CHAPTER 1: INTRODUCTION

Vibrio forms the most important bacterial groups among marine flora. Vibrios are ubiquitious in aquatic setting like estuaries and are part of the normal flora of marine coastal waters (Colwell, 1984). Vibrios are important in nutrient cycling and biogeochemical cycles, and play a role in the degradation of organic matter. Vibrios are one of the major links that transfer dissolved organic carbon to higher trophic levels in the marine food web (Mouriño-Pérez et al., 2003). Some vibrios such as *V.cholerae*, *V.mimicus* and *V.vulnificus* are able to grow in estuarine and freshwater habitats where salinities are at their optimum level. For example, *V.cholerae* that is related to the spread of cholera within human populations is associated with freshwater systems (Thompson et al., 2005).

Vibrios are said to prefer to stay attached on animals such as fish, plants and sediments (Grimes et al., 2009). A symbiotic relationship has clearly been observed between the squid *Euprymna scolopes* and its luminous bacterial symbiont *V. fischeri* (Nyholm et al., 2004). From a study, the fish intestine of *Sarotherodon galilaeus* contained 5×10^3 cfu g^{-1} of *V.cholerae* in its intestine content (Senderovich et al., 2010).

There are more than 63 *Vibrio* spp., of which at least 12 are important pathogens of humans and marine organisms (Thompson et al., 2004). Some of the well known human pathogens are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. *V. cholerae* is associated with worldwide epidemics of cholera (Colwell, 1984) whereas *V. parahaemolyticus* is an important agent for seafood-associated gastroenteritis. In contrast, *V. vulnificus* is an emerging foodborne pathogen that causes most of the

mortality in food-associated bacterial infections (Todd, 1989). These human pathogens and other vibrios can also cause infections in marine organisms (Jones and Oliver, 2009). Vibrios are most critical as seafood-associated pathogens, and some are capable of killing coral tissues (Vandenberghe et al., 2003), eels (Hoi et al., 1998), fish and shrimps (Liu and Chen, 2004). Since vibrios comprised of both human and animal pathogens, it is important to determine if there are specific triggers for their abundance and diversity.

Characteristics

V.cholerae is the type species of *Vibrio* and it is a gram negative facultative anaerobe that appears as a rod that is either straight or a single, rigid curve. Vibrios are chemoorganotrophs and they have facultative fermentative metabolism. Vibrios are motile with the aid of a singular polar flagellum (Serratore et al., 1999). Most vibrios have two circular chromosomes. Chr 1 which is the larger chromosome contains genes of ribosomal proteins, polar flagella and DNA replication machinery whereas Chr 2 which is the smaller gene is responsible for pathogenicity, antimicrobial resistance, host avoidance mechanism and are important for survival in a variety of environmental conditions. High genomic diversity shown by vibrios is one strong reason they have the ability to strive in highly diverse and rapidly changing environments (Grimes et al., 2009). For example, *V.cholerae* has shown to have a large 2.96 Mb chromosome I and a small 1.07 Mb chromosome II (Rasmussen et al., 2007). The causative agent of cholera disease, *Vibrio cholerae* can cause epidemic- and pandemic-scale cholera. The serogroups O1 and O139 are mostly responsible for this (Jabeen et al., 2008). Cholera pandemic by serotype O1 is known to start from 1881 (Chun et al., 1999). Serotype

O139 named Bengal caused major cholera epidemics in India in 1992, and epidemics of cholera are recurrent in Bangladesh (Alam et al., 2006).

Different genes in different *Vibrio* strains are responsible for different diseases. For example, cholera toxin (CT) in *V. cholera* and thermostable direct hemolysin (TDH) in *V. parahaemolyticus* (Thompson et al., 2004). Infections by pathogenic vibrios also depend on factors such as the animal's physiological condition and the surrounding environment.

Plasmids are also found in *Vibrio* at a high frequency, and may be ecologically important to the survival of these bacteria in the environment (Zhang et al., 2006). Most *V. fischeri* strains isolated from the *Euprymna scolopes* carry a large (>40kb) plasmid, and one small (<12kb) plasmid (Dunn et al., 2005). Plasmids such as pJM1 of *V.anguillarum* have been shown to play a role in *Vibrio* pathogenicity (Hazen et al., 2007).

Pathogenicity

Certain known species of *Vibrio* are pathogenic to human (Lopez et al., 1995). There are as many as 24 species of *Vibrio* identified to infect animals and plants. Some of the known pathogens are *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus*, *V. fluvialis*, *V. furnissii* and *V. metschnikovii*. Although many vibrios are non-pathogenic towards human (Thompson et al., 2004), there are about 12 *Vibrio* species which have been known to cause infection in humans alone (Grimes et al., 2009). The ways which humans contract infections by *Vibrio* are through consumption of raw seafood such as shrimp and oyster, accidental drinking or wound exposure to

Vibrio contaminated water (Ji Dong et al., 2003). *V.vulnificus* has been reported to cause 20 to 40 cases of primary septicemia in U.S. each year with a mortality rate of 50% among individuals with liver disease and elevated serum iron levels (Kaysner and De Paola, 2004). These primary septicemia that forms, usually affects humans with hepatic diseases, alcohol habit and diabetes mellitus (Lee et al., 1998).

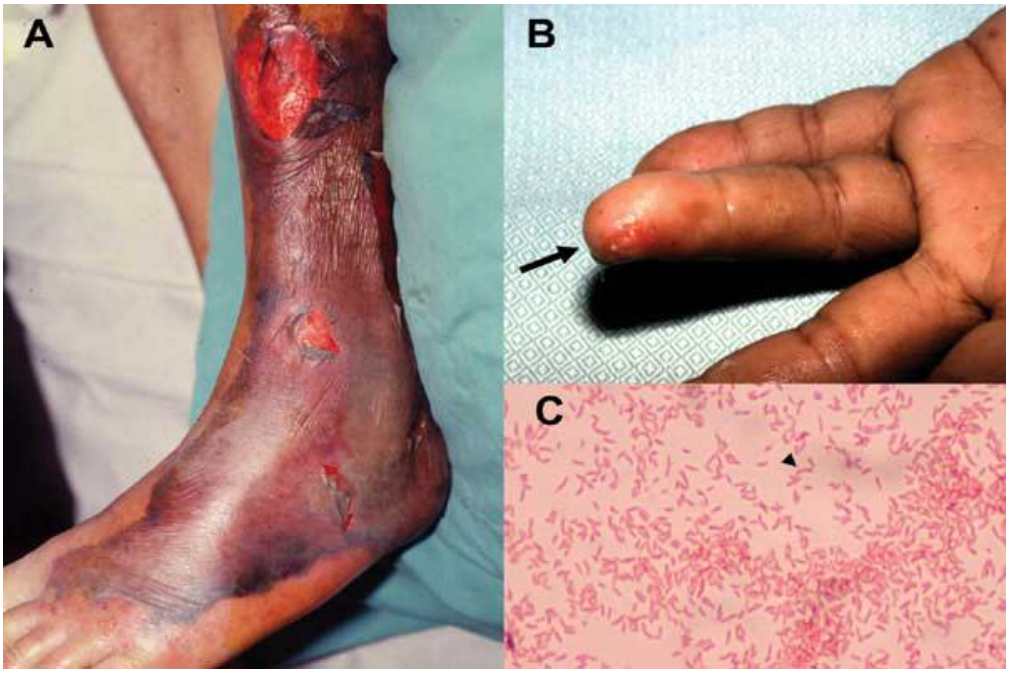

Figure 1: A) Skin lesions associated with *V. vulnificus* on the leg of a patient. B) *V. vulnificus* bacteremia developed one day after a fish bone injury on the finger. C) Gram-negative curved bacilli isolated from the blood sample of the patient (Photo source: Hsueh et al., 2004)

It was found that *Vibrio* infections do affect corals as certain *Vibrio* species are capable of killing coral tissues (Thompson et al., 2004). For example, *V. shiloi* has been identified to be responsible towards bacterial bleaching of corals (Banin et al., 2000).

Vibrio strains such as *V.mediterranei* and *V.coralliilyticus* are responsible for coral bleaching.

Figure 2: A pale yellow or white patch across the surface of the coral indicates yellow band disease. In this photograph of *Fungia* corals from the Indo Pacific, the banded areas show where bacteria from the genus *Vibrio* have killed zooxanthellae, the coral's source of energy. With depleted metabolic resources, the coral usually dies. (Source: James Cervino, Pace University)

Vibrio species such as *V. mestchnikovii* is also known to infect ducks and geese causing avian diseases (Ji Dong et al., 2003). Vibrios also play role as pathogens towards organisms reared in aquaculture. They cause internal symptoms like hemorrhaging and external symptoms like sluggish behavior (Thompson et al., 2004). Pathogenic *Vibrio* strains cause serious vibriosis in both wild and cultured fish and shellfish (Manjusha et al., 2005). In Malaysia, vibriosis outbreak was reported mainly in shrimp farms located in Kedah and Sabah (Musa et al., 2008).

Vibrio species cause diseases in marine fish and shellfish farming on a worldwide scale (Lopez et al., 1995). The seasonal and regional distributions of *V. parahaemolyticus* that cause infections towards man are closely related to the water temperature parameter (Watkins et al., 1976). Outbreaks of food poisoning caused by *V. parahaemolyticus* are common in Japan and Southeast Asia. It also occurs occasionally in other parts of the world (Marshall et al., 1999).

Presence of antimicrobial agents through leaching or continued usage in fish and shrimp farming for the purpose of disease treatment or prevention may have lead to the development of drug-resistant and multiple antibiotic resistance (MAR) in vibrios (Manjusha et al., 2005). Vibrios are known to be resistant to many common antibiotics. For example, *Vibrio cholera*e strains from poultry sources were found to be resistant to the antibiotics Penicillin, Ampicillin, Kanamycin, Erythromycin, Tetracycline and Rephampicin (Akond et al., 2008). Resistant factor allows them to survive in adverse environmental conditions.

Factors affecting distribution of Vibrio

The abundance of *Vibrio* varies according to temperature and other physio-chemical factors of the seawater. *V. cholerae* for example, survives in fresh water, and are proven to have the ability to pose the greatest threat of wound infections (Eiler et al., 2006).

(i) Temperature

Some research has suggested that temperature is the most significant factor determining population occurrence. Vibrios are more common in warm waters, especially when temperature exceed 17°C (Eiler et al., 2006). When seawater

temperature rises above 17°C (Eiler et al., 2006), *Vibrio* grows faster in both seawater and intestines of marine animals (Yoon et al., 2003). Many reports have stated that the ability of vibrios to be pathogenic to humans and marine animals are correlated to the rising seawater temperature as a result from global warming (Thompson et al., 2004).

(ii) Salinity

Vibrio spp. are able to tolerate a wide range of salinity from 2 to 30 ppt. NaCl requirement affects the distribution of *Vibrio* species in various aquatic environments (Eiler et al., 2006). Salinity also can cause a reversible inhibition effect on the adhesion or virulence ability of *V. cholerae* O139. For vibrios, the effects resulting from changes in salinity are stronger relative to pH (Sung et al., 2003).

