp farms have not been very sustainable (Blomqvist, 2002). Figure 16 in the appendices shows the typical layout of a shrimp farm in Sarawak. This shrimp farm is situated at Sadong Jaya.

2.1.1 Black Tiger shrimp (*Penaeus monodon*)

Penaeus Monodon is one of the families of penaeidae. Commercially it is also known as the jumbo tiger prawn. This is the largest commercially available shrimp with sizes reaching up to 330 mm or more (13 inches). *Penaeus monodon* is the species dominating production everywhere in Asia (except China and Japan). It makes up 58% of the world shrimp production. In most producing countries, the shrimps contribute only insignificantly to the nutrition of the population. However, the shrimps constitute an important export article and their production provides employment, especially in rural areas. Main consumers of shrimp are Japan, the USA and Western Europe, but as the

economies of developing or newly industrialized countries (especially in Asia) strengthen, their consumptions of shrimps are steadily increasing (Andreas, 1997).

2.2 Enumeration

The quantitative analysis of water quality is important to monitor its sanitary quality. Water quality may be analyzed by the amount and types of bacteria present in water samples. As it is not practical to test for all types of organisms; hence, it is a common practice to test for specific indicator organisms such as the *Escherichia coli (E. coli)* and *Vibrio spp.* (Atlas, 1998). Bacterial enumeration is one of such test.

Bacterial enumeration is the measurement of the number of bacterial cells per milliliter, gram or cubic meter of a sample (the units depends on the nature of the sample). The determination of the number of microorganisms in environmental samples is a much more complicated procedure, since it is impossible to directly count bacterial cells with the naked eye. Various methods that are suitable for the determination of microbial numbers in a sample had been develop and divided into a few different categories, such as viable cell counts, total counts, direct methods and indirect methods. A viable count is a method which involves counting cells that can be cultured and metabolically active. Meanwhile, the total counts methods involve counting all cells including dead or inactive cells. Direct methods of enumeration is involving in counting the actual cells or colonies of bacterial and for indirect methods is involving in estimating the number of cells based on cell mass, scattering of light through a culture (spectroscopy) or a statistical method called the MPN (most probable number) technique (Furlong Michelle, 2005).

2.2.1 Plate Count (Spread Plate) Technique

The plate count method is a direct count method used to determine the number of viable, heterotrophic cells per volume (or per milliliter) of fluid. It involves diluting down a sample (if the sample contains > 3,000 cells/ml) and evenly spreading a small amount across an agar plate, such as thiosulfate-citrate-nile salts-sucrose (TCBS) agar for *Vibrio spp.* Each cell that ends up on the plate will form a colony overnight. The colonies can then be easily counted and a few calculations can help the *Vibrio spp.* in determining the number of cells/ml of sample (Furlong Michelle, 2005).

TCBS agar is highly selective medium for the isolation of *V. cholerae, V. parahaemolyticus, V. alginolyticus* and *V. vulnificus*. TCBS agar medium consists of plant and animal proteins, a mixture of bile salts, one percent sodium chloride, sodium thiosulfate, ferric citrate, sucrose, and yeast extract (Hardy, 2000). After incubation at 35°C for 18-24 hours, the yellow colonies of sucrose fermenting *V. cholerae* and *V. alginolyticus* can be seen on the agar surface. Meanwhile, *V. parahaemolyticus* and *V. vulnificus* will form green colonies on TCBS agar, as both *Vibrio spp.* are non-sucrose fermenting.

2.2.2 Most Probable Number (MPN) Technique

Unlike the total plate count method, the most probable number (MPN) method is an indirect count method. Only viable organisms are enumerated by the MPN determination. MPN is a statistical technique that relies on estimating the number of bacteria based on positive or negative growth in replicate tubes at each dilution (Furlong Michelle, 2005).