BACTERIAL COMMUNITY AND DIVERSITY OF POST LARVAE WILD SHRIMPS ALONG MERBOK RIVER INFER USING METAGENOMIC APPROACH

SITI ZULIANA BINTI AHMAD

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BACTERIAL COMMUNITY AND DIVERSITY OF POST LARVAE WILD SHRIMPS ALONG MERBOK RIVER INFER USING METAGENOMIC APPROACH

by

SITI ZULIANA BINTI AHMAD

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LIST OF SYMBOLS & ABBREVIATIONS

°C  Degree Celsius
µL  Microlitre
CFU/g  Colony-forming unit per gram
mg/L  Milligram per litre
ppt  Parts per thousand
rpm  Rotation per minute
BHI  Brain heart infusion
BLAST  Basic Local Alignment Search Tool
CAMERA  Community Cyberinfrastructure for Advance Marine Microbial Ecology Research and Analysis
DO  Dissolved oxygen
DoE  Department of Environment
DoF  Department of Fisheries
EMP  Earth Microbiome Project
FAO  Food and Agriculture Organization
GenBank  Genetic sequence database
IMG/M  Integrated Microbial Genomes-Metagenomic
min  minute
MA  MacConkey agar
Meta-IDBA  Meta-Iterative De Bruijin graph \textit{de novo} short read assembler
MG-RAST  Metagenomic-Rapid Annotations using Subsystems Technology
NGS  Next generation sequencing
OTU  Operations taxonomic unit
SAL  Salinity
TVC  Total viable counts
TEMP  Temperature
TCBS  Thiosulfate-citrate-bile salts-sucrose
UNESCO  The United Nations Educational, Scientific and Cultural Organization
WD  Water depth
KOMUNITI DAN KEPELBAGAIAN BAKTERIA PADA UDANG DI PERINGKAT PASCA LARVA SEPAJANG SUNGAI MERBOK MENERUSI PENDEKATAN RUMUSAN METAGENOMIK

ABSTRAK

Kajian ini menerangkan penyelidikan terhadap komuniti bakteria dan kepelbagaian yang berkaitan dengan udang liar pada peringkat pasca larva di sepanjang Sungai Merbok yang kaya dengan paya bakau berdasarkan pendekatan metagenom. Terdapat kekurangan kajian yang dijalankan terhadap mikrobiom berkaitan dengan udang diperingkat pasca larva terutamanya dalam spesies liar. Selain itu, terdapat jurang pengetahuan di dalam hubungan diantara mikrobiom beserta hos terutamanya pasca larva udang dengan keadaan persekitaran. Matlamat kajian ini adalah bagi mengenalpasti kepelbagaian komuniti mikrob yang berkaitan dengan udang liar pada peringkat pasca larva dan untuk mengenalpasti faktor utama kelimpahan mikrobiom spesies pada pasca larva udang liar dan keseluruhan struktur komuniti mikrobiom. Objektif ini dicapai dengan menggunakan penjужukan generasi akan datang (NGS) dengan pendekatan metagenom amplikon gen 16S rRNA menggunakan platform Illumina Miseq. Ini disusuli dengan analisis bioinformatik menggunakan talian paip maklumat analisis penjужukan 16S rRNA. Kelimpahan relatif pada kepelbagaian bakteria di empat kawasan berbeza dibandingkan dengan menggunakan perisian PRIMER6 dan PERMANOVA+. Sampel diambil dari empat kawasan (stesen); St 1 hingga St 4 daripada habitat air tawar di bahagian hulu ke hiliran air payau menghala ke perairan laut. Beberapa parameter kualiti air telah diukur iaitu pH, saliniti, oksigen terlarut, kedalaman air, kekeruhan dan suhu permukaan. Udang
yang dijumpai dalam jumlah yang banyak menggambarkan parameter air berada pada kadar yang sesuai dalam menyokong kemandirian pasca larva udang. Data metagenom mengesan sebanyak 171, 722 unit taksonomi operasi (OTUs) bagi semua kawasan secara keseluruhan. Sebanyak 28 filum bakteria telah dikenalpasti pada mikrobiom udang yang didominasi oleh filum Proteobakteria, Actinobakteria, Firmicutes dan Bacteroidetes di semua empat kawasan. Sementara itu genus yang paling banyak adalah bakteria bukan patogen kepada udang misalnya *Streptomyces* spp., *Mesorhizobium* spp., *Rhizobium* spp., *Bacillus* spp. dan *Pseudomonas* spp.. Patogen bakteria *Vibrio* spp. yang boleh menyebabkan jangkitan pada udang hanya ditemui pada kawasan St 1 dan St 4 tetapi dalam kekerapan yang rendah. Kesimpulannya, pendekatan metagenomik jujukan amplikon 16S rRNA dapat mengenal pasti kelimpahan dan kepelbagaian bakteria yang berhubung dengan udang liar pada peringkat pasca larva di sepanjang Sungai Merbok. Walaupun analisis terhadap data metagenom yang kompleks sangat mencabar, ini dapat diatasi dengan pelbagai jenis saluran maklumat yang direka bagi memudahkan proses analisis. Merujuk kepada Sungai Merbok dan paya bakau sekitarnya, walaupun terdedah dengan pelbagai aktiviti manusia, ia masih mampu berfungsi sebagai tempat yang sesuai untuk udang bertelur dan membesar serta tidak mempengaruhi populasi udang liar pada peringkat pasca larva. Walaubagaimanapun, usaha pemuliharaan dan pemantauan terhadap populasi hidupan liar adalah diperlukan bagi mengatasi kehilangan kepelbagaian udang disebabkan oleh penyakit berjangkit dan memastikan sumber udang liar yang berdaya mapan.
ABSTRACT