(iii) Host organism

Studies have shown that marine host and vector organisms promote the survival of *Vibrio* and also influence their persistence and distribution in the environment (Eiler et al., 2006). *Vibrio* spp. is closely related to zooplankton. They are able to survive starvation and environmental stresses by absorbing nutrients from the biofilm on exoskeletons of marine organisms. In some cases, abundance of host organisms has been shown to have an effect on the distribution of some coastal *Vibrio* populations (Thompson et al., 2004). Although benthic marine environment may function as a reservoir of *Vibrio* spp., their ecological features are different from the ones discovered in the pelagic environment (Vezzulli et al., 2009).

(iv) *Vibrio* mortality

Sometimes, low numbers of vibrios observed in certain places are related to the selective **grazing** activity by heterotrophic nanoflagellates (Thompson et al., 2004). In a separate study, a novel marine siphovirus, the *Vibrio* phage SIO-2 has lytic properties towards a *Vibrio* sp. (Baudoux et al., 2012). In marine systems it is assumed that 20 to 40% of bacteria are killed by viruses on a daily basis (Suttle, 2005).

Isolation and Identification of *Vibrio* spp.

Culture based studies have shown that vibrios comprise about 10% of the easily culturable marine bacteria. The selective medium thiosulfate-citrate-bile salt-sucrose agar (TCBS) is used to grow vibrios (Thompson et al., 2004). There are two types of TCBS strains, one is classified as the salt strain which requires NaCl such as *V.parahaemolyticus* and the other as a non-salt strain such as *V.cholerae* (Uchiyama, 2000). Among the different methods that are available, membrane filtration is accepted as a precise and reliable method to enumerate *Vibrio* from water samples but values obtained by membrane filter are usually higher (30-50%) compared to those obtained by the plate count method (Lopez et al., 1995).

Vibrios can be differentiated according to biochemical tests. Characterisation of phenotype and identification of the genus *Vibrio* presented several difficulties due to its high biochemical diversity, and description of several new species has led to a constantly changing taxonomy of the Vibrionaceae (Vandenberghe et al., 2003). Research on the biochemical identification of *Vibrio* species has improved, and the set of biochemical tests has been updated after discoveries of new *Vibrio* species (Alsina and Blanch, 1994). Identification was based on percentages of positive and negative

scored in the different test done (Alsina and Blanch, 1994). Most of the members are oxidase positive, have catalase and can ferment glucose. *V. metschnikovii* differs from other *Vibrio* species in lacking cytochrome oxidase. Since the growth of *Vibrio* sp. is very much affected by the concentration of NaCl available, tolerance towards different salinity has been used as part of the identification step (Vanderzant et al., 1972).

Although research on *Vibrio* spp. in the natural environment is mostly with culture-dependent techniques, it is well known that many vibrios enter a state of unculturability when exposed to poor growth conditions such as nutrient limitation or starvation, pH, temperature and salinity variations. These vibrios appear to be inactive due to environmental stresses such as exposure to low temperature or adverse growth conditions. The bacteria cells at this point are undetected because they cannot grow on the conventional media even though their viability and pathogenicity are maintained (Eiler et al., 2006).

Serological methods based on antibodies that target flagellar H and LPS antigens have also been developed for the rapid identification of some pathogenic *Vibrio* sp. *V. cholerae* is subdivided into serogroups according to the somatic O antigen. Among the groups, O1 and O139 are reported to cause disease. Each of the O1 biotype is subdivided into major serotypes Inaba (VCI) and Ogawa (VCO).

Molecular techniques have also been applied for the identification and characterization of vibrios. Examples of techniques used are Fluorescent In-situ hybridisation (FISH), Pulsed Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP) and many more. However most molecular techniques have their own limitations in identification, cost and facilities needed for analysis.

These limitations affect certain field studies which involve large number of samples (Choopun et al., 2002).

Objectives

In Malaysia, research on *Vibrio* sp. is more focused on clinical samples especially pathogen strains such as *V. cholerae*. The quantification of environmental *Vibrio* abundance and diversity has not been done. *Vibrio* dynamics in Malaysian coastal waters is important because Malaysia has many coastal areas where recreational activities and fishery is being carried out. In view of the potential pathogenicity of vibrios, it is important to understand their distribution and diversity in relation to the environment. Therefore the objective of this study is to investigate the temporal variation of culturable vibrio abundance and diversity in our coastal waters.

CHAPTER 2: MATERIALS & METHOD

Physical parameters

Surface seawater samples were collected from both coastal areas of Port Klang, Selangor (03°00.1'N, 101° 23.4'E) and Port Dickson, Negeri Sembilan (02°29.5'N, 101° 50.3'E) (Figure 3). Port Klang is located in an estuary that is heavily developed whereas Port Dickson is locally well-known for its recreational beaches. Samples were collected monthly during high tide for about two years (from June 2008 till July 2010). In situ measurements such as temperature (± 0.1°C), and salinity level (± 0.1 ppt) were measured using a conductivity meter (YSI-30, USA) whereas pH was measured with a portable pH meter (Martini Mi-106, Romania). Triplicate samples for dissolved oxygen (DO) were also collected with 50 ml DO bottles, and DO was fixed in situ with manganous chloride and alkaline iodide reagents according to the Winkler's method (Grasshoff et al., 1999). One sample was also preserved with glutaraldehyde (1% final concentration) for bacterial total count whereas samples for bacteriological analyses were collected using sterile bottles. Samples were brought back to the lab in a cool box, and processed within three hours of sampling.

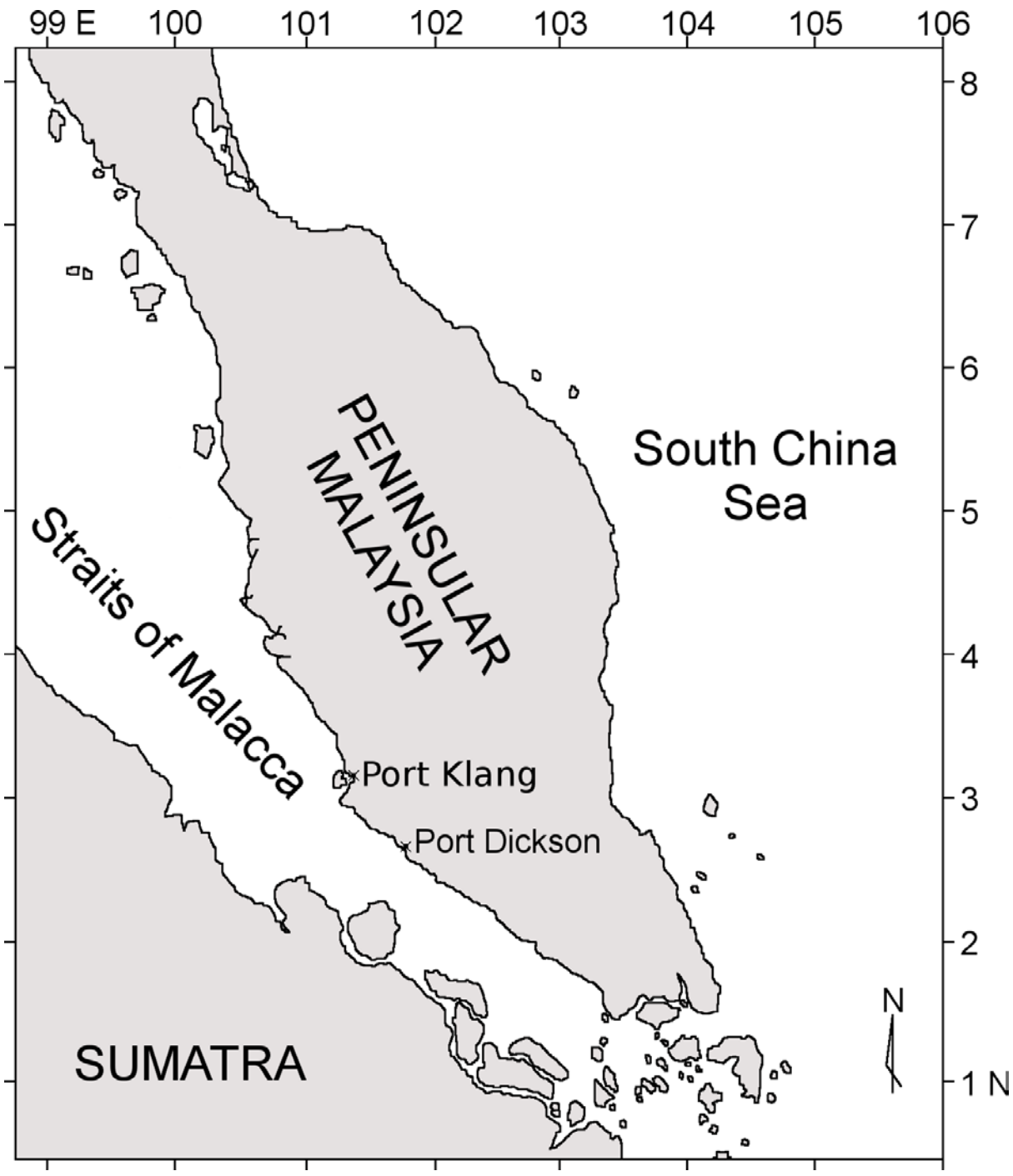

Fig. 3: Map showing the sampling stations.

In the laboratory, seawater samples were filtered through pre-combusted (500˚C for 3 hours) Whatman GF/F filters. The filters were used for total suspended solids (TSS) and chlorophyll *a* (Chl *a*) determination whereas the filtrate was kept frozen until dissolved inorganic nutrient analysis (nitrate+nitrite [NO_3+NO_2], ammonium [NH_4] and phosphate [PO_4]) (Parsons et al., 1984). For TSS, it was determined by the weight increase after drying the filter (50˚C for 72 hours) whereas Chl *a* was extracted overnight with 90% ice-cold acetone, and its absorbance was measured with a spectrophotometer (Beckman DU7500i, USA) (Parsons et al., 1984).

For bacterial abundance, samples were stained using 4'6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.1 µg L^{-1}) in the dark for 7 minutes (Kepner and Pratt, 1994) and filtered through a 0.2 µm pore size black polycarbonate membrane filter (Millipore, USA). At least 7 fields or 300 cells were counted using an epifluorescence microscope with a U-MWU filter cassette (excitation, 330 to 385 nm; dichroic mirror, 400 nm; barrier, 420 nm) (Olympus BX 60, Japan). Correction for autofluorescing picoplankton was carried out by observing the same field under U-MWG filter cassette (excitor 510-550 nm, dichroic mirror 510 nm, barrier 590 nm).

Vibrio Isolation

Vibrio spp. were isolated and enumerated via both spread plating and membrane filtration method. *Vibrio* spp. were isolated on Thiosulfate Citrate Bile salt Sucrose (TCBS) (Difco, USA) and TCBS+NaCl (3% NaCl final concentration) at 37°C for 24 hours. TCBS is the bacteriological medium of choice, and is a proven selective medium for the isolation of vibrios (Pfeffer and Oliver 2003; Thompson et al., 2004). All cfu on TCBS and TCBS+NaCl were counted as presumptive vibrios. Every cfu was purified via dilution streaking on new TCBS and TCBS+NaCl plates before identification via biochemical tests (Alsina and Blanch, 1994; Garrity et al., 2005) as shown in Appendix I. The average *CV* for isolation on TCBS was 12% for spread plating and 9% for the membrane filtration method.

The following biochemical tests for the identification of *Vibrio* isolates were performed according to Bergey and Holt (1994). Gram staining was carried out on each isolate before oxidase test. After which, the following tests were carried out: Kligler iron agar (KIA), gelatinase test, Voges-Proskauer test, salt tolerance test, lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase assays, O/129 Vibriostat sensitivity test, lactose and mannitol utilization tests and urease test. Biochemical tests for isolates that grew on TCBS+ NaCl plates were supplemented with 3% NaCl (final concentration).

