CHAPTER 1: INTRODUCTION

1.1 Microbial food web

Marine ecosystems are complex and dynamic. Understanding of the susceptibility of marine ecosystems to global environmental variability and climate change driven by greenhouse gases is essential. It requires several factors include marine physical, chemical and biological interactions including thresholds, negative and positive feedback mechanisms and other nonlinear interactions. The fluxes of matter and energy, and the microbes that mediate them, are of central importance in the ocean, yet remain poorly understood (DeLong and Karl 2005).

In the food web, microorganisms are capable of creating a sustained cycle of production and decomposition of organic matter, requiring only the input of sunlight or the chemicals released from rivers and from hot vents that occur near undersea volcanism. Phytoplankton and photosynthetic and chemosynthetic bacterioplankton convert carbon dioxide or bicarbonate and inorganic nitrogen and phosphorus into the organic constituents of their cells. Microflagellates eat heterotrophic and the smaller autotrophic bacteria. In so doing, they usually control the numbers of bacteria in the sea, and are consumed in turn by larger ciliated protozoa. Ciliates are a staple food of copepods and other mesozooplankton that are the food of larval fishes (Pomeroy *et al*. 2007).

Detailed field studies over the past three decades have established the current ‘microbial loop’ hypothesis wherein microbes have a central position in the conversion of dissolved organic matter into higher trophic levels (Figure 1.1).

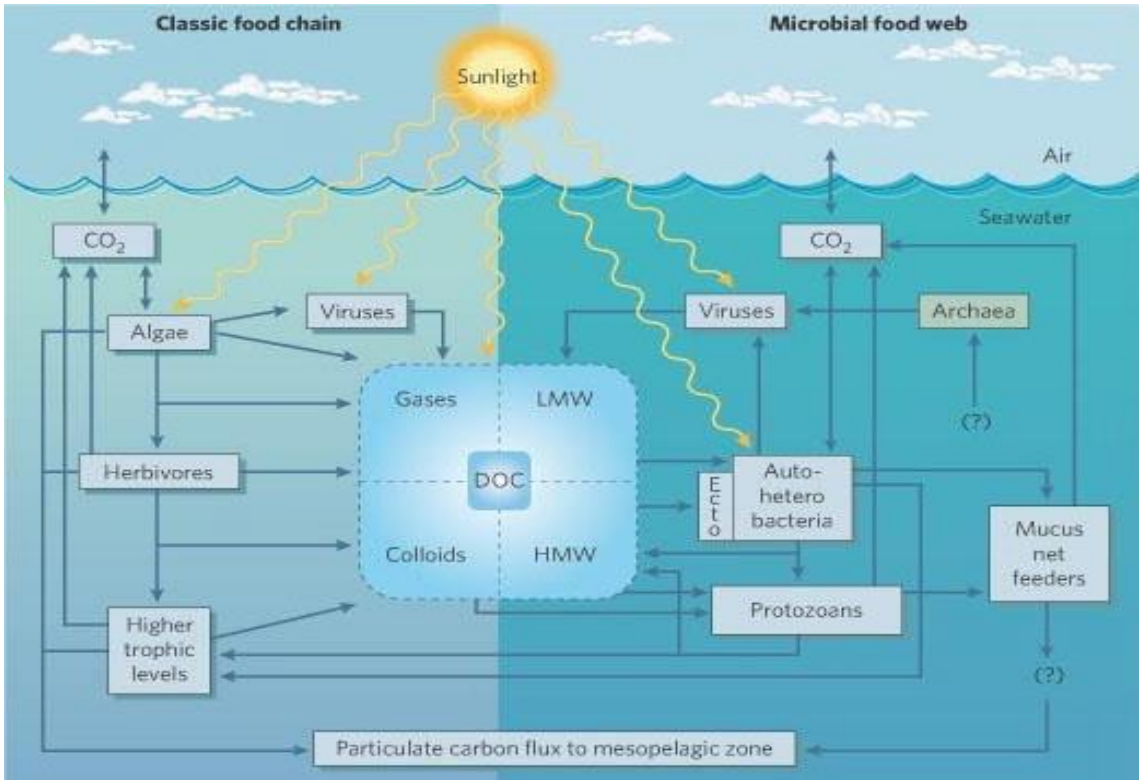

Figure 1.1: Marine microbial interactions in the upper ocean. Schematic representation of the ocean food web. On the left is the classic pathway of carbon and energy flow through photosynthetic Eukarya, to herbivores and on to higher trophic levels. Depicted on the right is the microbial food web, which uses energy stored in the non-living, detrital carbon pool to produce microbial biomass that can re-enter the classic pathway of carbon and energy flow. Cell-associated ectoenzymes (Ecto) enable bacteria to use high-molecular-weight (HMW) DOC in addition to the more traditional low-molecular-weight (LMW) and gaseous carbon substances. Also shown in the microbial food web are viral particles and Archaea. At the present time, there is only rudimentary knowledge of the role of Archaea in the oceanic food web. Shown at the bottom of this diagram is the downward flux of particulate carbon (and energy), which is now thought to fuel most subeuphotic zone processes. The classic algae-herbivore grazer pathway (left side) is most important in this regard. Adapted from Karl, D. M., 1994. Figure and legend from DeLong & Karl, 2005.

1.2 Attached and free-living bacteria

Bacteria are ubiquitous, abundant, diverse, and represent a large pool of genetic and physiological diversity in aquatic habitats. Bacteria are also the main consumers of dissolved organic matter and subsequently play a major role in carbon and nutrient cycling in aquatic ecosystems (Azam *et al*. 1994; DeLong and Karl 2005). In an aquatic environment, bacteria are separated into two main groups: attached and free-living bacteria (Murrell *et al*. 1999) as shown in Figure 1.2. These two groups are not independent communities but can be considered interacting as shifts between attached and free-living modes occur among them (Rieman and Winding 2001; Grossart *et al*. 2003). Bacterial attachment, detachment, and release of free-living progeny when attached (Stocker *et al*. 2008), are processes that contribute to the dynamic nature of the mode-shifts.

Generally, the proportion of attached bacteria in an aquatic environment increases with eutrophication, and can range from < 5% in oligotrophic to > 60% in eutrophic ecosystems (Bano *et al*. 1997; Crump *et al*. 1998; Garneau *et al*. 2009). One major reason is that eutrophic waters contain more organic-rich particles that are 'hotspots' for attached bacteria (Azam *et al*. 1994). However not all particle rich environments have high attached bacterial abundance as free-living bacteria can also contribute to particulate organic matter recycling (Lapoussiere *et al*. 2011). Attached bacteria are larger and more active than free living bacteria (Acinas *et al*. 1999; Lee *et al*. 2001; Mevel *et al*. 2008; Lapoussiere *et al*. 2011), but their abundance over total bacteria vary among different locations and seasons (Iriberri *et al*. 1987; Griffith *et al*. 1994).

There is also ambiguity whether particles can host distinct microbial communities where some studies are affirmative (Acinas *et al*. 1999; Crump *et al*. 1999; Ghiglione *et al*.2009) and others are contradictory (Hollibaugh *et al*. 2000; Ghiglione *et al*. 2007; Ortega-Retuerta *et al*. 2013). Due to the dynamic nature of the mode-shifts between attached and free-living bacteria, a majority of bacterial groups are ubiquitous. However niche segregation and therefore distinct bacterial populations are still possible as different metabolic capacities are required when growing on particulate versus dissolved matter. The bacterial community structure in different fractions are well studied in temperate waters (e.g. Crump *et al*. 1999; Bouvier and del Giorgio 2002; Ortega-Retuerta *et al*. 2013) and in subtropical waters (e.g. Zhang *et al*. 2007; Jing and Liu 2012) but is strikingly lacking in tropical waters.

Tropical waters cover about 40% of the global ocean. These waters are rich in marine biodiversity but knowledge of the bacterial diversity in this ecosystem remains limited, especially in the Southeast Asia region. The coastal waters in Southeast Asia have a persistently high suspended solid concentration (Bong and Lee 2008), and most of its major rivers carry a high suspended solids load (Moller *et al*. 2005; Lee and Bong 2006). It is therefore interesting to explore the dynamics of attached versus free-living bacteria in these waters. Moreover extensive works on the microbial ecology of our coastal waters (Lee and Bong 2008; Lee *et al*. 2009; Bong and Lee 2011; Lee and Bong 2012) have not resolved the role of attached and free-living bacteria in our coastal waters. Therefore results from this work fill an obvious data gap in this field. In tropical waters, temperatures show no marked seasonality, and are relatively high and stable (Lee *et al*. 2009). In temperate waters, it is difficult to distinguish between temperature and substrate supply as factors for attached bacterial abundance especially when both temperature and substrate co-vary (Griffith *et al*. 1994). Therefore ecological

relationships between substrate and attached bacterial dynamics would be more apparent in tropical waters.