The study describes the investigation of the bacterial community and diversity associated with post larvae wild shrimps along the mangrove-diverse Merbok River based on metagenomic approach. There are very few studies conducted on the microbiome associated with post larvae shrimps especially in wild species. Furthermore, there is knowledge gap of relationship between microbiome associated with host especially post larvae shrimps with environmental conditions. The goal of this study was identifying of microbial community diversity associated with the wild post larvae shrimps in Merbok Rivers and to determine key major abundance of the wild post larvae shrimp microbial species and overall microbial community structure. These objectives were addressed by using next generation sequencing of metagenomic 16S rRNA gene amplicon using the Illumina MiSeq platform. This was followed by the bioinformatics analysis using a 16S rRNA sequencing analysis pipeline. Relative abundance of bacterial diversity in the four different localities were compared by using a PRIMER6 and PERMANOVA+ softwares. Samples were obtained from four sites (stations); St 1 to St 4 from upstream freshwater habitat to downstream brackish water towards the marine waters. Water quality parameters were measured namely pH, salinity, dissolved oxygen, water depth, turbidity and surface temperature. Shrimps were found in abundance, a reflection that water parameters were in the range that were suitable to support post larvae shrimp survival. Metagenomic data detected a total
of 171, 722 operational taxonomic units (OTUs) for all sites. Twenty-eight bacterium phyla were detected, dominated by phyla Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes at all four sites. The most abundant genera were the non-pathogenic bacteria for shrimps as example *Streptomyces* spp., *Mesorhizobium* spp., *Rhizobium* spp., *Bacillus* spp. and *Pseudomonas* spp.. The pathogenic *Vibrio* spp. that can cause infection in shrimps was only found in St 1 and St 4 but in low frequencies. As a conclusion, metagenomics approach 16S rRNA amplicon sequencing is able to identify the abundance and diversity of bacteria associated with post larvae wild shrimps along the Merbok River. Although analysis of a complex metagenomics data is challenging, it can be addressed with various designed pipelines to simplify the process. As for the Merbok River and its mangrove surroundings, despite being exposed to various human activities, it still can serve as a good spawning and nursery sites of shrimps and did not affect post larvae wild shrimp population. However, conservation and surveillance efforts on the wildlife populations are required to avoid the loss of shrimp diversity due to disease infections and to ensure that wild shrimps sources are sustainable.
CHAPTER 1
INTRODUCTION

1.1 An overview of bacterial-host interaction in post larvae wild shrimp

Bacterial communities influence the environment and other organisms in more ways that we can imagine. It is present ubiquitously and important (in fact critical) in all terrestrial (Andam et al., 2016, Delgado-Baquerizo et al., 2016, Laforest-Lapointe et al., 2017) and aquatic ecosystems, in macro and microhabitats (Huber et al., 2007, Smith, 2007, Ladau et al., 2013, Dennis et al., 2019) under a myriad of environmental conditions. However, the different ecosystems often harbour specific bacterial diversity due to environmental and ecological factors. Bacteria can also be found associated with eukaryotic hosts such as in humans (Turnbaugh et al., 2007, Costello et al., 2009, He et al., 2015, Byrd et al., 2018, Forster et al., 2019), insects (Yun et al., 2014, Douglas, 2015, Lanan et al., 2016), fishes (Wu et al., 2012, Larsen et al., 2013, Tarnecki et al., 2017, Egerton et al., 2018), plants (Andreote, et al., 2009, Proenca et al., 2010, Hu et al., 2018) and other organisms.