Faecal coliform Isolation

Escherichia coli was enumerated in this study as a faecal pollution indicator (Cho et al., 2010). Both the spread plating and membrane filtration techniques were used, and the volume of inoculation was 0.1 ml and 1.0 ml, respectively. Sterile 0.45µm pore size nitrocellulose filters (Millipore, USA) were used in the membrane filtration technique, and *E. coli* was grown on MacConkey agar (Difco, USA) for 24 hours at 37ºC. The membrane filtration technique had a detection limit of > 1 colony forming unit per ml (cfu ml^{-1}), and was useful when *E. coli* counts were below the detection limit of spread plating (> 10 cfu ml^{-1}). All lactose fermenting microorganisms that appeared as pink colonies with or without a zone of precipitated bile were selected and purified for further tests. Colonies that were Gram negative, oxidase negative and with the IMViC (Indole, Methyl Red, Voges-Proskauer and Citrate utilization tests) profile of ++−− were identified as *E. coli* (Garrity et al., 2005). We replicated the isolation procedure, and the average coefficient of variation (*CV*) for spread plating and membrane filtration method were 21% and 9%, respectively. Details for each biochemical tests for both *Vibrio* spp. and *E. coli* identification are listed below:

Gram Staining

Using sterile technique, smear of each organism were prepared on clean slides. This was done by placing a drop of water on the slide. Then each organism was transferred separately to the drop of water with a sterile cooled loop. The organism was mixed and spread by means of a circular motion of the inoculating loop. Smear was allowed to air-dry and then was heat fixed. Gently, smears were flooded with methyl violet and let to stand for 1 minute before $NaHCO_3$ solution was added. It was then gently washed with tap water. Gently, smears were flooded with the Gram's iodine mordant and let to stand for 1 minute. It was then gently washed with tap water. Slides were decolourized with 95% ethyl alcohol. Reagent was added drop by drop until alcohol runs almost clear showing only a blue tinge. It was gently washed with tap water. Slides were then counterstain with safranin for 45 seconds. It was then gently washed with tap water. Slides were blot dry with bibulous paper and examined under oil immersion.

Methyl violet solution:

Reagent formula	Volume
Methyl violet	1 g
Distilled water	100 ml

$NaHCO_3$ solution

Reagent formula	Volume
Sodium Carbonate Hydrated	0.5 g
Distilled water	10 ml

Iodine Burke solution

Reagent formula	Volume
Potassium iodide	1.0 g
Iodine	0.5 g
Distilled water	100 ml

Acetone

Reagent formula	Volume
95% ethyl ethanol	35 ml
Acetone	15 ml

Saffranin solution

Reagent formula	Volume
Safrranin	1.0 g
Distilled water	50.0 ml

Kligler Iron Agar Test

Principle: Used for differentiating pure cultures of gram negative bacilli based on fermentation of dextrose and lactose and production of H_2S.

Formula per Liter:

Bacto Beef Extract	3 g
Bacto Yeast Extract	3 g
Bacto Peptone	15 g
Bacto Proteose Peptone	5 g
Bacto Lactose	10 g
Bacto Dextrose	1 g
Ferrous Sulfate	0.2 g
Sodium Chloride	5 g
Sodium Thiosulfate	0.3 g
Bacto Agar	12 g
Bacto Phenol Red	0.024 g

Final pH 7.4 $\pm$ 0.2 at 25°C

Method:

Exactly 55 grams of powder was suspended in 1 liter of distilled water. Mixture was heated to boil and dissolve completely. Mixture was then dispensed into tubes which were autoclaved at 121° C for 15 minutes. Tubes were allowed to cool by placing in slanting position. Tubes were inoculated and incubated at 35°C for 18-48h. Tubes were observed for its colour change, production of gas or production of H_2S

Oxidase Test

Principle: This test is to detect presence of cytochrome oxidase in bacteria.

Formula per Liter:

N,N,N',N'- Tetramethyl-p-phenylenediamine.2HCL	1 g
Distilled water	100 ml

Method:

Reagent was prepared according to the formula above and stored in a dark glass bottle. Small amount of culture was transferred to a sterile filter paper using a sterile wooden stick. About 1 drop of prepared oxidase reagent was added onto the culture.

Positive: Development of purple colour.

Negative: No colour change.

Gelatin Test

Principle: This test is used for detecting gelatin liquefaction by proteolytic microorganisms

Formula per liter:

Bacto Beef Extract	3 g
Bacto Peptone	5 g
Bacto Gelatin	120 g

Final pH 6.8 + 0.2 at 25°C

Method:

Exactly 128g of powder was suspended in 1 liter of distilled water. Mixture was warmed at 50-55° C to dissolve completely. About 5 ml was dispensed in test tubes and autoclaved at 121°C for 15 minutes. Using a sterile inoculation needle, heavy inoculums was stabbed into the tube and incubated at 35 ± 2°C for 48h. On the next day, tubes were gently transferred to a refrigerator and allowed to cool. Tubes were gently inverted to test for solidity by comparing to an uninoculated tube.

Positive: Tubes remain liquid.

Negative: Medium becomes solid after refrigeration

Salt Tolerance Test

Principle: Alkaline Peptone Water (APW) acts as an enrichment medium for isolation of *Vibrio* spp from water samples.

Formula per Liter:

Peptone	10 g
Sodium Chloride	20 g

Final pH 8.6 + 0.2 at 25°C

Method:

About 30 g of powder was added to 1 liter of distilled water. Sodium chloride concentration was increased according to the salt tolerance tested (3%, 6%, 8%, and 10%). For 0% salt tolerance, peptone broth was used instead of APW. Suspension was mixed well and distributed into test tubes and sterilized by autoclaving at 121° C for 15 minutes. Tubes were then incubated overnight and turbidity was checked the following day.

Positive: Turbid growth.

Negative: No change in the turbidity

Decarboxylase Test

Principle: This test is used for differentiating bacteria based on their ability to decarboxylate amino acids.

Formula per Liter:

Bacto Peptone	5 g
Bacto Beef Extract	5 g
Bacto Dextrose	0.5 g
Bacto BromCresol Purple	0.01 g
Cresol Red	0.005 g
Pyridoxal	0.005 g

Final pH 6.0 ± 0.2 at 25°C

Method:

Exactly 10.5 g powder was suspended in 1 liter of distilled water and heat to dissolve completely. Exactly 10 g of L-amino acid was added to the mixture. About 5ml was dispensed into screw capped test tubes and autoclaved at 121° C for 15 min. The prepared tubes were inoculated with 24h culture using sterile loop. Control tube was also inoculated. Aseptically all tubes were overlaid with 4-5mm sterile mineral oil. Tubes were incubated at 35 ± 2° C for up to 4 days and were observed daily for color change.

Positive: Purple colour

Negative: Yellow colour

O129 Disc Susceptibility Test

Principle: For the differentiation of vibrios from other Gram negative rods.

Each disc impregnated with:

a) 10ug of 2, 4- Diamino-6, 7-di-iso-propylpteridine phosphate.

b) 150ug of 2, 4- Diamino-6, 7-di-iso-propylpteridine phosphate.

Method:

Mueller Hinton Plates were inoculated with test organisms using sterile cotton swab according to MacFarland standard. Disc with both concentration were placed on the plates. The plates were then incubated at 35 $\pm$ 2° C for 24h.

Sensitive: Zone of inhibition present.

Resistant: No zone of inhibition.

Fermentation Test

Principle: For differentiating bacteria based on fermentation reaction

Formula per Liter:

Bacto Beef Extract	1 g
Bacto Protease Peptone No.3	10 g
Sodium Chloride	5 g
Bacto Phenol Red	0.018 g

Final pH 7.4 + 0.2 at 25°C

Method:

Exactly 16g of powder was dissolved in 1 liter of distilled water. About 10 g of desired carbohydrate was added to the basal medium. Mixture was then distributed into tubes. To detect gas production, inverted Durham tubes were placed in the tubes of the medium. Tubes were then autoclaved at 121° C for 15 minutes. Tubes were inoculated with pure culture and then incubated at 35 ± 2° C for 24h with caps loosened. Tubes were examined for acid production and gas production.

Positive: Medium changes to yellow

Negative: Medium remain as orange-red colour

Presence of bubble indicate positive for gas production

Urease Test

Principle: This test is used to differentiate microorganisms based on urease activity.

Formula per Liter:

Bacto Yeast Extract	0.1 g
Potassium Phosphate, Monobasic	9.1 g
Potassium Phosphate, Dibasic	9.5 g
Bacto Urea	20 g
Bacto Phenol Red	0.01 g

Final pH 6.8 + 0.1 at 25°C

Method:

Exactly 38.7 gram of powder was dissolved in 1 liter of distilled water. The mixture was allowed to dissolve completely. The solution was filter sterilized as it shouldn't be boiled or autoclaved. Aseptically 3 ml of solution was distributed into sterile bijoux bottles. The bottles were inoculated with heavy inoculums from a 24h culture. Tubes were shaken gently. Bottles were then incubated aerobically at 35 ± 2° C. Reaction was recorded after 48h of incubation.

Positive: Intense red/pink colour formed.

Negative: No colour change observed.

Indole Production Test

Peptone preparation:

Reagent Formula	Volume
Amino nitrogen	2.6 g
Sodium chloride	1.6 g

Final pH 6.3 + 0.2

About 1 gm of peptone broth powder was dissolved into 100 ml distilled water. The mixture was boiled until complete dissolution and placed into test tubes before autoclaving for sterilization.

Indole Kovacs reagent preparation:

Reagent Formula	Volume
p-Dimethylaminobenzaldehyde	1 g
Hydrochloric Acid, 37%	5 ml
Amyl Alcohol	15 ml

About 15 ml of amyl alcohol was added first before addition of p-Dimethylaminobenzaldehyde. After the dissolution of these compounds only then HCl was added.

Method:

About 5 ml of peptone broth was poured into test tubes. Peptone broth was lightly inoculated with the test organism. Culture was incubated at 24-48 hours at 35 °C. Exactly 10 drops of Kovac's reagent was added to the tube. The tube was then shaken gently.

Positive: Formation of pink band on surface of tube

Negative: No change

Voges-Proskauer Test

MR-VP broth preparation:

MRVP medium

Reagent Formula	Volume
Peptone	7.0 g
Glucose	5.0 g
Phosphate buffer	5.0 g

Final pH 6.9 + 0.2

Method:

About 3.4 gm of MR-VP broth powder was dissolved into 200 ml distilled water. The mixture was boiled until complete dissolution and placed into test tubes before autoclaving for sterilization.

Reagent preparation:

Voges-Proskauer Reagent A

Reagent formula	Volume
Absolute ethanol	50.0 ml
α- naphtol	2.5 g
Distilled water	50.0 ml

Voges-Proskauer Reagent B

Reagent formula	Volume
Potassium hydroxide	40.0 g
Distilled water	100.0 ml

Method:

About 5 ml of MR-VP broth was placed into each sterile test tube. The tubes were inoculated with organism of interest and incubated for 24 hours at 35° C. On the day of testing, 2.5 ml inoculated MR-VP broth was placed into a separate test tube for MR test. In the remaining 2.5 ml MR-VP broth, 15 drops of Voges-Proskauer reagent A was added followed immediately by 5 drops of Voges-Proskauer reagent B. The mixture was shaken gently.