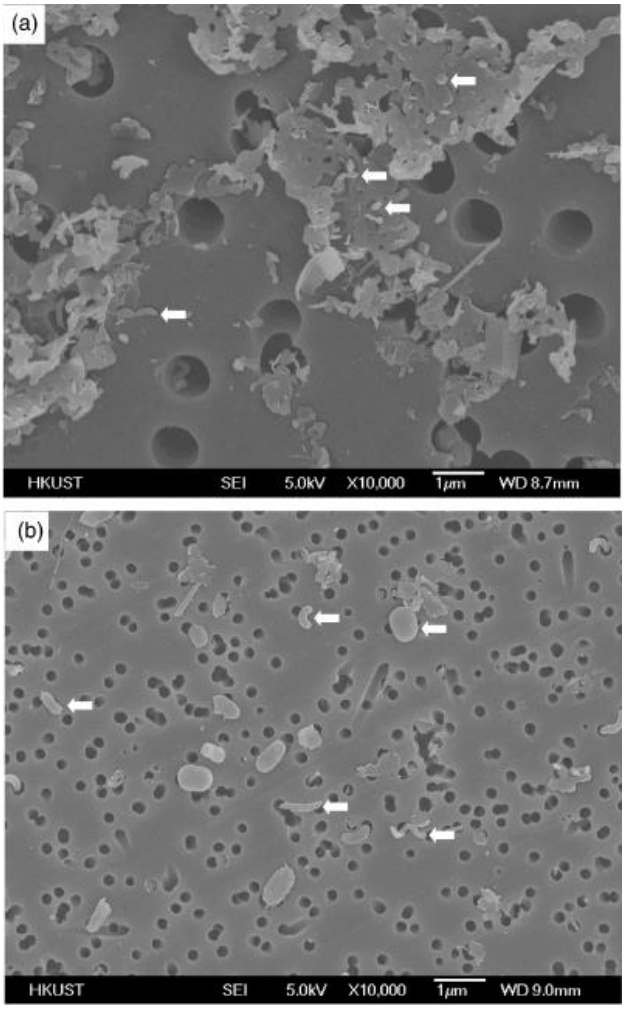

Figure 1.2: Scanning electron microscopy of particle-attached (a) and free-living (b) bacterial populations in surface sea water of Victoria Harbor. Some bacterial cells are indicated by arrows. Figure from Zhang *et al*. 2007.

1.3 Culture-independent approaches for microbial community studies

Before the 1990s, the diversity of bacteria was assessed by culture-dependent method such as phenotypic tests and numerical taxonomy of isolates cultivated on microbiological media (Fry 2000). However, Staley and Konopka (1985) found that studies of microbial communities in natural environments using cultivation methods have reached a limitation. Only 0.001% to 0.1% of marine bacteria have been cultured (Oren 2004), and most of the marine microbial community remains unknown.

In 1990s, the analysis of bacteria in the environment has shifted from culture-dependent to culture-independent approaches by using phenotypic or genotypic methods. Culture-independent molecular approaches in characterizing microbial communities have become the norm due to the fact that more than 90% of the microbes present in the environment are difficult to culture or are unculturable (Schloss and Handelsman 2005).

The most popular phenotypic method is phospholipids fatty acid (PLFA) analysis while genotypic methods are DNA-based. PLFA analysis characterized the phospholipids present in the periplasma of cells. Genotypic methods are where a total community genome DNA is extracted from environmental samples and analyzed directly through techniques such as "shot-gun cloning", "shot-gun sequencing" or "DNA probe hybridization". Besides analyzing the whole genome, specific genetic markers can also be amplified using polymerase chain reaction (PCR). Subsequently, the PCR products are analyzed by sequencing, hybridization, DNA melting behavior or length polymorphism (Nocker *et al*. 2007).

The mainstay of the culture-independent techniques for microbial identification is genotypic fingerprinting which is based on the 16S ribosomal RNA (16S rRNA). This method has been extensively used with accessibility of databases such as Ribosomal Database Project (RDP) (Maidak *et al*. 2000), and Basic Local Alignment Search Tool

(BLAST) search program at the National Center for Biotechnology Information (NCBI) (Johnson *et al*. 2008). However, genotypic methods have their own limitations such as DNA extraction method efficiency, PCR bias and sequencing accuracy.

One of the useful tools that generate genotypic fingerprinting is denaturing gradient gel electrophoresis (DGGE), which is based on separation of the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (Muyzer and Smalla 1998). By analyzing bands that migrate to different positions in the polyacrylamide gel, we can analyze the environmental samples diversity and monitor changes in microbial community (Muyzer 1999; Gurtner *et al*. 2000; Díez *et al*. 2001; Ogino *et al*. 2008).

The newly developed technique for microbial community profiling is high-throughput pyrosequencing which generate several thousand sequences per sample and reduce the cost and time of sequencing dramatically (Huse *et al*. 2008). The high sensitivity of pyrosequencing provides very comprehensive information of the microbial composition (Quince *et al*. 2008), is sensitive and gives highly reproducible results (Sogin *et al*. 2006) and novel organisms to be detected (Cardenas and Tiedje 2008). This helps in better understanding of microbial community structure. However high-throughput method has its limitation where sequencing error can occur and cause false estimation of diversity (Kunin *et al*. 2010).

Various techniques and technologies are used in different applications. Each approach has different specificity and limitation. Choice of approach in every research is based on the technologies available, stage of technologies development, and logistics. In this study, the bacterial community structure in attached and free-living fractions was determined via 16S rRNA PCR-DGGE (Muyzer *et al*. 1993). Although 16S rRNA

PCR-DGGE does not provide a comprehensive bacterial community structure, it is still useful to show the spatial and temporal variability in a community and can help identify the predominant species in a community (Miller *et al*. 1999).

1.4 Objectives of the study

Basically, the objectives of this study were:

i. To investigate the dynamics of attached and free-living bacterial abundance over a period of 18 months in tropical coastal waters.

ii. To sample both oligotrophic and eutrophic ecosystems and hypothesize that attached bacteria are predominant in eutrophic waters.

iii. To address whether attached and free-living bacteria differ phylogenetically.

CHAPTER 2: MATERIALS AND METHODS

2.1 Sampling

We sampled monthly from October 2009 until April 2011 (n = 18) at two stations along the west coast of Peninsular Malaysia i.e. eutrophic estuarine waters at Klang (03°00.1'N, 101°23.4'E) and oligotrophic coastal waters at Port Dickson (02°29.5'N, 101°50.3'E) (Figure 2.1). Seawater samples were collected about 0.1 m below surface seawater during high tide levels.

In-situ measurement of temperature was carried out using a digital thermometer (Comark, PDT 300, USA). For chemical analyses, seawater was collected using an acid-cleaned bucket whereas sterile bottles were used to collect samples for bacterial identification. Samples for bacterial counts were preserved with glutaraldehyde (1% final concentration). All the samples were kept in a cooler box until processing in the laboratory within three hours.

2.2 Dissolved oxygen (DO)

Seawater samples were collected in 50 ml DO bottles and fixed immediately with manganous chloride ($MnCl_2$) and alkaline iodide (KI) solutions. DO concentration was then determined by the Winkler titration method (Grasshoff *et al*., 1999).