Shrimps is also not excluded from interacting with bacteria (Liu et al., 2011, Dabadé et al., 2016, Xue et al., 2018). The association of the host shrimp with bacteria can lead to both positive and negative impacts. In positive ways, numerous bacteria species help to protect the host from pathogens. As examples, *Streptomyces* spp. and *Bacillus* spp. that are commonly naturally present in the host are important to strengthen the shrimp and fish immune systems. Together with another species, *Pseudomonas* spp., they are important to combat against *Vibrio* spp. (El-Rhman et al., 2009, Far et al., 2013). Thus, in many aquaculture systems, *Pseudomonas* spp., *Streptomyces* spp. and *Bacillus* spp., are systematically included to neutralise invasive
pathogens as probiotics. Other bacteria that are commonly used as probiotics in shrimp aquaculture are *Nitromonas* spp. and *Nitrobacter* spp. (Wang et al., 2005, Chi et al., 2017). Bacteria are also important as indicators to determine the health status of the hosts (Zhang et al., 2014, Xiong et al., 2014). High abundance of *Vibrio* spp. and *Photobacterium* spp. in the shrimp host can indicate an unhealthy shrimp, infected with diseases such as vibriosis (Vaseeharan et al., 2003, Rivas et al., 2013). On the other hand, some bacteria such as *Lactobacillus* spp. *Saccharomyces* spp. and *Streptococcus thermophilus* are important bacteria to the shrimp gut (Rengpipat et al., 1998, Merrifield et al., 2014). Their functions include nutrition absorption, digestion and metabolism besides combatting against pathogens. This reveals that bacteria diversity plays important roles to the shrimps to maintain the host health and body functions.

By contrast, other groups of bacteria can also cause mortality to the infected host and makes it worse by spreading the disease to other individuals or other organisms. Aquatic organisms like fishes, crustacean and gastropods are sensitive to environmental changes in their habitats (Cornejo-Granados et al., 2017, Kinnula et al., 2017, Mioduchowska et al., 2018, Alfiansah et al., 2018, Altaf et al., 2018). This is because in aquatic environments, spreading of bacteria is rapid and therefore infection from diseases (Olafsen, 2001, Cabral, 2010) can easily occur. Aquaculture and agriculture facilities, housing estates and other human activities can pose great risks to the environment in spreading of pathogenic bacteria which in consequence can affect growth and health of aquatic organisms. The post larvae stage is exposed to a higher risk in being infected than adult shrimps because of the undeveloped immune system (FAO 2005-2019, Rungrassamee et al., 2013, Hossain et al., 2017). Any perturbations from the surrounding environment could negatively impact the wild populations.
Thus, understanding the bacterial diversity associated with the host is important to indicate the host health status. Coupled with information on its interaction with the environment, the data would be critical for strategizing management of the environment and its living community. Most shrimp-microbiome studies have been focused on an aquaculture setting because of the commercial values and disease invasion. Examples of economically important shrimp species that have been investigated for its microbiome interaction include the giant freshwater prawn, Macrobrachium rosenbergii, (Nhan et al., 2010, Mujeeb et al., 2017) Pacific white leg shrimp, Litopenaeus vannamei, (Zhang et al., 2014) banana shrimp, Fenneropenaeus merguiensis (Oxley et al., 2002) and black tiger shrimp, Penaeus monodon (Vaseeharan and Ramasamy, 2003, Iehata et al., 2017).

Consequently, less attention is given to studies of wild shrimp populations and the bacterial diversity associated with them is not fully understood. This information is important to reduce risk of wild shrimp exposed to the potential of bacterial diseases in shrimp especially at the early stage. As example, detection of pathogenic bacteria such as Vibrio spp. in shrimps will give crucial information and strong evidence of shrimps are potentially infected by a vibriosis disease such as acute hepatopancreas necrosis disease (AHPND).

After all, bacterial diversity can be influenced by environmental factors. Any disturbance in the natural ecosystem can cause an imbalance of bacterial diversity which may lead to an increase in pathogenic bacteria in the host and aquatic ecosystem which is of high concern. This is particularly in areas where fisheries industry is important for the livelihood of the people. Although shrimp aquaculture production is rapidly expanding, capture fisheries is still contributing the major part of the shrimp industry (Chowdhury et al., 2012, Pauly and Zeller, 2016). Thus, loss of species in
natural habitats will not only affect the shrimp diversity and any related ecological functions but also the industry and as a food source. Furthermore, keeping it free from pathogenic bacteria is important to avoid bacterial infection in human consumers.

Molecular approaches have advanced rapidly in the last few decades. Currently, advanced methods such metagenomics is being applied in many branches of science such as medical, life sciences, earth sciences, agriculture, aquaculture, and many more (Forster et al., 2015, Andújar et al., 2015, Tarnecki et al., 2017, Martínez-Porchas et al., 2017, Hackman, 2018). Metagenomics is the study of microbial genome directly from their natural habitat without isolation and laboratory cultivation of individual species (Tringe & Rubin, 2005, Sullam et al., 2012). It is increasingly utilised for various applications. For example, the metagenome approach was used to study gut microbiota in wild marine fishes (Egerton et al., 2018). During the initial stages of gut bacterial diversity studies, many researchers had documented low levels of diversity (Yoshimizu and Kimura, 1976). However, through more advanced molecular techniques including next generation sequencing technologies, identification have become efficient (Zarkasi et al., 2014). This method has increased bacterial species discovery although the knowledge on their roles/functions are still lacking (Egerton et al., 2018).