Positive: Formation of orange/red colour

Negative: No colour change

Methyl Red Test

Material:

- MR-VP Broth
- Methyl Red Indicator

Methyl red indicator

Reagent formula	Volume
Methyl red	0.04 g
Absolute ethanol (ethyl alcohol)	60 ml
Sterile deionized water	40 ml

Method:

Exactly 2.5 ml of MR-VP broth which was transferred and incubated for 2 days was used. On the day of testing, methyl red reagent was allowed to warm to room temperature. 5 drops of methyl red reagent was added to each tube. The tubes were shaken and the results were interpreted.

Positive: Formation of red colour

Negative: No colour change

Citrate Utilization test

Simmon's citrate agar was prepared following its formula. It was then autoclaved. The test tubes with the agar were then slanted to form the agar slant when it solidifies. Using sterile technique, organism of interest was inoculated appropriately by means of streak inoculation. All cultures were incubated for 24 to 48 hours at 37° C.

Simmons Citrate Agar

Formula	Volume
Sodium chloride	5.0 g
Sodium citrate, tribasic	2.0 g
Sodium ammonium phosphate	0.8 g
Magnesium sulphate	0.2 g
Ammonium dihydrogen phosphate	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g

Final pH 7.0 + 0.2

Exactly 2.3 gm of powder was dissolved in 100 ml of distilled water. The mixture was boiled until complete dissolution and placed into test tubes before autoclaving for sterilization.

Positive: Agar changes to blue colour

Negative: Agar remains blue colour

Preparation of stock culture

For short term storage, cultures are streaked on prepared slants of NA + 2% NaCl and stored at 4°C. For long term storage, the cultures are first grown in trypticase soy broth (TSB) + 2% NaCl and let to grow overnight at 37°C. Then 80% of sterile glycerol is added into tubes. The tubes are then frozen at -70°C (liquid nitrogen) for 15 minutes and stored in a -80^0C freezer.

Glycerol Stock

Material:

- Nutrient Broth
- Glycerol 100%

Method:

Exactly 0.8 ml of nutrient broth was pipetted into microfuges. Desired organisms were inoculated into it. Triplicates were done for each culture. The cultures were incubated at 37°C for 1 day. The next day, 0.2 ml of glycerol was added into each tube .The tubes were vortexed well. The tubes were then placed in liquid nitrogen for 30 minutes before storing in the freezer.

Nutrient broth (NB) preparation**:**

Formula	Volume
Peptone	5.0 g
Sodium chloride	5.0 g
Yeast extract	2.0 g
‘Lab-Lemco’ powder	1.0 g

Final pH 7.4 + 0.2

Method:

About 1.3 gm of powder was dissolved in 100 ml of distilled water. The mixture was boiled until complete dissolution and placed into 20 ml universal bottles before autoclaving for sterilization.

Nutrient slant preparation:

Material:

- Nutrient agar

Nutrient agar preparation:

Nutrient agar:

Formula	Volume
Nutrient broth	1.3 g
Agar	1.5 g
Distilled water	100 ml

Method:

Exactly 10 ml of nutrient agar was placed into universal bottle to prepare slant. Desired organism was streaked onto the agar. The caps of the bottle were loosened before it was incubated at 37° C. When growth was present, the stock was kept in freezer.

Statistical Analysis

Statistical analysis was performed using PAST (Hammer et al., 2001). All values were reported as mean ± standard deviation (S.D.) unless mentioned otherwise. Differences between the two sites were tested via Student's *t*-test whereas correlation analysis was carried out to determine factors that might affect the distribution and abundance of vibrios. Before statistical analysis, *Vibrio* spp. and *E. coli* counts were transformed by the following equation: log (n + 1) whereas total bacterial count was log transformed. Diversity of *Vibrio* spp. was calculated as the Shannon's diversity index.

CHAPTER 3: RESULT

Seawater temperature at both stations, were on average high (30°C) and stable (coefficient of variation, CV < 4%), which is usual of tropical waters (Table 1). pH was similar between both stations (7.08 – 8.04 at Port Klang and 7.00 – 8.07 at Port Dickson) whereas salinity was significantly lower at Port Klang (Student's $t = 2.08$, $df = 36$, $p < 0.05$). Salinity was also more variable at Port Klang (CV = 23%) than Port Dickson (CV = 14%). Salinity measurements at Port Klang were typical of an estuarine system where river influx resulted in lower salinity, and wider range. DO was also lower at Port Klang (Student's $t = 5.43$, $df = 39$, $p < 0.001$), and together with the higher dissolved inorganic nutrient concentrations (Student's t-test > 2.05, $df > 20$, $p < 0.01$) reflected the eutrophic nature of Port Klang waters (Lee et al., 2009). The eutrophic waters at Port Klang also supported higher phytoplankton biomass (via Chl *a* concentration) and bacterial abundance. Sample photomicrographs of the DAPI stained slides are available in the Appendix. However the differences were not statistically significant, probably due to the large fluctuations of both variables (Chl *a* CV = 189%, bacterial abundance CV = 88%).

Table 1. Mean ± S.D. of variables measured in this study. Student's t-tests were carried out to compare values from Klang and Port Dickson, and *, ** and *** indicate the significant p values of < 0.05, < 0.01 and < 0.001, respectively.

	Klang (n = 21)	Port Dickson (n = 21)
Temperature	30.2 ± 0.8	30.1 ± 1.2
Salinity (ppt) *	23.9 ± 5.5	26.9 ± 3.8
pH	7.47 ± 0.23	7.54 ± 0.42
Dissolved oxygen (µM)***	164 ± 35	227 ± 40
Total suspended solid (mg l^{-1})	57.6 ± 23.8	55.6 ± 25.3
Chlorophyll *a* (µg L^{-1})	6.31 ± 11.94	2.10 ± 1.07
Bacterial abundance (× 10^6 cells ml^{-1})	5.0 ± 9.5	2.6 ± 4.1
NH_4 (µM)**	26.89 ± 36.42	1.76 ± 1.68
NO_3+NO_2 (µM)***	6.61 ± 3.35	1.37 ± 0.87
PO_4 (µM)**	2.48 ± 1.81	1.06 ± 0.85

Throughout the two year sampling period, *E.coli* counts fluctuated widely at Port Klang (range up to 200 cfu ml^{-1}) (average = 44 ± 12 cfu ml^{-1}, n=21), and was more than one order higher than Port Dickson (range up to 3 cfu ml^{-1}) (average = 1.0 ± 0.3 cfu ml^{-1}, n=21) (Student's *t*-test: $t = 6.14$, $df = 24$, $p < 0.001$) (Figure 4). The peaks observed at Port Klang were not in tandem with Port Dickson. At Port Klang, *E. coli* was observed in 81% of the sampling, whereas at Port Dickson, *E. coli* was observed in 71% of the sampling.

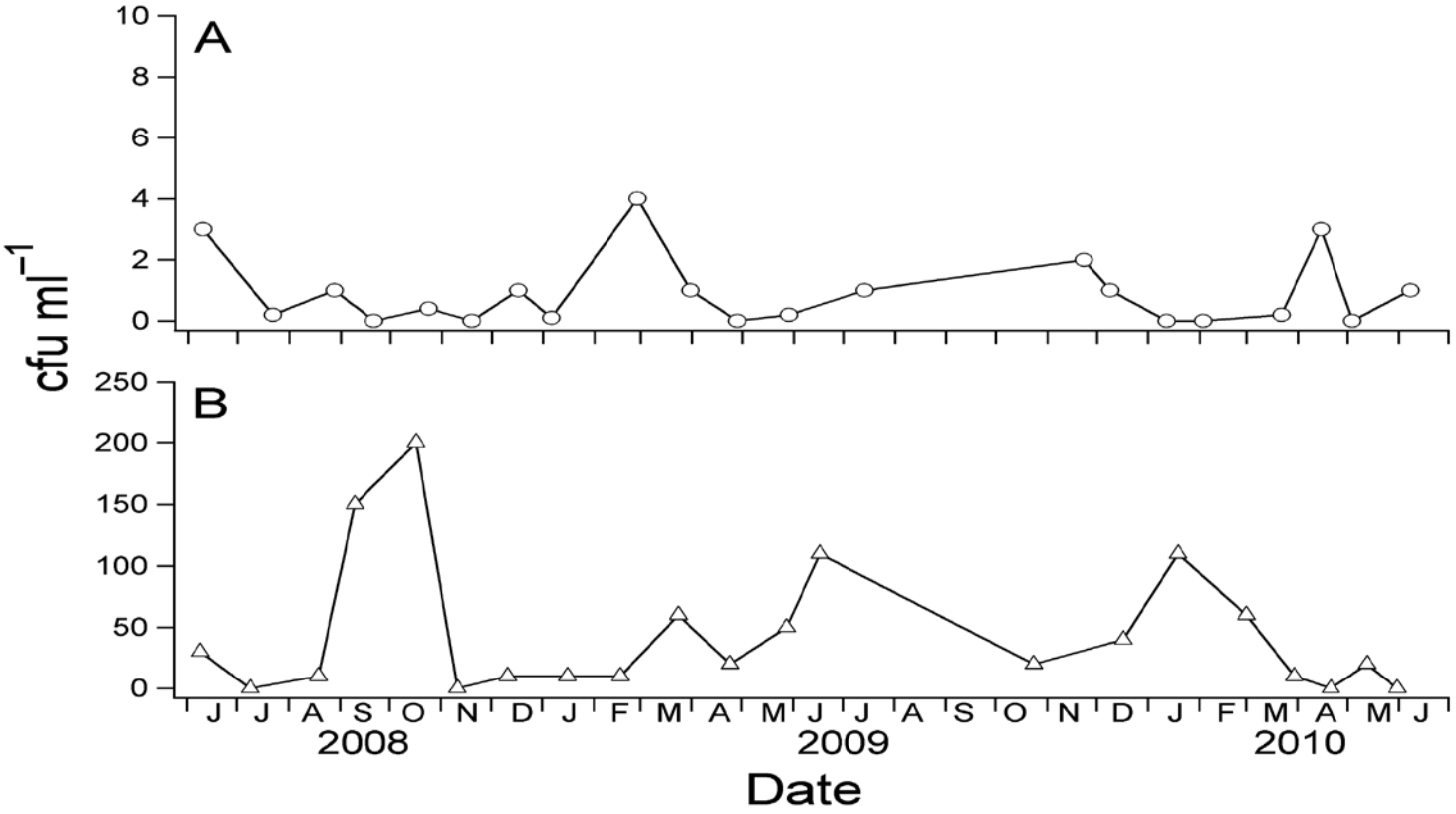

Fig. 4: Temporal variation of *Escherichia coli* counts (cfu ml^{-1}) at both Port Dickson and Port Klang.