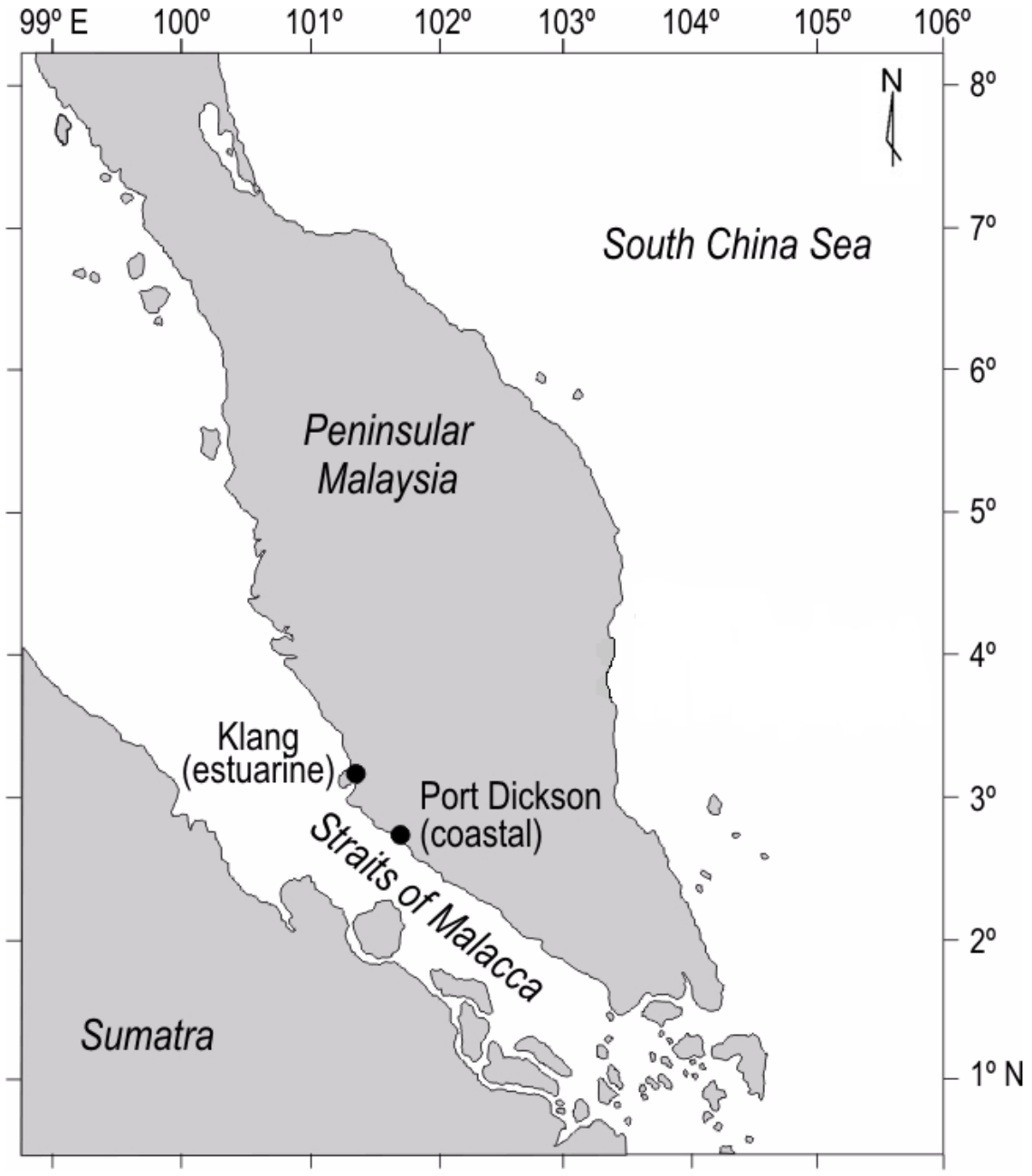

Figure 2.1: Map showing the location of the sampling stations; Klang (03°00.1'N, 101°23.4'E) and Port Dickson (02°29.5'N, 101°50.3'E).

2.3 Total suspended solids (TSS)

In the laboratory, 500 ml of seawater sample (V) was measured and filtered through a pre-combusted (500°C for 3 hours) and pre-weighed (W1) Whatman GF/F filter. The filtrate was kept frozen (−20°C) for nutrient analysis whereas the filter paper was dried at 50°C until a constant reading (W2). TSS was measured as the weight increase in a GF/F filter by using the following equation:

$$TSS\ (mg\ L^{-1}) = (W1 - W2)/V$$

2.4 Chlorophyll *a* concentration

500 ml of seawater sample was filtered through a pre-combusted (500°C for 3 hours) Whatman GF/F filter, and the filter was kept frozen (−20°C) until analysis. GF/F filter for chlorophyll *a* (Chl *a*) was extracted overnight with 90% ice-cold acetone at −20°C. The absorbance of the chlorophyll pigments were measured at 750, 665, 664, 647, 630 510, and 480 nm (using the trichromatic method) with a spectrophotometer (Beckman, DU®7500i, USA). One drop of 1 N HCl was added for phaeopigment correction, and Chl *a* concentration was then calculated according to Parsons *et al*. (1984).

2.5 Nutrient analysis

Dissolved inorganic nutrients (ammonium [NH_4], nitrite [NO_2], nitrate [NO_3], phosphate [PO_4], and silicate [SiO_4]) were measured in triplicates according to Parsons *et al*. (1984). Ammonium was measured via phenate colorimetry where alkaline citrate medium and sodium hypochlorite reacted with ammonium to form a blue indophenol colour which was further intensified with sodium nitroprusside. Absorbance was measured at 640 nm with a spectrophotometer (Hitachi, U-1900, Japan). Nitrite was measured via reaction with sulfanilamide in an acid solution to form diazo compound that was reacted with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly coloured azo dye. Absorbance was measured spectrophotometrically at 543 nm. For nitrate measurement, nitrate was reduced by a column containing cadmium fillings coated with metallic copper, before being measured as nitrite. The increase in nitrite was assumed to be proportional with nitrate concentration.

Phosphate was allowed to react with a composite reagent containing molybdic acid, ascorbic acid and trivalent antimony. The resulting complex was reduced to form phosphomolybdenum blue where the absorbance was measured at 880 nm. Silicate was measured via reaction with molybdate under conditions, resulting in the formation of silicomolybdate, phosphomolybdate and arsenomolybdate complexes. A reducing solution, containing metol and oxalic acid, was then added to reduce the silicomolybdate complexes to give a blue colour solution whereas phosphomolybdate or arsenomolybdate was decomposed simultaneously. Absorbance was measured at 810 nm.

2.6 Bacterial abundance

Bacterial abundance was determined by epifluorescence microscopy on samples filtered onto a black polycarbonate filter (0.2 μm pore size, Millipore, USA), and then stained with 4'6-diamidino-2-phenylindole (DAPI, 1 μg mL^{-1} final concentration) for 7 minutes in the dark (Kepner and Pratt 1994). Slides were kept frozen for not more than three days before enumeration. To enumerate the stained bacteria, the slides were viewed under epifluorescence microscope (Olympus BX60, Japan) with a U-MWU filter cassette (excitor 330-385 nm, dichroic mirror 400 nm, barrier 420 nm) and a minimum of 10 microscopic fields or 300 cells were counted. Each field was also viewed under the U-MWG filter cassette (excitor 510-550 nm, dichroic mirror 570 nm, barrier 590 nm) to exclude the autofluorescing picoplankton. Cell counting was performed using a digital imaging system (analySIS version 3.2, Soft Imaging System, Münster, Germany).

2.7 Bacterial viability

Due to logistic constraint, only six occasions (January - June 2010) were carried out bacterial viable counts and were measured at each station by the LIVE/DEAD® *Bac*Light Bacterial Viability Kits (L13152, Molecular Probes, Eugene, OR). The kits can quantitatively distinguish live and dead bacteria in minutes. In the Bacterial Viability Kits, mixtures of SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide are utilized. When used alone, the SYTO 9 stain generally labels all bacteria with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and propidium

iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide.

According to manufacturer's instruction (Molecular Probes, 1997), samples were filtered onto a black polycarbonate filter (0.2 μm pore size, Millipore, USA), and then stained for 15 minutes in the dark with 2X stock solution containing SYTO 9 stain (6 μM final concentration) and propidium iodide (30 μM final concentration). Slides were kept frozen for not more than 3 days before enumeration. A minimum of 10 fields or 300 cells were counted for each sample using an epifluorescence microscope (Olympus, BX60F-3, Tokyo) with a U-MWB filter cassette (excitor 460-490 nm, dichroic mirror 500 nm, barrier 520 nm) for live cells (fluorescent green), and a U−MWG filter cassette (excitor 510-550 nm, dichroic mirror 570 nm, barrier 590 nm) for dead cells (fluorescent red). Cell counting was performed using a digital imaging system (analySIS version 3.2, Soft Imaging System, Münster, Germany).

2.8 Differential filtration

Unfiltered seawater was directly filtered through 0.2 μm polycarbonate filters to retain total bacteria for enumeration with epifluorescence microscopy. Seawater sample was also filtered through 1.0 μm polycarbonate filter (Whatman, USA) to separate the free-living from the attached bacteria. The filtrate was then filtered onto 0.2 μm polycarbonate filters to retain free-living bacteria for enumeration with epifluorescence microscopy. The difference between total bacterial counts and free-living bacteria was the attached bacterial abundance. Similarly to determine the 16S rRNA PCR-DGGE based bacterial community structure, 1.0 μm polycarbonate filter was used to retain

particle-attached bacteria for DNA extraction. The filtrate was then filtered onto 0.2 μm polycarbonate filters to retain free-living bacteria for DNA extraction. Unfiltered seawater was also directly filtered through 0.2 μm polycarbonate filters to retain total bacteria for DNA extraction. All these filters were kept frozen (−20°C) before use. Only eight samples (October 2009 - June 2010) at each site were collected for the 16S rRNA PCR-DGGE analysis due to logistic constraint.