Traditionally, microorganisms were identified and classified through microscopic before pure culture technique was introduced. This cultural-dependent method is a new evolution to microbiology field research. It gives a more microorganisms group can be studied through isolated and pure culture growth. Even though, this method is limited to culturable organisms and inadequate in microorganism’s diversity research, it is still use until today for screening and identification of targeted culturable species (Lagier et al., 2015). Through cultivation
and identification of bacteria in six species of fish (Canadian rock cod, sole, lantern fish, red rock fish, Norwegian mackerel and USA Smelt), Sanchez et al. (2012) successfully isolated 20 strains of bacteria from three phyla; Actinobacteria, Proteobacteria, and Firmicutes associated with fish. However, studies based on a metagenomics approach of 16S rRNA sequencing, identified 12 phyla dominated by phyla Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Fusobacteria associated with the ornamental bluestreak cleaner wrasse, *Labroides dimidiatus* (Nurul et al., 2019) while 28 phyla were detected in the gut of the Atlantic salmon, (*Salmo larna* L.) (Zarkasi et al., 2014).

These studies highlighted that the metagenomics approach was more efficient in identifying bacterial diversity than culturable techniques. However, culture-dependent approach is critical when the objective is for detailed information on targeted bacterial species such as *Rahnella aquatilis* (Enterobacteriaceae), and *Photobacterium damselae* (Vibrionaceae), *Salmonella* sp. in commercial fishes (Ampofo and Clerk, 2010, Alikunhi et al., 2017). Another advantage of the cultural technique is it is not costly and does not require advanced facilities.

During the pioneering period of bacterial studies, bacterial diversity was detected by cultural dependent method. However, not all types of bacteria could be detected by cultural methods as most bacteria are not amenable to laboratory culture and a lot of diversity lay hidden within the uncultured bacterial samples (Handelsman et al., 2004). Thus, in any cultural experiment, only a small fraction of the bacteria diversity could be detected and not the full spectrum. Fortunately, advances in technology has enabled further exploration in bacteria diversity. In the last few decades, new molecular technologies have emerged to identify microorganisms. During the early development of the molecular method, microorganisms was identified
through DNA and sequenced by using Sanger sequencing technologies (Mardis, 2011). Sanger sequencing is the latest technologies have introduced new ways of sequencing called next generation sequencing (NGS) for microorganismal identification. This culture independent approach can sequence a bulk of samples and the sample analysis on bacterial taxonomy be conducted simultaneously. Through this metagenomics approach, more bacterial species can be detected and helps to broaden the study of microbial communities and function of the ecosystem.

This study was conducted in the Merbok River, Kedah to identify the bacterial diversity associated with wild shrimps at the post larvae stages. This river forms part of the Sungai Merbok Permanent Mangrove Forest Reserve, considered to be the most diverse mangrove area (in terms of mangrove species per unit area), globally (Ong et al., 1991). Mangrove forests are sites for food, shelter and recreation for numerous species from all the major groups of animals- fishes, birds, mammals, amphibia, reptile and invertebrates and others. These include residents as well migratory ones which transit here (example migratory birds) for food and rest. More interestingly they are important spawning and nursery sites of many aquatic animals including shrimps. Its high diversity is a source of income in the fisheries and aquaculture sectors for the locals and also attract non locals to reside here to earn their living. Thus, artisanal fishing, aquaculture, agriculture, and eco-tourism activities are very important along the river and the surroundings land area have many farms and even oil palm plantations.

The river can be divided into the upper stream, middle stream and lower stream. The upper stream of the river has many shrimp ponds, and surrounded by plantations, residences and industrial activities. The middle stream is mostly surrounded with mangrove areas while the lower reaches are gaining importance as an
ecotourism destination. Aquaculture and artisanal fishing activities are common features of the Merbok River. By utilising the metagenomics approach, a broad spectrum of bacteria diversity that is associated with post larvae wild shrimps and affect human activities can be identified in the Merbok River.

1.2 Problem statement

There have been very few studies on the microbiome of post larvae shrimps especially in wild species. Studies have generally been focused on commercially important cultured shrimps with no/little information on wild and less commercially important shrimp species. This knowledge gap on bacteria associated with wild shrimps in a mangrove area needs to be addressed for their protection during this part of their life cycle. Understanding the microbial community diversity and its potential to affect post larvae shrimp community structure is important for understanding the environmental influence that may affect the post wild shrimp larvae. Although there have been numerous studies on microbiome of shrimps (Hood and Mayers, 1974, Zhang et al., 2014, Zheng et al., 2017, Hou et al., 2018), none have been conducted in the mangrove biodiversity hotspot of Merbok River (or any other areas in Malaysia) in Malaysia. Many of the earlier studies have utilised the traditional cultivation method which is limiting in elucidating a comprehensive bacterial diversity. Most bacteria species require specific conditions to survive, thus the culturing technique is highly challenging to study bacterial diversity (Dickson et al., 2014).