In contrast to *E. coli* counts, presumptive vibrios were within the same order at both Port Klang and Port Dickson (Figure 5), ranging from 40 to 610 cfu ml^{-1} and from < 10 to 760 cfu ml^{-1}, respectively. At both stations, vibrio abundance isolated on TCBS was consistently higher than TCBS+NaCl (Figure 5). Further analysis using TCBS counts showed there were more presumptive vibrios at Port Dickson (206 ± 30 cfu ml^{-1}) than Port Klang (170 ± 41 cfu ml^{-1}) (Student's *t*-test: $t = 1.77$, $df = 34$, $p < 0.05$). From these presumptive vibrios, 78% were identified as *Vibrio* spp. at Port Dickson whereas 60% were *Vibrio* spp. at Port Klang. The abundance of *Vibrio* spp. were also higher at Port Dickson (160 ± 21 cfu ml^{-1}) than Port Klang (101 ± 32 cfu ml^{-1}) (Student's *t*-test: $t = 3.08$, $df = 36$, $p < 0.01$) (Figure 6). Although both Port Dickson and Port Klang are located > 100 km away from each other, and have fundamentally different ecosystems with different trophic status, we observed that *Vibrio* spp. from both these stations varied in tandem. There was a recurring pattern of higher *Vibrio* spp. abundance in the period from December until March.

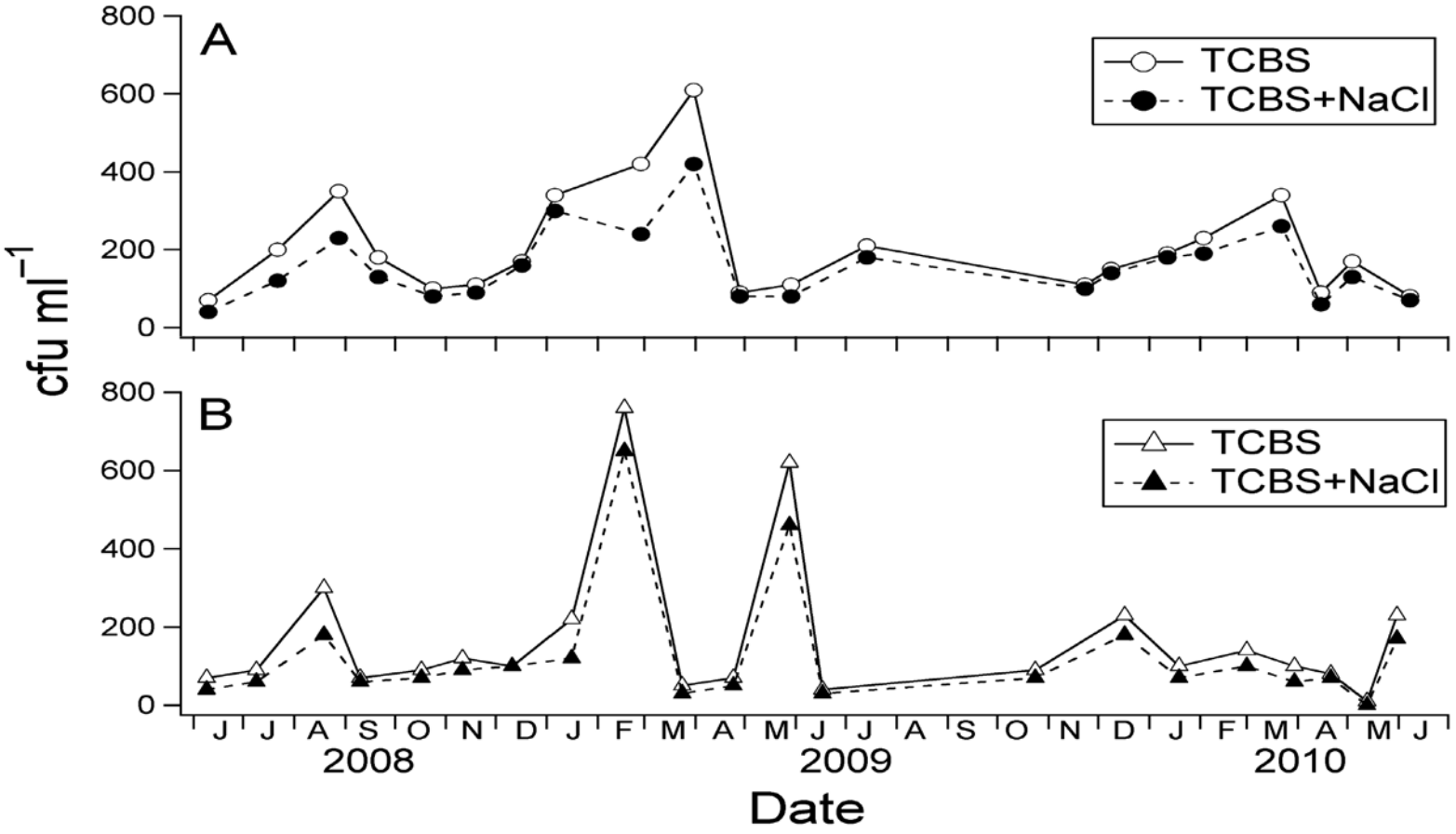

Fig. 5: Temporal variation of presumptive vibrios on both TCBS and TCBS+NaCl (cfu ml^{-1}) at both Port Dickson and Port Klang.

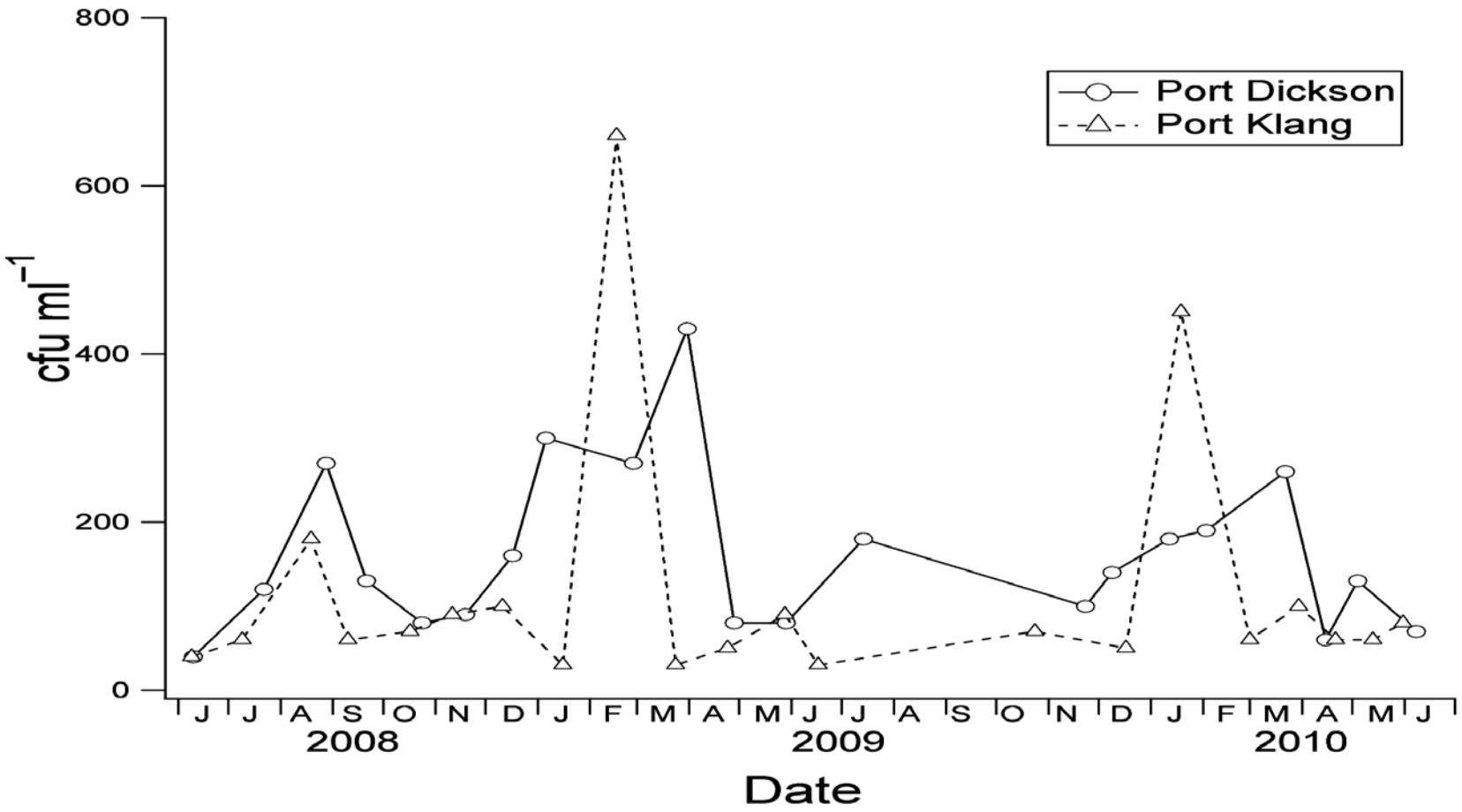

Fig. 6: Temporal variation of *Vibrio* spp. counts (cfu ml^{-1}) at both Port Dickson and Port Klang.

A total of 16 *Vibrio* spp. were identified in this study, of which 15 species were found in Port Dickson (Table 3) and 11 species at Port Klang (Table 2). Shannon's diversity index was higher at Port Dickson (1.62 ± 0.08, n=21) than Port Klang (1.11 ± 0.09, n=21) (Student's *t*-test: $t = 5.79$, $df = 40$, $p < 0.001$). There were 10 common species but one species (*V. parahaemolyticus*) that was found only at Port Klang. In contrast, a total of five species (*V. fluvialis, V, harveyi, V. logei, V. metschnikovii* and *V. splendidus II*) were unique to Port Dickson. The predominant species at both sites was *V.alginolyticus*, which was also the main species contributing to the increase in *Vibrio* spp. abundance from December until March.

Table 2. *Vibrio* species isolated at Port Klang (cfu ml^{-1})

Species	Jun-08	Jul-08	Aug-08	Sep-08	Oct-08	Nov-08	Dec-08	Jan-09	Feb-09	Mac-09	Apr-09	May-09	Jun-09	Oct-09	Nov-09	Dec-09	Jan-10	Mac-10	Mac 10	Apr-10	Jun-10
V.alginolyticus	10	40	80	20	40	40	–	10	420	–	20	40	20	40	20	230	10	50	20	20	30
V.anguillarum	–	–	10	10	–	–	–	–	70	–	–	–	–	20	–	70	–	–	–	–	–
V.cholerae	–	–	–	–	–	–	–	–	20	–	–	–	–	–	–	–	–	–	–	–	–
V.damsela	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–	–	–
V.furnisii	–	–	10	–	10	–	–	–	–	–	–	–	–	–	–	–	–	20	–	10	20
V.mimicus	10	–	30	–	–	–	20	–	30	10	20	40	–	–	10	40	20	–	20	10	10
V.mytili	–	–	10	–	–	–	–	–	40	–	–	–	–	–	–	–	20	10	–	20	–
V.nereis	20	–	20	10	10	40	20	20	20	–	–	10	10	–	–	20	–	–	–	–	–
V.ordalii	–	–	–	–	–	–	–	–	10	–	–	–	–	–	–	–	–	–	–	–	–
V.parahaemolyticus	–	–	–	10	–	–	10	–	20	–	–	–	–	–	–	–	–	–	–	–	–
V.splendidus I	–	20	20	10	10	10	50	–	30	20	10	–	–	10	20	90	10	10	20	–	20
Shannon's Diversity Index	1.04	0.64	1.63	1.56	1.15	0.96	1.22	0.64	1.36	0.64	1.06	0.96	0.64	0.96	1.06	1.31	1.33	1.36	1.10	1.33	1.32

Table 3. *Vibrio* species isolated at Port Dickson (cfu ml^{-1})