2.9 DNA extraction

DNA extraction was performed for total, attached and free-living bacterial community. Both 1.0 μm polycarbonate filter and 0.2 μm polycarbonate filters were extracted according to Bostrom *et al*. (2004). The filters were cut into small pieces and transferred into microcentrifuge tubes. Cell lysis was carried out with adding 525 μL lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl pH 9.0) and 11 μL lysozyme (1 mg mL^{-1}, final concentration, Sigma-Aldrich) into the filtered samples. The samples were subsequently incubated at 37°C for 30 minutes. Then 60 μL sodium dodecyl sulfate (SDS) (1%, final concentration, Sigma-Aldrich) and 3 μL proteinase K (100 μg mL^{-1}, final concentration, Biolabs) were added, and the samples were incubated at 55°C for 12 hours. The filter was removed and washed with 500 μL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). All the lysate was pooled together. Fifty milliliters Baker's yeast tRNA (50 μg per sample, Roche) was used as coprecipitant. DNA was precipitated with 1/10 volume of 3 M NaAc, pH 5.2, and 0.6 volume isopropanol, and was frozen at -20°C for 1 hour. The pellet was washed with 70% ethanol, dried on a clean Schott towel, dissolved in TE, and kept at -20°C.

2.10 Amplification of the 16S rDNA

Extracted DNA was purified with phenol-chloroform before it was used as a template in PCR amplification. Partial 16S rDNA genes were amplified using the primer pair 341F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') (Winter *et al*. 2007). A 40-bp-long GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') was attached to the 5' end of primer 341F to obtain PCR fragments suitable for DGGE analysis. Basically, for a total of 50 µL reaction mixture, 5 µL of 10x *Taq* buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 1% Trinton X-100; Finnzymes), 2 µL of 50 mM $MgCl_2$ (2 mM final concentration; Finnzymes), 1.25 µL of a 10 mM deoxynucleoside triphosphate mix (final concentration 250 µM each; catalog no. F-560L, Finnzymes), 0.5 µL of 10 µM primers 341F and 907R (0.1 µM final concentration for each; AITBIOTECH), 0.625 µL of 2 U μL^{-1} *Taq* polymerase (catalog no. F-503L, Finnzymes), and 5 µl of DNA templates were used. PCR conditions were performed with an initial denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, elongation at 72°C for 1 min, and a final elongation step at 72°C for 30 minutes. The PCR was performed with thermal cycler (Applied Biosystems, 2720, Singapore). PCR fragments were cleaned and concentrated using a QIAquick PCR purification kit (QIAGEN, Germany) according to the manufacturer's instructions. Standard agarose gel electrophoresis was used to size and quantify the PCR fragments.

2.11 Denaturing gradient gel electrophoresis and sequencing

DGGE was performed for total, attached and free-living bacterial community. PCR amplicons were resolved via DGGE on a 8% (w/v) polyacrylamide gel containing a 25% to 65% denaturant gradient, where 100% was defined as 7 M urea with 40% (v/v) formamide. Electrophoresis was performed at 80 V and 60°C for 12 hours in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) using a DCode system (Bio-Rad, Hercules, USA). After electrophoresis, the gel was stained with SYBR Gold (1:10,000 dilution of stock solution, Molecular Probes, USA) for 30 minutes before observation under Geliance 200 Imaging System (Perkin-Elmer, USA). Selected visible bands were excised from the DGGE gel. The gel slices were incubated in 100 µL of TE buffer for at least 12 hours at 37°C to extract the PCR fragments. Extracted PCR fragments were reamplified using the corresponding primers without GC-clamps under identical PCR conditions as described above. Reamplified PCR products were purified and sent for sequencing at the AITBIOTECH, Singapore. The nucleotide sequences were submitted to both the BLASTn search program (Altschul *et al*. 1990) of the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project II (RDP-II) (Cole *et al*. 2005) for identification of the closest related bacteria. Sequences from this study were deposited in GenBank under the accession codes: JX193773−JX193793.

2.12 Statistical analyses

All data unless noted otherwise, were reported as mean ± standard deviation (SD). Student's *t*-test was used to test for differences between the two stations whereas correlation analysis was used to determine possible relationships among the variables measured. In order to compare the bacterial community structure, DGGE bands at the

same vertical position were assumed as one unique operational taxonomic unit (OTU). The DGGE banding patterns were transformed into presence / absence matrix data for cluster analysis and distance between each DGGE profiles was calculated using Morisita's coefficient index. All the statistical tests were carried out with PAST (Hammer *et al*. 2001).

CHAPTER 3: RESULTS

3.1 Environmental characteristics

Average surface seawater temperature was around 30°C at both Klang and Port Dickson (Table 3.1). Total suspended solids (TSS) was significantly higher at Klang than at Port Dickson (Student's *t*-test: $t = 1.76$, $df = 30$, $P < 0.05$) whereas dissolved oxygen (DO) concentration was significantly lower at Klang (Student's *t*-test: $t = 8.62$, $df = 31$, $P < 0.001$). The concentration of dissolved inorganic nutrients (NH_4, NO_2, NO_3, PO_4 and SiO_4) was higher at Klang than at Port Dickson (Student's *t*-test: $t > 2.35$, $df > 17$, $P < 0.05$), except for PO_4 which was around 1.11 ± 0.67 and 0.80 ± 0.82 µM at Klang and Port Dickson, respectively. Chl *a* concentration which is commonly used as a proxy for phytoplankton biomass, fluctuated over a wide range at Klang (CV > 40%) relative to Port Dickson (CV < 28%). At Klang, Chl *a* concentration ranged from 1.93 to 6.85 µg L^{-1}, and peaked in Oct 2009, Apr 2010, Sep 2010 and Feb 2011. In contrast, Chl *a* concentration at Port Dickson was lower (Student's *t*-test: $t = 2.53$, $df = 25$, $P < 0.01$), and consistently < 4 µg L^{-1} (Figure 3.1). Similar to Chl *a* concentration, total bacterial abundance was higher at Klang (Student's *t*-test: $t = 4.87$, $df = 19$, $P < 0.001$) (Figure 3.2). Total bacterial abundance varied within a narrow range at Port Dickson whereas total bacterial abundance at Klang fluctuated over a wider range, and peaked concurrently with Chl *a* concentration. Of the total bacteria, 25 ± 13% and 44 ± 22% were free-living bacteria at Klang and Port Dickson, respectively. With the LIVE/DEAD BacLight Bacterial Viability Kit, a range of 5 – 39% of total bacterial abundance at Klang and Port Dickson were viable (Figure 3.3). The percentage of viable cells at Port Dickson was 24 ± 13%, and slightly higher than Klang (16 ± 9%). Similarly, the percentage of viable attached bacteria was higher at Port Dickson (16 ± 14%) than Klang (10 ± 9%). In contrast, the percentage of viable free-living bacteria

over total bacterial abundance at both Klang and Port Dickson were 6 ± 4% and 8 ± 4%, respectively.

Table 3.1: Physico-chemical characteristics of the sampling stations in this study. Values are means (± SD) of surface water temperature, DO, TSS, ammonium (NH_4), nitrite (NO_2), nitrate (NO_3), phosphate (PO_4) and silicate (SiO_4), Chl *a* and bacterial abundance. Student's *t*-test was used to compare the two stations, and * is $P < 0.05$, ** is $P < 0.01$, *** is $P < 0.001$.

Station	Klang (n = 18)	Port Dickson (n = 18)
Temperature (°C)	29.9 ± 0.7	29.7 ± 1.1
TSS (mg L^{-1}) *	59 ± 16	50 ± 11
DO (µM)***	144 ± 26	209 ± 19
NH_4 (µM) *	22.87 ± 38.54	1.51 ± 1.01
NO_2 (µM) ***	2.82 ± 1.55	0.12 ± 0.11
NO_3 (µM) ***	6.29 ± 2.88	1.38 ± 1.02
PO_4 (µM)	1.11 ± 0.67	0.80 ± 0.82
SiO_4 (µM) ***	26.57 ± 17.78	9.00 ± 5.05
Chl a (µg L^{-1}) **	3.58 ± 1.44	2.62 ± 0.71
Bacterial abundance (× 10^6 cells mL^{-1}) ***	2.7 ± 1.0	1.4 ± 0.3