Metagenomics is an advanced tool to study the microbial community diversity compared to the conventional cultivation techniques as a broader range of microbiota species can be identified and studied. The metagenomics method could extract and
sequence multiple DNA samples simultaneously which could permit species level identification (if voucher sequences are available in the databases) in different environmental conditions (Felczykowska et al., 2015). It can provide a better understanding of bacterial diversity and functions, with higher sensitivity and is less time consuming. Thus, a metagenomic study would not only address the knowledge gap in wild shrimp microbiome of this region but also ensure optimal data.

Based on these knowledge gaps, this study was aimed at addressing the following objectives;

1.3 Objectives

The objectives of this study are:

1. Identifying of microbial community diversity associated with the wild post larvae shrimps in Merbok Rivers.
2. To determine key major abundance of the wild post larvae shrimp microbial species and overall microbial community diversity.
CHAPTER 2
LITERATURE REVIEW

2.1 Bacterial diversity

Microorganisms which among others include bacteria are the most abundant and diverse form of life. They take on many critical roles to support other living organisms. Bacteria can be found in nearly all habitat types from common surroundings to more extreme/specialised environments such as deep ocean sediments (Li et al., 1999, Wu et al., 2019), glaciers (Zhang et al., 2002, Calvillo-Medina et al., 2019), volcanic vents (Huber et al., 2003, Huber et al., 2007, Bendia et al., 2018), mangroves (Priya et al., 2018, Pramanik et al., 2019) as well as in the internal and external environments of various hosts.

Even though, bacteria can be found everywhere but the species present may differ among ecosystems and geographical locations. Among and even within aquatic ecosystems i.e marine, brackish and freshwater environments, different compositions of bacterial diversity may occur. As an example, Betaproteobacteria are commonly found in freshwater environment but almost absent in marine environment. This has been attributed to the influence of physicochemical conditions of water quality on bacterial species presence.

A study conducted by Lozupone and Knight (2007) revealed that the phylogenetic relationships of bacterial communities based on the 16S rRNA sequences showed a clustering governed by salinity even though the samples had originated from different environments with extreme ranges of temperature and pH. Another study carried out in five high-altitude lake ecosystems but with different salinity concentrations also showed variations in bacterial diversity (Liu et al., 2013). Furthermore, pH could also influence bacterial compositions in both soil and water.
(Bååth and Kritzberg, 2015). Neutral pH in the soil have been reported to harbour greater bacterial diversity than acidic pH soil. Results from Cho et al. (2016) indicated that bacterial diversity, evenness, and richness were higher at neutral pH based on the four major phyla (Actinobacteria, Bacteroidetes, Proteobacteria, and Cyanobacteria) compared to acidic soil where almost half of the communities belonged to phylum proteobacteria.

In addition, pH could also influence some bacterial species distribution such as Acidobacteria (Jones et al., 2009). This group can be found abundantly in acidic soil compared to neutral pH soil. This shows that pH can be influenced by the presence of specific bacterial groups in the ecosystem. Thus, human activities in agriculture, plantation and aquaculture which affect the temperature, pH and other associated parameters (including nutrients) can disturb the equilibrium balance of bacterial diversity and lead to differences in the abundances of many classes and phyla present in the ecosystem as a result of changes in the physicochemical conditions.

Even though bacteria are single celled organisms with the simplest structure of life, the taxonomic classification are still undergoing revision to this day. Historically, during the early days of taxonomic classification, organisms were very broadly divided into either the plant or animal kingdom. Thus, at the initial stage of bacteria taxonomic studies, they were classified as plants because of their morphology (Schleifer, 2008). In 1800s Haeckel discovered single-celled protists and proposed another kingdom, protists (Corliss, 1998). However, in 1938, Copeland proposed improved the kingdom classification and classified bacteria into monera kingdom (Copeland, 1938). This Tree of life was further revised by Whittaker (Whittaker and Margulis, 1978) who recognized fungi as another kingdom. Based on this, they proposed a 5-kingdom scheme which consisted of kingdom animalia, plantae, fungi, protista and monera.
Following on a molecular approach, mainly using ribosomal RNA (rRNA) sequences to infer phylogenies, the bacteria classification was expanded into a domain system called the three-domain system (Woese et al., 1990). The three-domain system was made up of eukarya, archaea and bacteria. In the late 1990s, a six-kingdom system was introduced which separated bacteria into two subdivisions which are Eubacteria and Archaeabacteria (Cavalier-Smith, 1998).