Species	Jun-08	Jul-08	Aug-08	Sep-08	Oct-08	Nov-08	Dec-08	Jan-09	Feb-09	Mac-09	Apr-09	May-09	Jul-09	Nov-09	Dec-09	Jan-10	Feb-10	Mac-10	Apr-10	May-10	Jun-10
V.alginolyticus	10	40	70	30	20	–	70	160	120	190	30	30	70	20	30	80	20	90	30	40	40
V.anguillarum	–	10	10	–	10	30	10	20	20	20	–	–	40	20	–	20	–	10	–	20	–
V.cholerae	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–	–	–	–
V.damsela	–	30	10	–	–	10	–	10	10	–	–	–	–	–	–	–	–	–	–	–	10
V.fluvialis	–	10	20	10	–	–	–	10	–	40	20	–	10	20	20	10	20	20	–	–	–
V.furnisii	–	–	–	10	–	10	10	20	–	–	–	–	–	–	30	20	30	40	20	–	10
V.harveyi	20	20	60	30	10	10	10	20	20	40	–	30	20	–	–	10	30	20	–	10	–
V.logei	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–	10	–	–	–	–	–
V.metschnikovii	–	10	–	–	10	–	–	10	–	–	–	–	–	–	–	–	–	–	–	–	–
V.mimicus	10	–	20	10	–	10	20	–	50	30	–	10	–	10	10	–	50	20	–	10	–
V.mytili	–	–	–	–	–	–	20	10	30	20	–	–	–	–	–	10	–	–	–	10	–
V.nereis	–	–	30	10	10	–	–	10	20	20	10	–	10	–	40	–	–	–	–	20	–
V.ordalii	–	–	–	–	10	–	20	–	–	10	10	–	10	–	–	–	–	–	–	–	–
V.splendidus I	–	–	50	30	10	20	–	30	–	60	10	10	10	20	10	20	20	60	10	20	10
V.splendidus II	–	–	–	–	–	–	–	–	–	–	–	–	10	–	–	–	10	–	–	–	–
Shannon's Diversity Index	1.04	1.63	1.87	1.80	1.91	1.68	1.66	1.67	1.62	1.78	1.49	1.26	1.75	1.75	1.67	1.74	1.96	1.71	1.01	1.82	1.15

In order to assess the vibrio community structure at both our stations, we carried out an analysis of similarities (ANOSIM) after transforming the cfu data into a presence / absence matrix. Using the Bray Curtis coefficient, ANOSIM shows that the vibrio community structure at Port Dickson was significantly different from Port Klang ($R = 0.334$, $p < 0.001$). The major taxon responsible for the differences in the vibrio community profiles was determined via similarity percentage (SIMPER). SIMPER test showed tha*t V. harveyi*, *V. anguillarum* and *V. fluvialis* were the most important for the difference between Port Dickson and Port Klang, and accounted for a cumulative 35% dissimilarity (Table 4).

Table 4. Average dissimilarity and cumulative contribution of taxa responsible for the differences in the vibrio community profiles as determined from SIMPER.

Taxon	Average dissimilarity	Cumulative %
V. harveyi	7.57	14.3
V. anguillarum	5.49	24.6
V. fluvialis	5.34	34.7
V. nereis	5.17	44.4
V .furnisii	4.98	53.8
V. mimicus	4.94	63.1
V. splendidus I	3.75	70.2
V. mytili	3.55	76.9
V. damsela	2.97	82.5
V. ordalii	2.42	87.0
V. alginolyticus	1.93	90.7
V. metschnikovii	1.30	93.1
V. parahaemolyticus	1.21	95.4
V. logei	0.91	97.1
V. splendidus II	0.82	98.7
V. cholerae	0.70	100.0

CHAPTER 4: DISCUSSION

The physico-chemical variables measured showed that both Port Klang and Port Dickson were essentially different, and were characteristic of eutrophic and oligotrophic waters, respectively. Port Klang is an estuarine system with nutrient and organic matter input from the Klang river. Rapid development, land clearing, agriculture and industrialization in its catchment area caused the deterioration of water quality at Port Klang (Lee et al., 2009). *E. coli* was also detected in 70 and 80% of the samples at Port Dickson and Port Klang, respectively and confirmed the prevalence of faecal pollution in our coastal waters (Chua et al., 2000). However, *E. coli* counts at Port Dickson were within the Malaysia Interim Marine Water Quality Standard (Department of Environment, 2009), and still suitable for recreational activities.

The culture-dependent method was used to enumerate *Vibrio* spp. even though culture-independent methods generally show a higher recovery of *Vibrio* spp. (Brayton et al., 1983). Culture-dependent method may also underestimate *Vibrio* spp. population due to 'viable but non-culturable' states (Roszak and Colwell, 1987). However as isolating and culturing *Vibrio* spp. on TCBS is used extensively, it provides a standard approach for comparison with other studies. In this study, > 60% of the presumptive vibrios isolated were identified as *Vibrio* spp. which was similar to the percentage obtained by Pfeffer and Oliver (2003). The abundance of *Vibrio* spp. observed at both Port Klang and Port Dickson were also within the range of other studies (e.g. Pfeffer et al., 2003; Gopal et al., 2005; Eiler et al., 2006; Wetz et al., 2008; Turner et al., 2009).

In this study, we observed a recurring pattern of higher *Vibrio* spp. abundance from December until March at both Port Klang and Port Dickson. This period coincided with the North-East monsoon (Dec – Mar) which brings a higher average precipitation. As higher precipitation transports more nutrients into coastal waters, the nutrient input stimulates higher levels of primary and bacterial production (Lee and Bong 2008; Lee et al., 2009), and could also support more vibrios. This was however not observed in our correlation analysis. More frequent sampling may be able to detect these environmental patterns. Heavy rains during the rainy season in Mozambique also brought a high load of land run-off into coastal waters that coincided with an increase in culturable *Vibrio* (Collin et al., 2013). The vibrio 'bloom' that we observed in this study seemed to be a recurring natural phenomenon, and might be relevant to other tropical and subtropical waters. This seasonality brings important implications to the management of coastal resources and activities, primarily due to the fact that some *Vibrio* spp. are pathogenic to both humans and aquatic organisms. For example, the harvesting of benthic organisms as food resource could be modulated to reduce health risks (Collin et al., 2013), or the massive use of antibiotics in intensive aquaculture to prevent proliferation of vibrios (Naylor et al., 1998) could be controlled for more effective antibiotic application.

The increase of *Vibrio* spp. during the North-East monsoon was mainly supported by an increase in *V. alginolyticus,* which was also the predominant vibrio at both Port Klang and Port Dickson. *V. alginolyticus* is a human pathogen, particularly for otitis and wound infections (Morris and Black, 1985), and is also a fish pathogen (Toranzo et al., 2005; Xiao et al., 2009).

Therefore, *V. alginolyticus* bloom posed a public health risk, and could be detrimental to mariculture and aquaculture industries that source seawater from nearby coasts. Others have also reported *V. alginolyticus* to be the predominant vibrio in their studies e.g. sub-tropical coastal waters of India (Gopal et al., 2005) and Thailand (Thongchankaew et al., 2011). Observations from tropics and sub-tropics contrasted with temperate regions where the predominant are *V. splendidus* and *V. anguillarum* (Eiler et al., 2006). The difference in the predominant vibrio could have reflected a form of diversity structuring according to latitudes (Fuhrman et al., 2008), although more studies should be carried out to confirm this especially in the tropics.

In a survey of seafood samples in Malaysia, Elhadi et al. (2004) reported eight potentially pathogenic *Vibrio* spp. of which seven were detected in this study with the exception being *V. vulnificus*. Human pathogens e.g. *V. cholerae* and *V. parahaemolyticus* were also isolated in this study. However their risk for human health could not be interpreted as we did not determine if these isolates were toxigenic. In this study, *Vibrio* spp. diversity increased with *Vibrio* spp. abundance ($R^2 = 0.653$, df = 34, $p < 0.001$). However the positive correlation was until a threshold of about 200 cfu ml^{-1} after which diversity decreased (Figure 7).

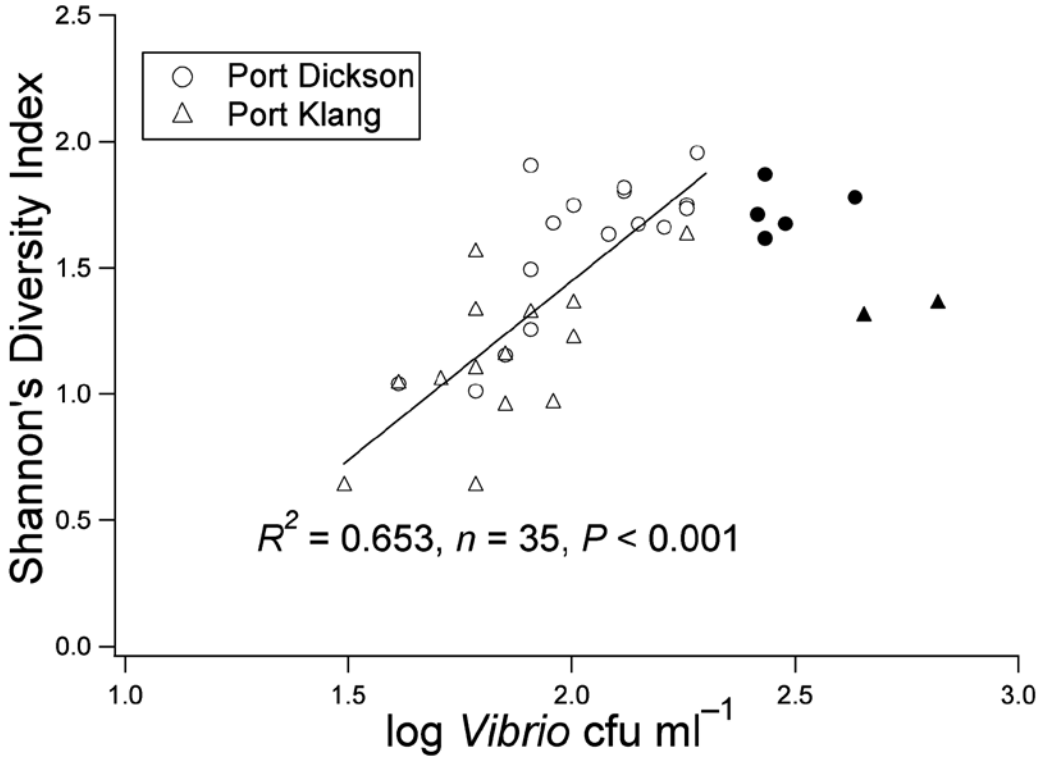

Fig. 7: Relationship between *Vibrio* spp. diversity (as shown by Shannon's diversity index) versus *Vibrio* spp. abundance (log Vibrio cfu ml^{-1}). Filled symbols were used in the correlation analysis, and the regression line is also shown.