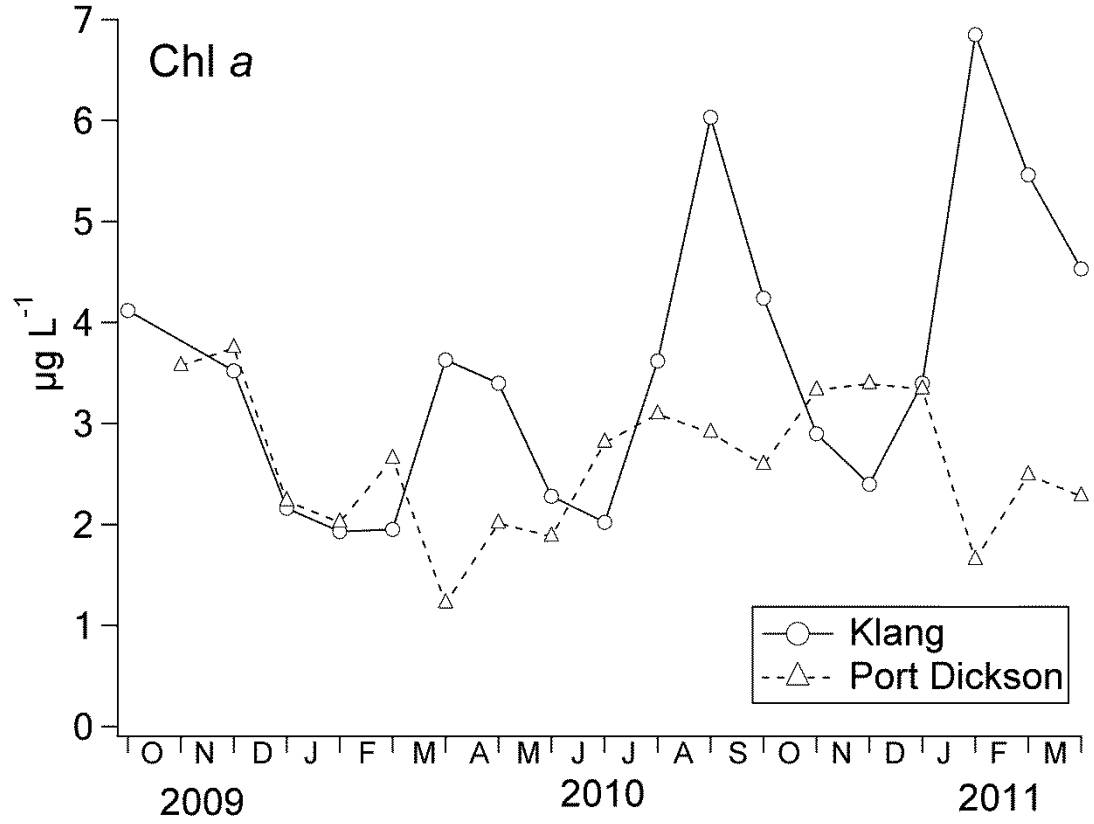

Figure 3.1: Temporal variation of Chl *a* at Klang and Port Dickson.

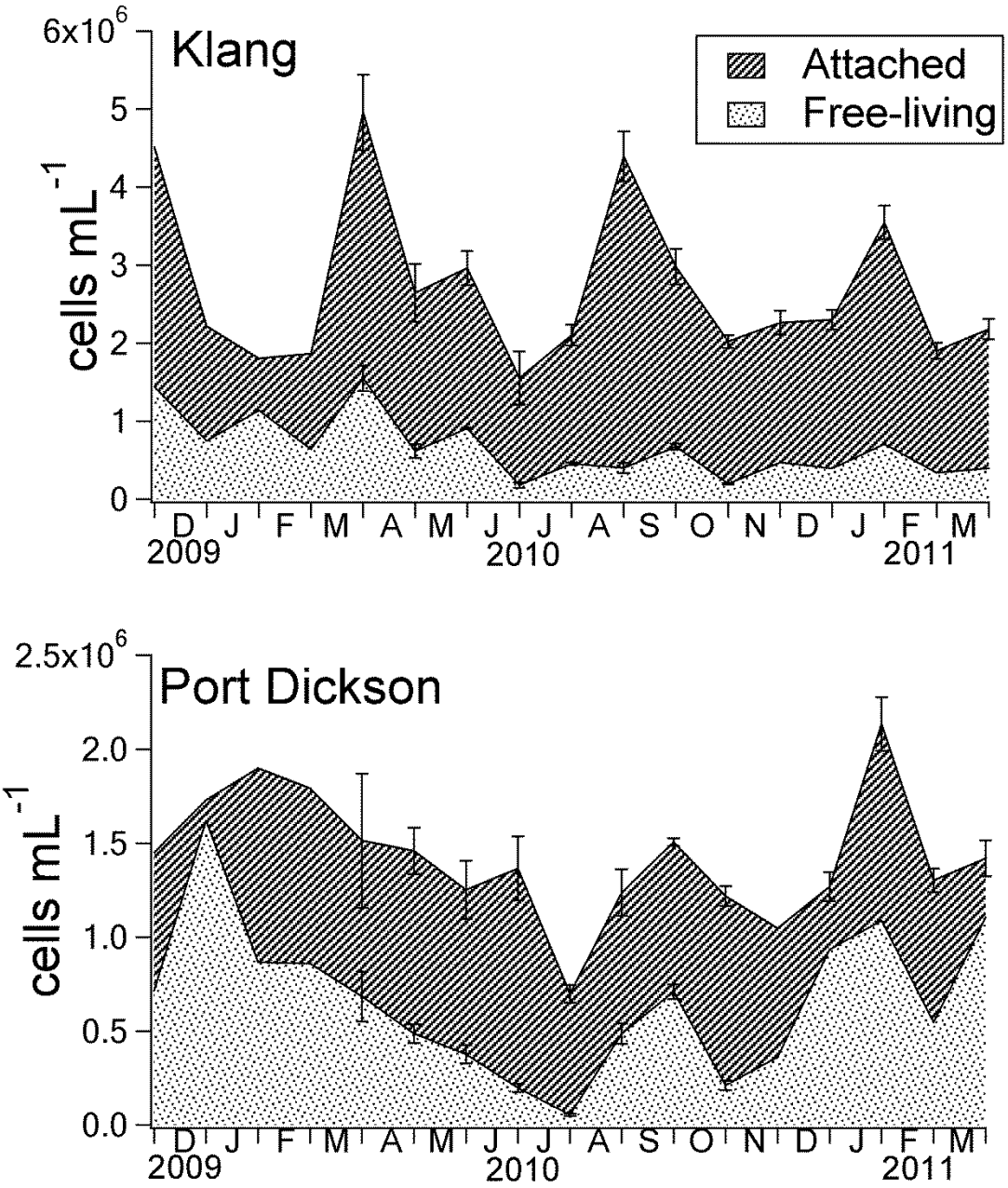

Figure 3.2: Temporal variation of attached and free-living bacterial abundance at Klang and Port Dickson. Measurements were replicated from March 2010 onwards, and error bars (± SD) are shown except when values are smaller than the symbol.

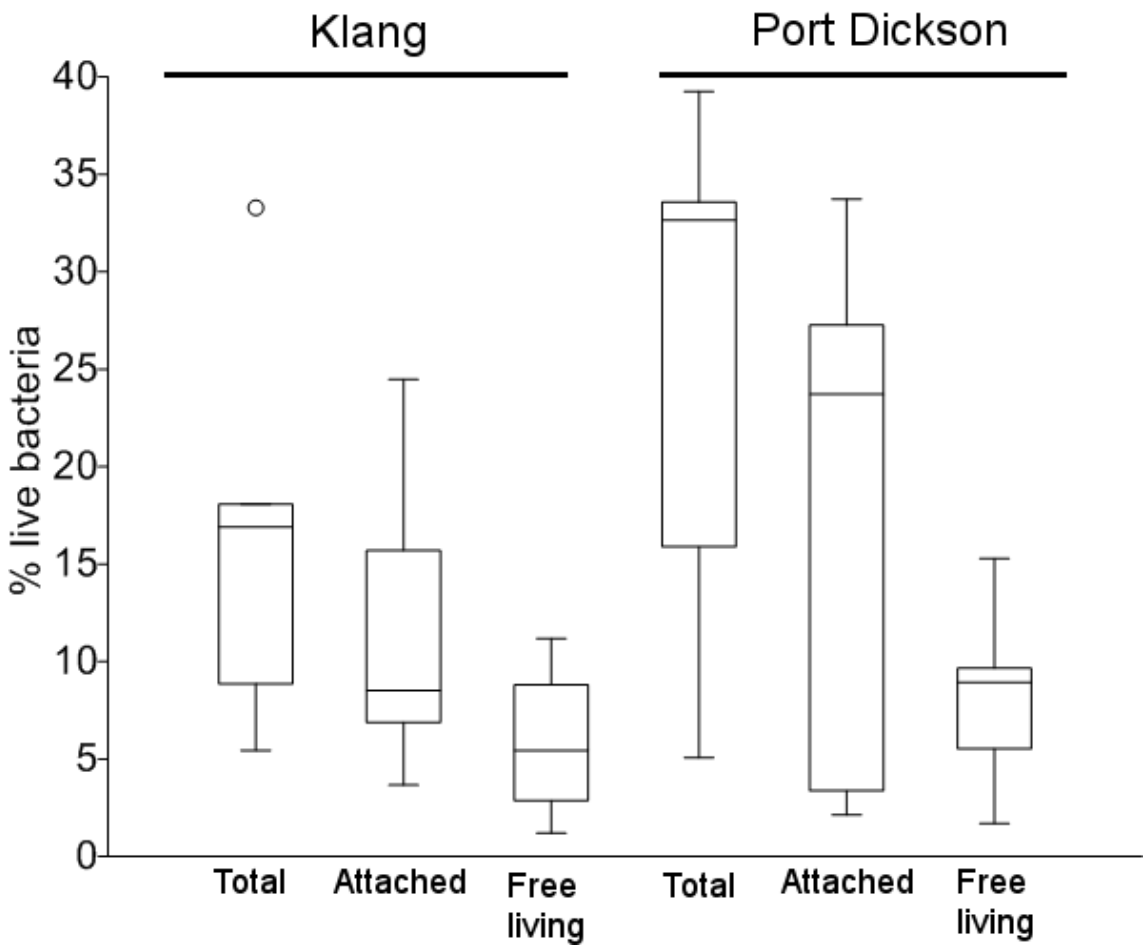

Figure 3.3: Box-and-whisker plots showing the range and the median of live bacteria (%) measured in the different fractions. Outliers are also shown as open circles.