Through sequencing technologies, bacteria are now widely identified by comparing with bacteria taxonomic reference databases such as SILVA, Ribosomal database project (RDP), Greengenes, and NCBI (Balvočiūtė and Huson, 2017). This sequencing strategy has revealed that class Alphaproteobacteria, which has more than 100 sequenced representatives, is one of the most ecologically diverse classes. The dominant bacteria orders from class Alphaproteobacteria are represented by Rickettsiales, Rhodospirillales, Sphingomonadales, Caulobacterales, Rhodobacterales and Rhizobiales (Philippot et al., 2010). In the aquatic ecosystem, alphaproteobacteria makes up around 35% of the bacteria communities in marine surface water. Rickettsiales are found mostly in surface water, whereas relatives from the orders Rhizobiales and Burkholderiales are found mostly in soil (Morris et al., 2002, Rusch et al., 2007, Philippot et al., 2010).

Recently, advances in technology have permitted more in-depth studies on microbial diversity including bacterial diversity. The Earth Microbiome Project (EMP) is the biggest microbial diversity survey. Its initial analysis has revealed an approximately six million bacterial taxonomic units (genus or species level taxa) from various environments in 10,000 of the 200,000 targeted samples which successfully detected bacteria and archaea (Gilbert et al., 2014). These (EMP) projects were not
only limited to aspects of bacterial diversity, but the microbiologists also seek to understand the beneficial and harmful effects that they bring.

Many branches of microbiological studies have since developed from these investigations including in the fields of medical microbiology, pharmaceutical microbiology, food microbiology, industrial microbiology and environmental microbiology (Lloyd-Price et al., 2016, Stadler and Love, 2016, Porcellato et al., 2018, Barzkar et al., 2018, Bergkessel et al., 2016). Being very minute, working on these taxa was very challenging in the early days but with advanced technology, the limitations to study microorganisms can and has been reduced and more detailed studies could be accomplished.

2.2 Bacterial-host interaction

The bacterial community is able to thrive in a wide range of environments by utilising various efficient and adaptation mechanisms. Generally, these have taken the form of bacterial-bacterial and bacterial-host interactions. In addition to connecting with the abiotic environment, bacteria often interact with other biotic organisms inhabiting the same area such as with plants and animals (Santhanam et al., 2014, Kai, 2016, Braga et al., 2016, Ely and Smets, 2019). The bacteria communities that are found in seeds, roots, leaves, stems, tubers, ovules and fruits also differ depending on their functions (Zinniel et al., 2002).

These interactions can be beneficial, harmful or does not affect the host (Freestone, 2013). In a beneficial interaction, bacteria such as *Pseudomonas fluorescens*, *Rhizobia* spp. and *Frankia* spp. help to promote root development and plant growth (Bhattacharyya and Jha 2012, Glick, 2012, Jansson and Hofmockel
2018), provide nutrients (Bais et al., 2006, Jacoby et al., 2017) and tolerance to abiotic stresses (Meena et al., 2017, Hirt et al., 2019), as well as protect plants from pathogenic bacteria (Berendsen et al., 2012, Schirawski and Perlin, 2018). However, pathogenic bacteria such as *Pseudomonas syringae* and *Xanthomonas campestris* can suppress the plant immune system enabling it to invade and cause diseases (Chisholm et al., 2006, Dodds and Rathjen, 2010). Infection in plants can cause great losses to the agriculture industry. Similarly, animals are important hosts to bacteria (McFall-Ngai, 2002, Thurber et al., 2011, Alegado and King, 2014).

### 2.3 Bacterial and shrimp interaction

By monetary value, shrimp is the most important commodity in the international seafood trade. Due to its high market value and demand, the shrimp industry has grown exponentially, and shrimp aquaculture has become popular in the fisheries industry. The global production of farmed shrimp in 2017 was estimated between 2.9–3.5 million tonnes increasing by 6% compared to the previous year (FAO market report, 2018). Nearly 75 - 80% of the production originate in the Asia-Pacific. Shrimps from family Penaeidae have the highest economic value compared to other families. These are sourced from the wild as well as cultured. The main target species in shrimp aquaculture are giant tiger prawn/green tiger prawn (*P. monodon*), Pacific whiteleg shrimp (*L. vannamei*), and giant freshwater shrimp (*M. rosenbergii*).

Besides the aquaculture industry, wild caught also contributes to the fisheries industry. Loneragan et al. (2005) reported that 35% from all caught shrimps in the 1990s were white shrimp, *P. merguiensis*. Others include the white shrimps (*P. indicus, P. penicillatus* and *P. latisulcatus*), sharp-rostrum shrimp (*Parapeneopsis*...
hardwickii, Parapeneopsis coromandelica, Parapeneopsis hungerfordii and Parapeneopsis gracilima), pink shrimps/greasyback shrimps (M. affinis, M. ensis and M. intermedius, rainbow shrimps (Parapeneopsis sculptilis), giant tiger prawn/green tiger prawn (P. monodon and P. semisulcatus (Loneragan et al., 2005). Varied methods such as trawling, trammel nets, pushnets and bagnets are used in shrimp harvesting and this activity is done throughout the year.