Favourable conditions e.g. nutrient inputs for vibrio 'bloom' (i.e. > 200 cfu ml^{-1}) decreased overall diversity as only certain vibrios could adapt, and dominated the community structure. It is well accepted that eutrophication, nutrient inputs or pollution cause a reduction of biological diversity (Piola and Johnston, 2008). Interestingly, Gregoracci et al. (2012) proposed a similar threshold of vibrio counts (> 200 cfu ml^{-1}) to indicate polluted seawater. Although vibrios are indigenous to the sea and some vibrios are pathogenic (Thompson et al., 2004), there has been no attempt to address their presence and to employ them in water quality assessments. Instead, *E. coli* is still used in many countries as pollution indicators for coastal waters even though its presence does not reflect marine pathogens e.g. *V. parahaemolyticus* (Lee et al., 2011). In this study, *E. coli* counts were also not indicative of *Vibrio* spp. abundance ($R^2 = -0.100$). As vibrios are indigenous marine bacteria, and are easily cultured, a vibrio threshold of 200 cfu ml^{-1} could be used to indicate polluted seawater and the occurrence of a vibrio bloom concurrent with decreased vibrio diversity.

We measured the abundance and diversity of culturable vibrios in oligotrophic and eutrophic waters as a model to better understand how environmental variables affect vibrios. We found that the abundance of *Vibrio* spp. was higher at Port Dickson than Port Klang. Akin to *Vibrio* spp. abundance, culturable vibrio diversity was also higher at Port Dickson. Using univariate analysis (Table 5), *Vibrio* spp. seemed to thrive in cleaner waters e.g. higher DO, and lower bacterial abundance.

Table 5.Correlation of variables measured against *Vibrio* abundance and diversity. * and ** indicate the significant p values of < 0.05 and < 0.01, respectively. A negative sign denotes an inverse relationship.

	Temperature (°C)	Salinity (ppt)	pH	Dissolved oxygen (µM)	Total suspended solids (mg l^{-1})	Chl *a* (µg l^{-1})	Bacteria (cells ml^{-1})	NH_4 (µM)	NO_3+NO_2 (µM)	PO_4 (µM)
Vibrio abundance	−0.025	0.136	−0.084	0.326*	−0.224	−0.138	−0.333*	−0.130	−0.207	−0.228
Vibrio diversity	0.061	0.061	−0.065	0.376*	−0.275	−0.040	−0.256	−0.383*	−0.401**	−0.336*

Culturable vibrio diversity was also higher with DO, and with lower concentrations of dissolved inorganic nutrients. In this study, the trophic state of the sampling stations was a more important factor for both vibrio abundance and diversity. In contrast, temperature and salinity did not correlate with both vibrio abundance and diversity, even though they are important in temperate waters (Eiler et al., 2006; Turner et al., 2009). As temperature is relatively stable in tropical waters, it is known that bacteria is not affected nor limited by it (Pomeroy and Wiebe, 2001). From the only other work carried out in tropical waters, Gregoracci et al. (2012) reported that vibrio counts correlated positively with phosphate but negatively with salinity. As understanding how environmental variables affect vibrios is important, more work should be carried out in tropical waters. This will help determine whether these observations are site-specific or could be applied over wider regions.

This is a first study on the tropical coastal waters along the Straits of Malacca where we provided a time series analysis of abundance and diversity of culturable *Vibrio* spp. Our study shows seasonality of a vibrio bloom during the North-East monsoon, and how vibrio diversity reduces when vibrio abundance breaches 200 cfu ml^{-1}. The results of this study not only provide information in a local environment but also have a broader benefit of giving insight into the seasonality of vibrios in coastal waters worldwide. As vibrios are indigenous marine microorganisms and some are pathogenic, they pose a potential danger to public health. The recurring vibrio bloom allows us to better evaluate the public health risks of vibrios, and should be implemented into future water quality management programs.

CHAPTER 5: CONCLUSION

Both *vibrio* abundance and diversity were higher in cleaner waters with higher dissolved oxygen and lower nutrient concentrations. *V. alginolyticus* was the predominant species in this study. We observed a recurring *vibrio* 'bloom' during the North-East Monsoon. Continuous study on tropical coastal water has to be done to monitor the trend of *Vibrio* spp in Malaysia. This is important to curb issues related to healthcare as there were pathogens seen on certain months in our study.

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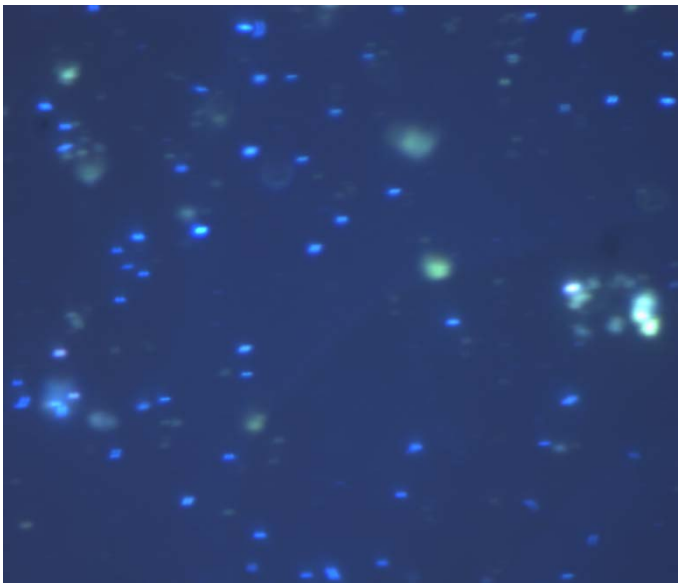
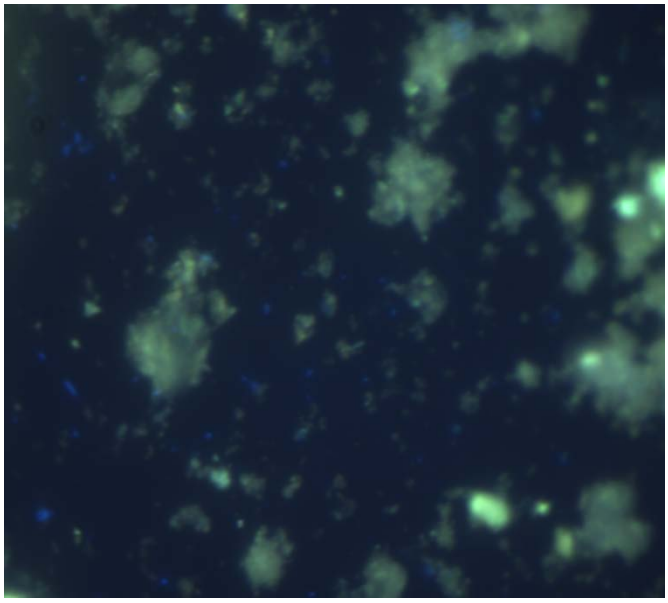
APPENDIX A

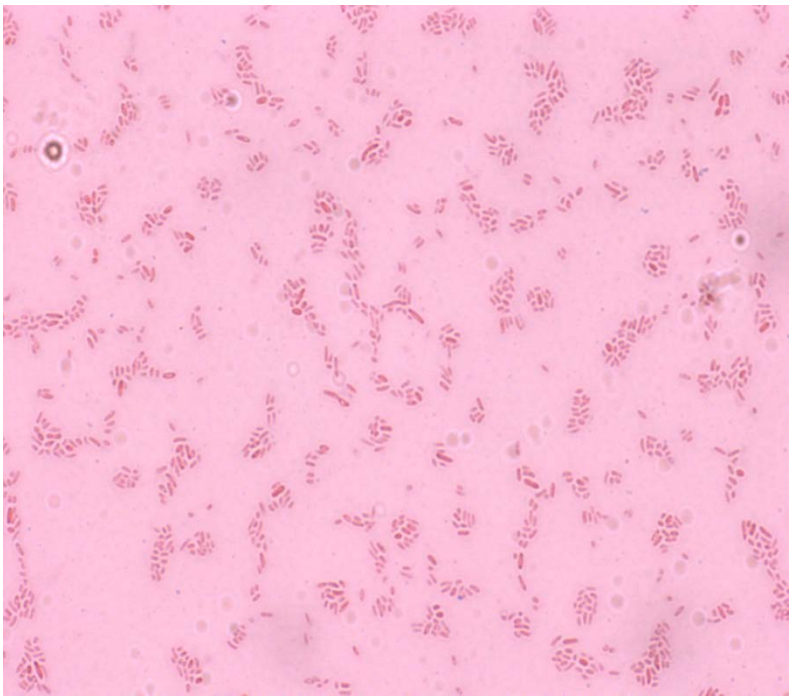


Microphotograph of DAPI stained sample from Port Dickson. Bacteria are stained fluorescent blue.



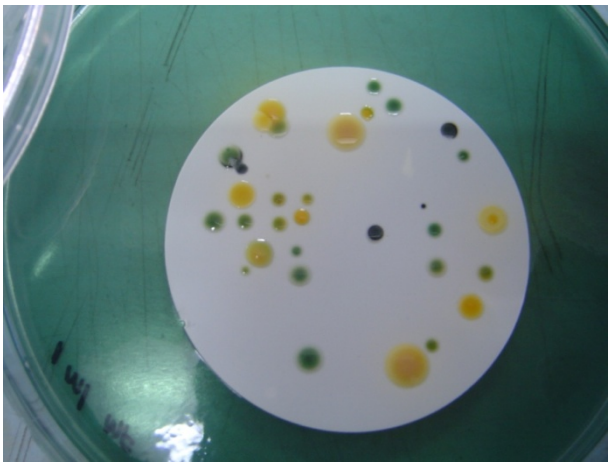
Microphotograph of DAPI stained sample from Klang. Bacteria are stained fluorescent blue.

APPENDIX B

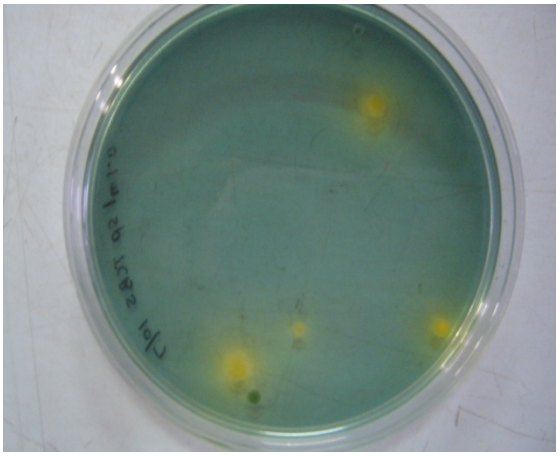


Microphotograph of a Gram stained *Vibrio* sp.