3.2 16S rRNA PCR-DGGE based bacterial community structure

DGGE analysis of partial 16S rDNA was used to investigate total, attached and free-living bacterial community structure at both Klang and Port Dickson (Figure 3.4). A total of twenty operational taxonomic units (OTUs) or phylotypes were identified based on the migration pattern. The number of OTUs per lane ranged from four to eleven. Among the 20 OTUs, seven OTUs were common for both Klang and Port Dickson whereas eight OTUs appeared only in Klang and another five OTUs appeared only in Port Dickson. In order to determine the phylogenetic affiliation of bacterial community at Klang and Port Dickson, we excised distinct DGGE bands of 12 different OTUs for nucleotide sequencing. We were however not successful in amplifying other distinct OTUs. Eleven OTUs were > 96% identical to sequences in the NCBI and RDP-II databases (Table 3.2) whereas OTU2 showed 88% similarity. We replicated one DGGE band (band 8) five times and from the sequencing results, confirmed that matching bands corresponded to an identical genotype. Bands from different stations but with similar migration patterns were also replicated (i.e. bands 10 and 12), and sequencing results also showed identical genotypes. Therefore DGGE bands at the same vertical position could be assumed as one unique OTU.

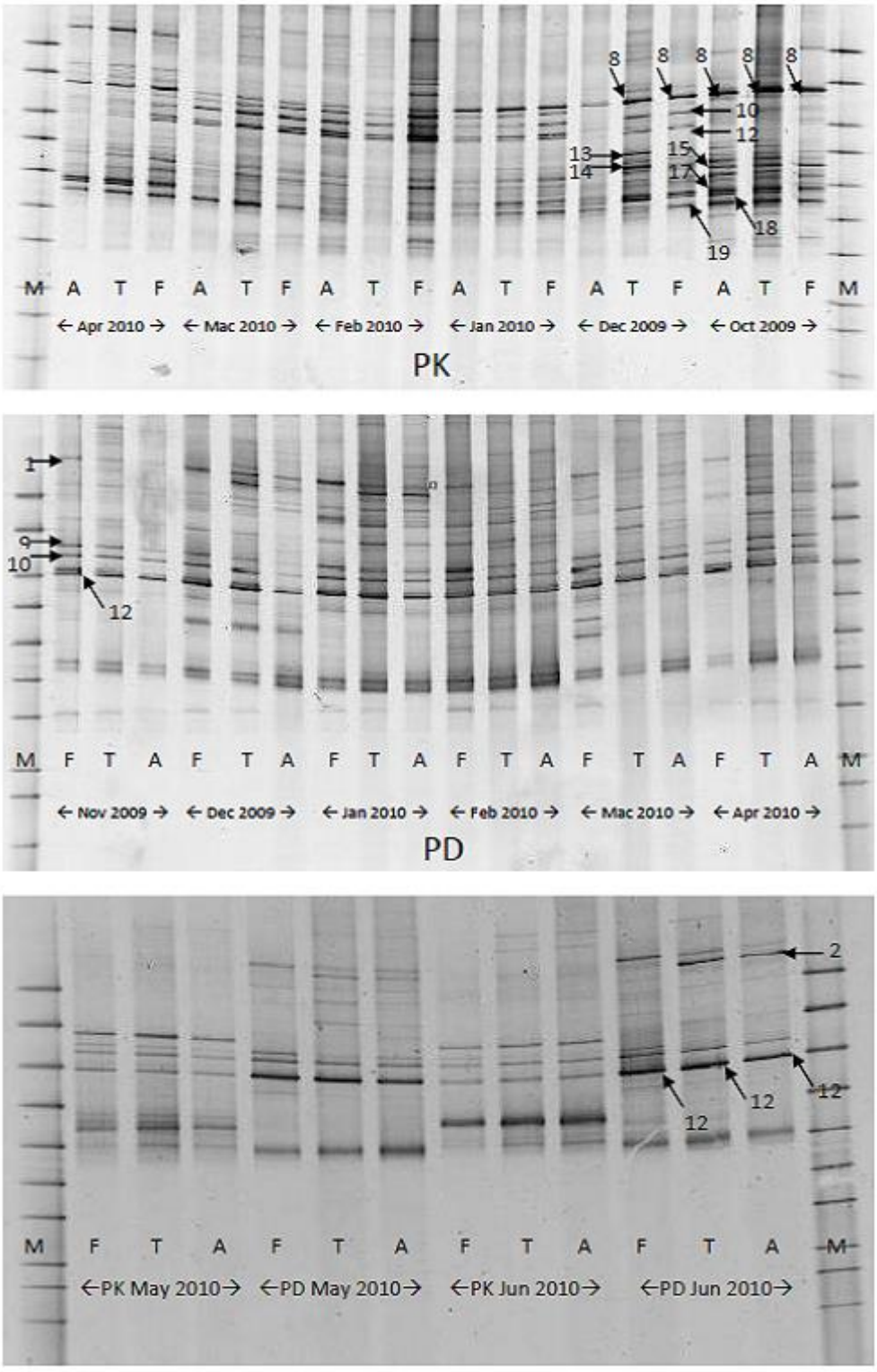

Figure 3.4: 16S rRNA PCR-DGGE profile of total (T), attached (A) and free-living (F) bacteria at Klang (PK) and Port Dickson (PD) from October 2009 to June 2010. Lanes M indicate marker whereas the numbers 1-19 indicate the excised bands from which sequences were determined.

Table 3.2: Phylogenetic affiliation and similarity to the closest relative of amplified 16S rRNA gene sequences excised from numbered bands shown in Figure 3.4. [a] Common OTUs for Klang and Port Dickson samples; [b] OTUs that only appeared in Klang samples; [c] OTUs that only appeared in Port Dickson samples; [d] Unique OTUs for attached bacteria; [e] Location from which sequence was retrieved either Klang or Port Dickson or both stations.

Band number	GenBank accession number	Location[e]	Closest match in Genebank database	Phylogenetic affiliation	Nucleotide similarity
1[a]	JX193773	Port Dickson	Uncultured *Flavobacterium* sp. clone ARTE1_211 (GU230407)	Cytophaga-Flavobacteria-Bacteroides	99%
2[c,d]	JX193774	Port Dickson	Uncultured cyanobacterium clone SGSO654 (GQ347890)	Cyanobacteria	88%
8[b]	JX193778	Klang	Beta proteobacterium TEG21 gene (AB607291)	β-Proteobacteria	99%
9[a]	JX193780	Port Dickson	Uncultured bacterium clone S23_389 (EF572290)	Actinobacteria	98%
10[a]	JX193782	Both	Uncultured *Marinovum* sp. clone SHWH_night1_16S_703 (FJ744909)	α-Proteobacteria	99%

Table 3.2, continued.

Band number	GenBank accession number	Location[e]	Closest match in Genebank database	Phylogenetic affiliation	Nucleotide similarity
12[a]	JX193786	Both	Uncultured *Roseobacter* sp. DGGE band DI-12 (AY919600)	α-Proteobacteria	100%
13[b]	JX193788	Klang	Uncultured delta bacterium clone SHWH_night1_16S_590 (FJ744834)	δ-Proteobacteria	98%
14[a,d]	JX193789	Klang	Uncultured delta bacterium clone SHWH_night1_16S_590 (FJ744834)	δ-Proteobacteria	97%
15[b]	JX193790	Klang	*Comamonas* sp. JL4 (JF740040)	β-Proteobacteria	96%
17[b]	JX193791	Klang	Uncultured alpha proteobacterium clone CD205F06 (DQ200551)	α-Proteobacteria	98%
18[b]	JX193792	Klang	Uncultured *Pelomonas* sp. clone MJ26 (GU212806)	β-Proteobacteria	99%
19[a]	JX193793	Klang	*Donghicola xiamenensis* strain Y-2 (DQ120728)	α-Proteobacteria	99%

The OTUs (*n*, %) identified in this study belonged to the following six classes: α-proteobacteria (4, 33.3%); β-proteobacteria (3, 25%); δ-proteobacteria (2, 16%); cytophaga-flavobacterium-bacteriodetes (CFB) group bacteria (1, 8.3%); cyanobacteria (1, 8.3%); and actinobacteria (1, 8.3%). Among the common OTUs present at both Klang and Port Dickson, α-proteobacteria was the most abundant (OTU10, 12 and 19). The closest relative of OTU19 is a novel marine bacterium *Donghicola xiamenesis* whereas the other two were *Roseobacter* sp. (OTU12) and *Marinovum* sp. (OTU10). The other common OTUs for the two stations were from the CFB group (OTU1), δ-proteobacteria (OTU14) and actinobacteria (OTU9). OTUs unique to Klang were from β-proteobacteria (OTU8, OTU15, OTU18), δ-proteobacteria (OTU13) and α-proteobacteria (OTU17). In contrast, only OTU2 (cyanobacteria) was unique to Port Dickson. There were also two OTUs (OTU2 and OTU14) that were found exclusively in the DNA from attached bacteria.