Understanding bacterial-host interaction particularly for economically important species such as shrimps is very important, whether in an aquaculture setting or for the protection of the natural environment where the culture facilities are located. Documented studies on bacteria-shrimp interaction began in the 1970s (Hood and Mayers, 1974). During the early stage, these studies were conducted by using conventional culture-dependent technique to characterize bacterial gut communities, such as those reported in cultured juvenile northern white shrimp, Peneaus setiferus gut (Hood and Mayers, 1974). Yasuda and Kitao. (1980) also studied the bacteria-shrimp interaction in wild and cultured kuruma shrimp, Peneaeus japonicus.

Bacteria diversity can vary significantly under different environmental conditions, including in the abiotic habitat or the biotic host. Thus, bacterial community in shrimps can be different depending on health status, environmental conditions and growth stages (Zheng et al., 2017, Hou et al., 2018). In a study conducted in a culture environment, the dominant bacteria phyla found in juvenile pacific whiteleg shrimp, L. vannamei shrimps were recorded to be Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes, Planctomycetes, and Actinobacteria (Zhang et al., 2014). Within these phyla, several genera were suggested as good indicators of healthy shrimps such as genera Flavobacteriia, (Bacteroidetes), Francisellaceae (Gammaproteobacteria), Rhodospirillaceae (Alphaproteobacteria),
*Pseudomonaceae* (Gammaproteobacteria), *Coxiellaceae* (Gammaproteobacteria), *Alphaproteobacteria* and *Betaproteobacteria* while *Rhodobacteriaceae, Erythrobacteraceae, Bacteroidetes, Cyanobacteria* and *Rickettsiales* act as indicators of diseased shrimps (Xiong et al., 2014). Similarly, wild and cultured shrimps showed differences in shrimp diversity. Several genera such as *Faecalibacterium, Bacteroides* and *Bifidobacterium*, and species, such as *Coprococcus eutactus* and *Faecalibacterium prausnitzii* were only found in cultured but not wild shrimps (Cornejo-Granados et al., 2017).

Furthermore, differences were also observed at different growth stages in the gut of the black tiger shrimp (*P. monodon*) and Pacific whiteleg shrimp, *L. vannamei* (Rungrassamee et al., 2013, Huang et al., 2016). Bacteroidetes and Alphaproteobacteria phyla were found in abundance in post larvae stages but not at the juvenile stage. However, Gammaproteobacteria was recorded in all growth stages in the shrimp gut wall (Rungrassamee et al., 2013, Huang et al., 2016). **Figure 2.1** shows the varies shrimps at the post larvae stages; 1. Penaeid shrimp 2. Sergestid shrimp and 3. Mysid shrimp
Similarly, bacterial community diversity is also very much influenced by the external environment. The optimum balance of physicochemical factors (salinity, total phosphate and total nitrogen concentrations, as well as temperature, and pH) are important to ensure the levels and composition of bacterial diversity promotes shrimp health (Chen et al., 2018, Ponce-Palafox et al., 2019). Changes of water quality can disturb the balance of physicochemical factors needed by the shrimp. This will cause stress to the shrimp and if this occurs, it is easily attacked by opportunistic bacteria. This balance of physicochemical factors can be disturbed by pollutants and human activities (Halliday et al., 2013). Thus, recreational activities not only affect the water quality of a habitat but also change the bacterial diversity it supports (Ibekwe et al.,
Disturbances can cause a rise in pathogenic bacteria and vice versa, a decrease of non-pathogenic bacteria in the environment. This situation can result in the aquatic organisms to be more susceptible to bacterial and viral infections. This is exacerbated by the hydrodynamics of the habitat that allow pathogens to broadly disperse the diseases (Krkošek, 2017).

Bacterial and viral infections are very common occurrences in cultured fish and shrimps. In Malaysia, species that are commonly cultured are the native giant tiger prawn (*P. monodon*) and non-native Pacific white leg shrimp (*L. vannamei*). In 2011, the National Fish Health Research Center (NaFisH) reported that cultured Pacific white leg shrimp from Perak showed signs of *Vibrio parahaemolyticus* infections that infected post larvae, juvenile and broodstocks shrimp (Kua et al., 2016). *Vibrio parahaemolyticus* is responsible to cause Acute Hepatopancreatic Necrosis Disease (AHND) in both non-native shrimp and native shrimp, giant tiger prawn (*P. monodon*) (Devadas et al., 2018). This problem is very worrying as not only does it impact the cultured organisms, but also the wild inhabitants in the vicinity (Cook et al., 2008). This is because the pathogenic bacteria from the cultured organisms could be introduced to wild populations, potentially threatening or endangering the wild populations of aquatic organisms that share the same ecosystem (Gozlan et al., 2006). This raises the issue of environmental impacts and ecosystem health by the aquaculture industry (Caruso, 2014).