APPENDIX C

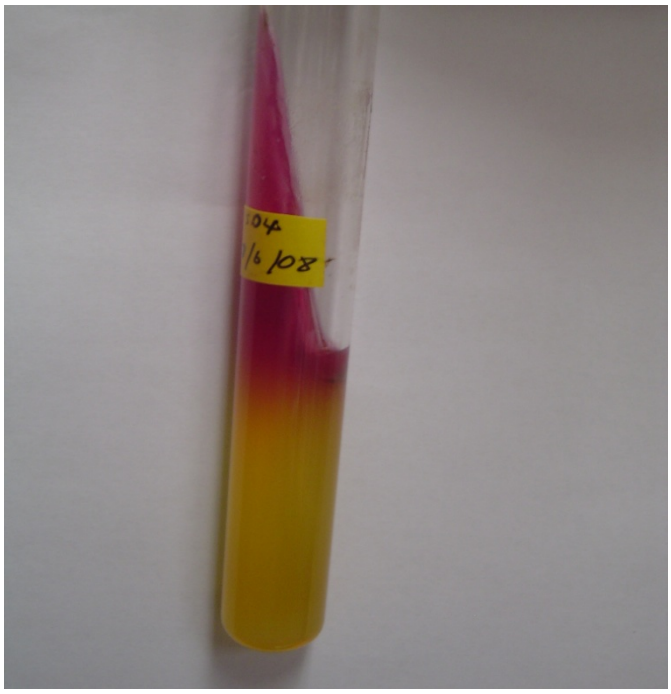


Photograph showing representative results of membrane filtration method on TCBS



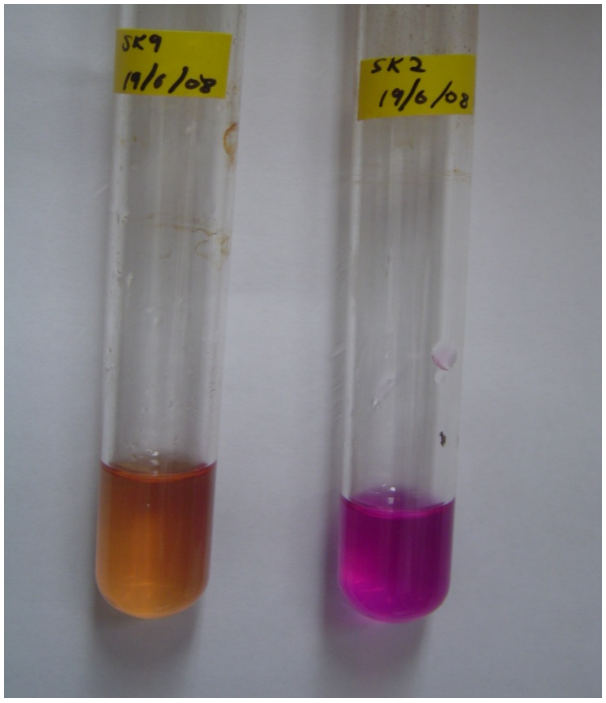
Photograph showing representative results of spread plating method on TCBS

APPENDIX D



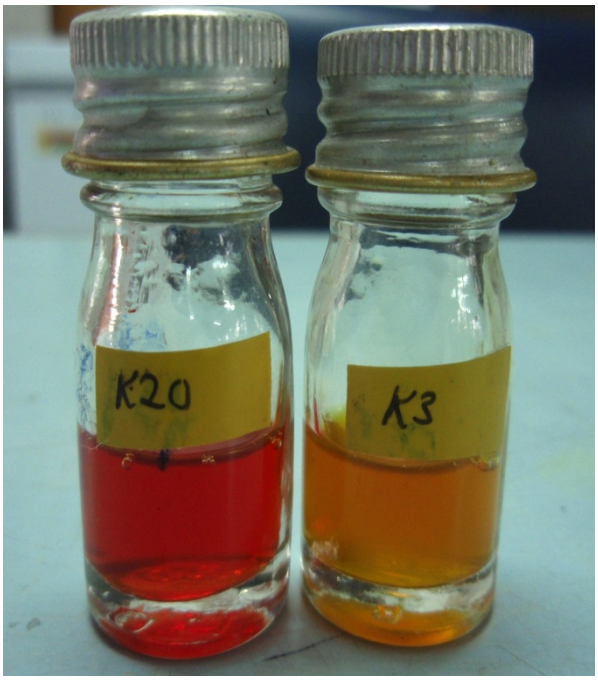
Photograph of a Kligler Iron agar test. This tube is interpreted as A/K, no gas and no H_2S production

APPENDIX E



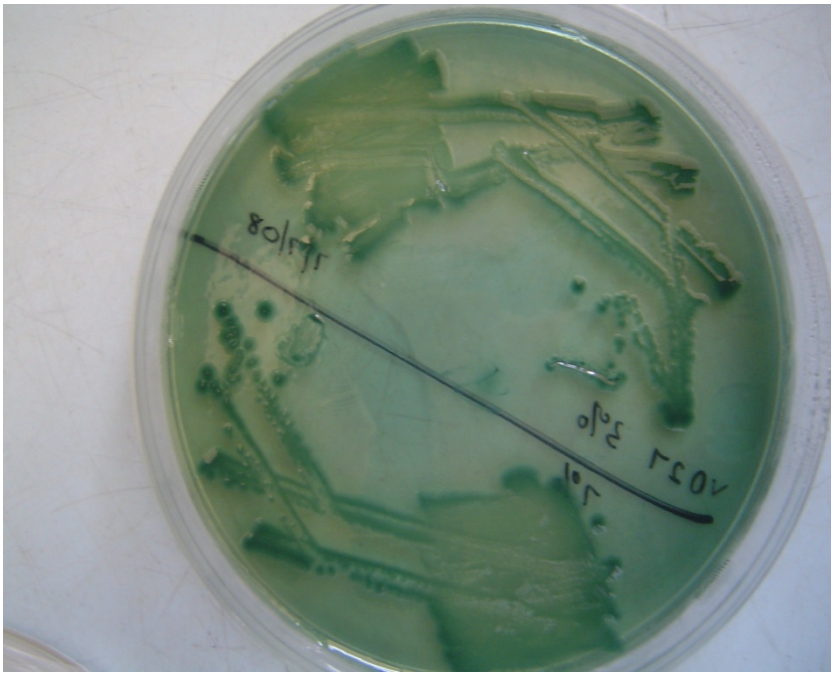
Photograph of a urease test. Left tube indicates negative result while right tube is interpreted as positive

APPENDIX F



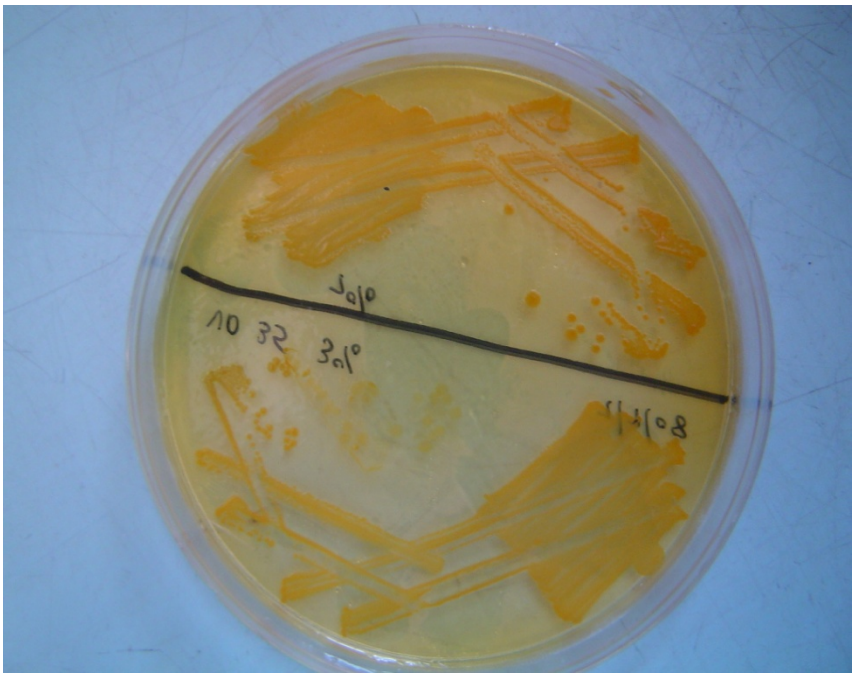
Photograph of a Carbohydrate (Mannitol) Fermentation Test. Result is interpreted as positive for yellow colour and negative if it remains red

APPENDIX G



Photograph of a green colony of *Vibrio* identified as *V.splendidus* in this study

APPENDIX H



Photograph of a yellow colony of *Vibrio* identified as *V.alginolyticus* in this study

APPENDIX I

IDENTIFICATION KEY USED TO IDENTIFY *Vibrio* Spp ISOLATED IN THIS STUDY (Based on Alsina & Blanch, 1994)

Species	KIA	Oxi	Ure	VP	Gel	0%	3%	6%	8%	10%	Man	Lac	Arg	Orn	Lys	O129 (150µg)	O129 (10µg)
V.harveyi	K/A	+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	+
V.costicola	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.damsela	K/A	+	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+
V.mytili	K/A	+	-	-	+	-	+	+	-	-	+	+	+	-	+	+	+
V.splendidus I	K/A	+	-	-	+	-	+	+	-	-	+	-	-	+	+	+	-
V.splendidus II	K/A	+	-	-	+	-	+	-	-	-	+	-	-	+	+	+	-
V.splendidus III	K/A	+	-	-	+	-	+	+	-	-	+	+	-	+	+	+	-
V. ordalii	K/A	+	-	-	+	-	+	+	+	-	+	-	+	-	+	+	-
V.metschnikovii	K/A	-	-	-	+	-	+	+	+	-	+	+	+	-	+	-	-
V.furnisii	K/A	+	-	-	-	-	+	+	-	-	+	-	+	-	-	+	+
V.cholerae	K/A	+	+	-	+	+	+	+	-	-	+	-	+	-	-	+	+
V.mediterranei	K/A	+	-	-	+	-	+	-	-	-	+	+	+	-	+	+	+
V.tubiashii	K/A	+	-	-	+	-	+	+	-	-	+	+	+	-	+	+	+
V.nereis	K/A	+	-	-	+	-	+	-	-	-	+	+	+	-	+	+	+
V.anguillarum	K/A	+	-	-	-	-	+	-	-	-	+	+	-	+	+	+	+
V.aestuarinus	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.diazotrophicus	A/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.fluvialis	K/A	+	-	-	-	+	+	+	-	-	+	-	+	-	+	-	-
V.fischeri	K/A	+	-	-	+	-	+	+	-	-	-	-	+	-	-	+	+
V.cincinnatiensis	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.marinus	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.vulnificus	K/A	+	-	-	+	-	+	+	-	-	-	-	+	-	-	+	+

V.mediterranei	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.campbellii	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.orientalis	K/A	+	-	-	+	-	+	+	-	-	+	+	+	+	-	+	+
V.alginolyticus	K/A	+	-	-	+	-	+	+	-	-	+	-	+	-	-	+	+
V.parahaemolyticus	K/A	+	-	-	+	-	+	-	+	-	+	-	+	-	-	+	+
V.mimicus	K/A	+	-	-	+	-	+	+	+	+	+	-	+	-	-	+	-

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Bulletin of Marine Science Decision for Manuscript #2013-1034R

bmsassistant@rsmas.miami.edu <bmsassistant@rsmas.miami.edu> Thu, Jul 18, 2013 at 12:29 AM
Reply-To: bmsassistant@rsmas.miami.edu
To: lee@um.edu.my
Cc: leechoonweng@yahoo.com

Dear Dr. Lee,

I am very pleased to inform you that your manuscript, "SEASONALITY AND DIVERSITY OF CULTURABLE VIBRIOS IN TROPICAL COASTAL WATERS" has been accepted and is now in line for publication in the Bulletin of Marine Science. We will likely make some minor changes in wording during copy editing, but otherwise we plan to publish the revised manuscript as submitted. When typesetting is complete, you will receive your galley proof and reprint order form in Adobe Portable Document Format (PDF).

Following the return of the galley proof to BMS, your article will be published in the "Fast Track" section of BMS Online. In most cases, your paper will appear "live" within 72 hours after the galley proof is received. This enables your research to be available to the scientific community as soon as possible, reducing the risk of delay that may be caused by the print process. You will be notified by e-mail when your article is available in Fast Track.

Again, congratulations and many thanks for submitting this manuscript to the journal.

Best regards,

Joseph E Serafy
Editor
Bulletin of Marine Science