A binary matrix was constructed based on OTU presence and absence to visualize the relationships of the different DGGE profiles obtained. Using Morisita's index of similarity, cluster analysis was performed and visualized in a dendrogram. Cluster analysis showed that the DGGE profiles at Klang and Port Dickson were placed in different groups at a low similarity index of 0.36 (Figure 3.5). All the DGGE profiles at Klang were grouped at a similarity level of 0.48 whereas at Port Dickson, branching occurred at a similarity level of 0.64. With the exception of the Nov 2009 DGGE profile, all the DGGE profiles at Port Dickson were grouped at > 0.85 similarity index. In fact, DGGE profiles at Port Dickson were the same in Jan, Feb and Apr 2010. In contrast, most of the DGGE profiles at Klang were grouped at similarity indices < 0.80. The only exception being the DGGE profiles in May and Jun 2010 (similarity index > 0.88). When the DGGE profiles from total, attached and free-living bacteria were compared, most had the same bands but at different intensity.

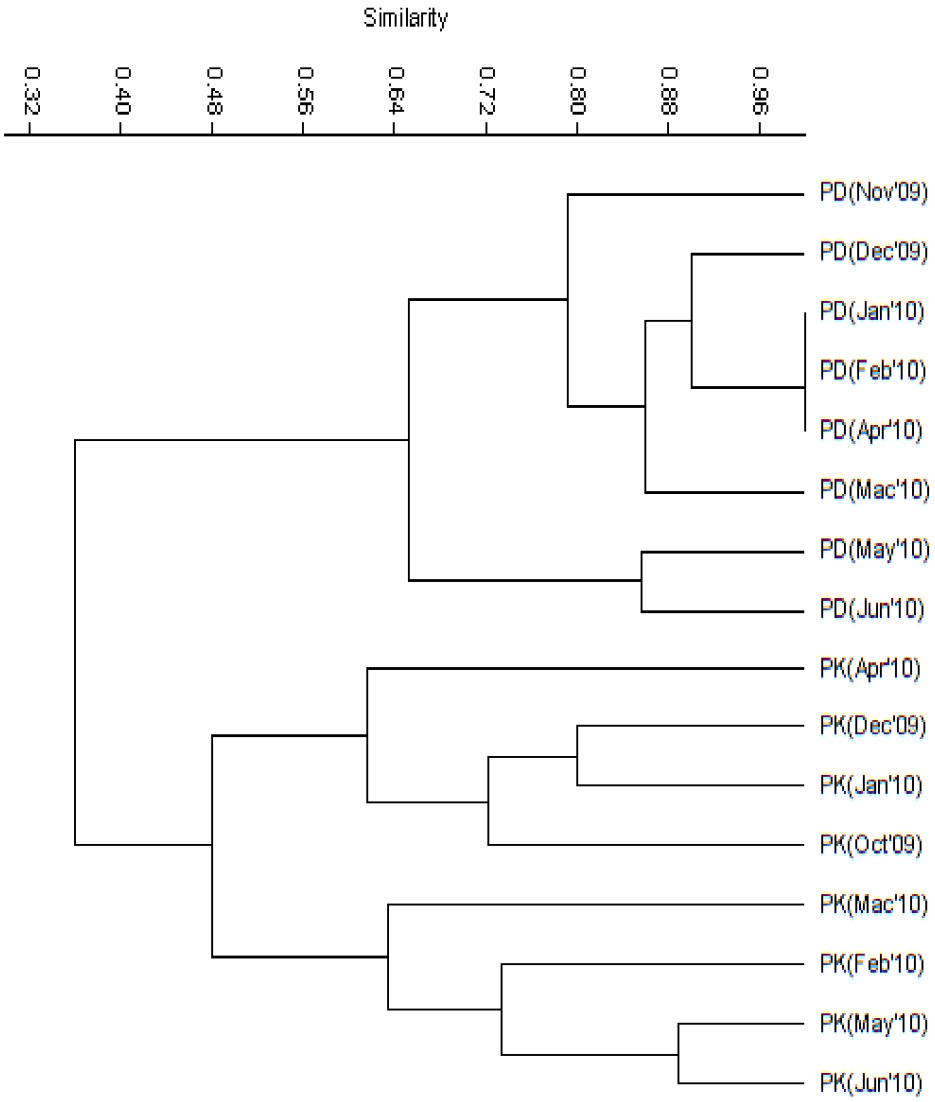

Figure 3.5: Cluster analysis of the 16S rRNA PCR-DGGE profile from the total bacterial community at both stations using the Morisita's index of similarity.

CHAPTER 4: DISCUSSION

4.1 Environmental characteristics

Surface seawater temperature observed in this study was similar at both stations, and typical of tropical waters (Lee *et al*. 2009). In contrast, DO levels were lower and TSS concentrations were higher at Klang. All other dissolved inorganic nutrient concentrations measured were also higher at Klang, with the exception of PO_4. Our results clearly indicated the high level of eutrophication at Klang, which is attributed to land clearing activities and rapid development taking place upstream (Lee and Bong 2006). Our results also showed wide fluctuations of Chl *a* concentration in Klang. In an estuary, episodic nutrient input via river flow is a key factor for the stimulation of phytoplankton production, and increase in Chl *a* concentration (Lee and Bong 2008). On the contrary, Chl *a* concentration at Port Dickson was generally low and stable, and within the range of previously recorded values (Lee and Bong 2008). The higher Chl *a* concentration in Klang reflected a higher phytoplankton biomass. We know that primary productivity and dissolved organic matter concentration are higher at Klang than Port Dickson (Lee and Bong 2008; Lee *et al*. 2009). As bacteria and phytoplankton are coupled in these coastal waters (Lee and Bong 2008), the higher concentrations of primary producers and dissolved organic matter are probable reasons for the higher bacterial abundance in Klang.

At Klang, attached bacteria formed a large fraction of total bacteria (75 ± 13%). This is typical of eutrophic waters where attached bacteria may account for > 60% of total bacterial abundance (Bano *et al*. 1997; Crump *et al*. 1998; Garneau *et al*. 2009). The habitat for attached bacteria was more likely linked to Chl *a* (i.e. phytoplankton) ($R^2 = 0.277$, $df = 16$, $p < 0.05$) than TSS. Although both Chl *a* and TSS were collected on filters of similar pore-size, correlation analysis showed that both these variables were

independent of each other ($p > 0.10$). The preference for Chl *a* as opposed to TSS was probably due to the fact that the TSS in both Klang and Port Dickson are nutritionally poor with < 6% organic matter content (Bong and Lee 2008). Our observation is especially relevant given the high sediment load in the coastal waters of South-East Asia, and reaffirms that the contribution of attached bacteria depends on the source, quality and age of particles (Hollibaugh *et al*. 2000; Kernegger *et al*. 2009).

At Klang, both attached and free-living bacteria increased as total bacterial abundance increase (Figure 4.1). Changes in attached bacterial abundance accounted for about 76% variation in total bacterial abundance whereas changes in free-living bacteria accounted for only 34%. Therefore at Klang, the contribution of attached bacteria towards the dynamics of total bacterial abundance was important. In contrast, our results suggested that free-living bacteria were more important towards the dynamics of total bacterial abundance at Port Dickson. Only free-living bacteria correlated with total bacterial abundance at Port Dickson, and changes in free-living bacterial abundance accounted for about 68% variation in total bacterial abundance. The differences in the proportion of attached and free-living bacteria observed between eutrophic and oligotrophic sites show the need to separate the contribution of attached and free-living bacteria when investigating the dynamics of bacterial distribution. This is especially true for large ecosystem studies encompassing waters of different trophic states.

In this study, we also assessed the viability of the bacterial cells in both attached and free-living fractions. At both stations, we observed that there were more viable cells in the attached than free-living fractions, and this concurs with the generally accepted idea that attached bacteria are usually more active (Lapoussière *et al*. 2011). As free-living bacteria in Port Dickson accounted for most of the variation in total bacterial abundance, we presumed that more of the free-living bacteria in Port Dickson were viable. However, there was no statistically significant difference in the percentage of

viable bacteria in total, attached and free-living fractions between both Klang and Port Dickson. One possible reason for the unexpected results from the LIVE/DEAD BacLight kit could be due to the presence of intermediate states that is relative to the degree of cytoplasmic membrane damage (Berney *et al*. 2007). Another is the differential permeability of intact membranes of different bacterial strains (Grégori *et al*. 2001). Further investigation employing a different method for example using staining/destaining procedure (Manini and Danovaro 2006) or identification of visible nucleoid region (Zweifel and Hagström 1995) should be carried out for the resolution of viable, intermediate and non-viable bacteria in total, attached and free-living fractions.

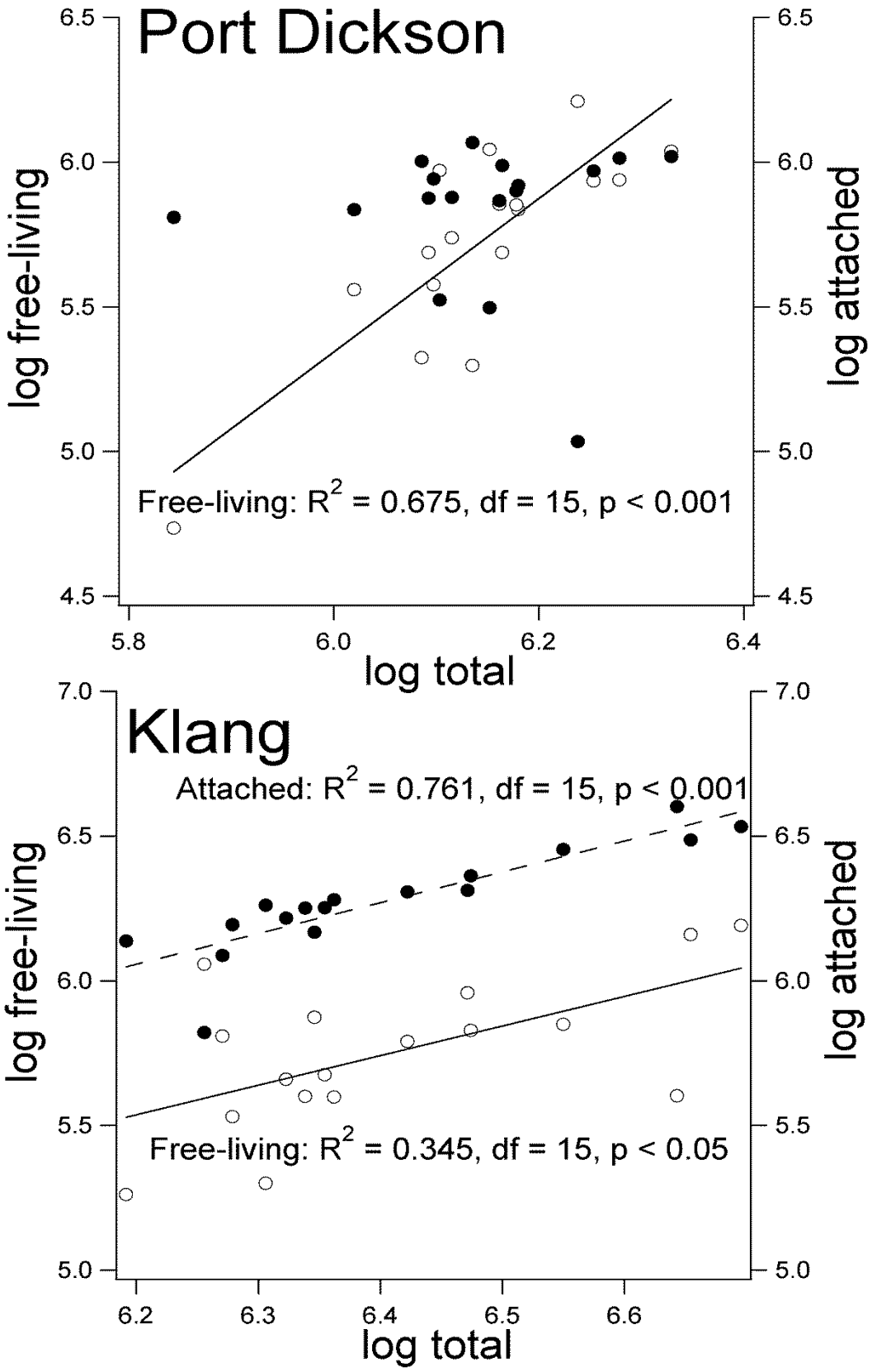

Figure 4.1: Correlation analyses between free-living bacteria (left y-axis, open symbol), attached bacteria (right y-axis, closed symbol) and total bacterial abundance for both Port Dickson and Klang. Linear regression slope is also shown.

4.2 16S rRNA PCR-DGGE based bacterial community structure

In this study, the DGGE method was adopted. However DGGE profile is not exhaustive enough to indicate bacterial community, and detects only changes in the dominant bacterial phylotypes (Suzuki and Giovannoni 1996; Muyzer *et al.* 1998). In the environment, it is the predominant phylotype that effects the most (Bouvier and del Giorgio 2002). Thus DGGE based technique is still a relevant and useful technique for preliminary screening and microbial profiling of predominant species (Lalande *et al*. 2013). Multiple samples can be analysed concurrently to monitor changes and patterns in microbial populations (Muyzer *et al.* 1998). Thus the bacterial community structure presented in this study was likely a profile of the predominant bacterial species. For an in-depth characterisation of the bacterial community structure, a next generation sequencing platform such as pyrosequencing should be employed (Matcher *et al.* 2011).

Proteobacteria were the predominant bacteria identified (75%) in this study where the most prevalent class was α-proteobacteria, followed by β-proteobacteria and δ-proteobacteria. Generally, marine systems are dominated by α-proteobacteria, whereas β-proteobacteria are often the dominant group in freshwater systems (Glöckner *et al.*, 1999). This would explain why β-proteobacteria was only detected at the estuarine Klang where there is riverine input. Other sequences belonged to the δ-proteobacteria, CFB group, cyanobacteria and actinobacteria, and were closely related to the bacterial diversity of coastal and estuarine systems (Crump *et al*. 1999; Bouvier and del Giorgio 2002; Zhang *et al*. 2007; Ortega-Retuerta *et al.* 2013). We also found one phylotype (OTU2) with only 88% sequence similarity to available database, possibly representing a novel bacterial lineage.

From the cluster analysis of a binary matrix based on OTU presence-absence, bacterial community structure at Klang and Port Dickson were distinctly different (similarity index = 0.36), suggesting spatial variation in the bacterial community

structure between these two stations. Several studies have shown that bacterial community structure correlates with environmental parameters including temperature (Sakami 2008), salinity (Crump *et al.* 1999), Chl *a* (Ortega-Retuerta *et al.* 2013), suspended solid, turbidity, pH and nitrogen (Zhang *et al.* 2007). As some these environmental variables (e.g. TSS, NH_4, NO_3, Chl *a*) were significantly higher at Klang than Port Dickson, the differences in these environmental variables could explain the spatial variation in bacterial community structure observed at both stations. Within each station, we observed that the bacterial community structure at Klang was more variable (grouped at a similarity index of 0.48) than Port Dickson (similarity index = 0.65). As Klang is an estuary, the variability of the bacterial community is probably a result of episodic inputs from both riverine freshwater and seawater.

In this study, we found similar phylotypes in the bacterial communities of attached and free-living fractions at both Klang and Port Dickson. Other than two OTUs (uncultured cyanobacterium and delta bacterium), the other OTUs were found in both attached and free-living modes. As it remains debatable whether particles host specific microbial communities, our findings seemed to suggest that there are no specific microbial communities attached to particles. It is more likely that marine bacteria experience either habitat shift between attached and free-living modes or that there is a co-occurrence of a phylotype in the different modes (Hollibaugh *et al*. 2000; Ghiglione *et al*. 2007; Ortega-Retuerta *et al*. 2013). Differences between attached and free-living bacteria are attributed to the composition of the particles especially where a high degree of specialization is required (Acinas *et al*. 1999; Crump *et al*. 1999; Ghiglione *et al*. 2009; Ortega-Retuerta *et al*. 2013). In this study, attached bacteria are associated with Chl *a.* As both bacterial biomass and activity are coupled to phytoplankton biomass and production (Lee and Bong 2008), there would not be any distinction between free-living and attached bacteria when both are relying on essentially the same substrate.

CHAPTER 5: CONCLUSION

In conclusion, we showed that attached bacteria formed a significant proportion of the bacterial population in eutrophic as opposed to oligotrophic ecosystems. Although the coastal waters at both Port Dickson and Klang have a relatively high suspended solids load, we showed that the attached bacteria have a preference for phytoplankton based particles. Our results implied that we should acknowledge the importance of attached bacteria in eutrophic water where they could play a major role in carbon and nutrient cycling. This is important as bacterial activity can cause net heterotrophy in tropical coastal waters (Lee *et al*. 2009). Finally, from the 16S rRNA PCR-DGGE results, we showed clear spatial differences in the bacterial community structure. However there was no difference phylogenetically between attached and free-living fractions.