Furthermore, infection by pathogenic bacteria can result in a loss in shrimp production and food safety issues on humans. Shrimps can be contaminated from metals, marine toxins, and infectious agents (Heidarieh et al., 2013, Putth and Polchana, 2016, Nicolas et al., 2017). Infectious agents associated with food-borne illness include bacteria, viruses, and parasites, and the illnesses caused by these agents
range from mild gastroenteritis to life-threatening syndromes. Fish, molluscs, and crustaceans can be affected by pathogens from various sources and culture areas of seafood contaminated with human sewage can be a major cause.

Outbreaks of seafood-associated illness linked to polluted waters have been reported due to *Vibrio* spp. such as *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Listeria monocytogenes*, *Clostridium botulinum*, *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter fetus*, Group A *streptococcus*, and *Salmonella enterica* serotype Typhi (Butt et al., 2004, Iwamoto et al., 2010, Bakr et al., 2011). This diverse group of pathogens manifests in a wide variety of clinical syndromes and effects. The condition is worsened when seafood is consumed raw or prepared in ways that do not kill the organisms. Ahmed et al. (2016) revealed that *V. alginolyticus* was isolated in a cultured sample of *P. monodon* from local market in Korea. Previous study also reported presence of *V. parahaemolyticus* in white prawn (*P. indicus*), and coastal mud shrimp (*Solenocera crassicornis*) from local market in Malaysia (Letchumanan et al., 2015). These species are known as opportunistic pathogens to humans and aquatic animals.

However, cultured shrimps have been shown to harbour different bacterial diversity in various cultural systems. In aquaculture farming of *L. vannamei*, bacterial community composition is abundantly composed of phyla Actinobacteria, Proteobacteria and Bacteroidetes (Tang et al., 2014). This is corroborated by Alfiansah et al. (2018) in their study in Indonesia which stated that *L. vannamei* cultured in ponds are composed of Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes. However, the dominant bacteria found in red spot king prawn, *L. stylirostris* cultured
in a biofloc system were Proteobacteria, Bacteroidetes and Cyanobacteria (Cardona et al., 2016). Phylum Proteobacteria seemed to be the major group present in shrimps.

2.4 Bacteria communities associated with mangrove ecosystem

Mangrove is an ecosystem that constitutes less than 1% all tropical forests worldwide. Nevertheless, it is important for human utilisation as a source of food, fuel wood and building materials. In addition, it provides various ecosystem services including global carbon exchange, and coastline protection (Clusener-Godt and Tomazic, 2016). Furthermore, mangrove areas also serve as nursery area and habitat for aquatic living organisms and act as natural filters for nutrients and toxicants. A key, although unseen force that facilitate in the critical ecosystem functions of a mangrove ecosystem are the microbial communities that it supports. These microscopic communities are responsible to convert nutrients from dead mangrove vegetation into phosphorus, nitrogen and other useful nutrients (Sahoo and Dhal, 2008).

Several major groups of beneficial bacteria have been identified in the mangrove ecosystem. For example, the genera *Azotobacter* and *Azospirillum* are the most common nitrogen fixation bacteria found in white mangrove (*Avicennia marina*) (Baskar and Prabakaran, 2015) while *Vibrio campbelli*, *Listonella anguillarum*, *V. aestuarianus*, and *Phyllobacterium* spp. are also involved as nitrogen fixation bacteria and have been isolated from other mangrove species for example red mangrove (*Rhizophora mangle*), black mangrove (*Avicennia germinans* (L.)) and white mangrove (*Laguncularia racemose*) in the mangrove area of Balandra Bay, Mexico (Holguin et al., 1992, Rojas et al., 2001). *Vibrio proteolyticus* has been identified as
the most active phosphate solubilising bacteria isolated from the mangrove tree roots of black mangrove (*Avicennia germinans* (L.)) and white mangrove (*Laguncularia racemose* (L.)) Gaertn (Vazquez et al., 2000). Sulphate reducing bacteria are common anaerobic microorganisms present in mangroves that are important for removing sulphate and heavy metals from waste streams. An analysis of 16S rRNA sequences revealed that most of the sulphate reducing bacteria belong to class Deltaproteobacteria (Muyzer and Stams, 2008). Most *Rhodobacter* and *Rhodopseudomonas* are common photosynthetic anoxygenic bacteria genera inhabiting mangroves, typically found on the root surface of the trees. These groups of bacteria contribute immensely to the mangrove ecosystem productivity (Sahoo and Dhal, 2008). In addition to their direct relationships with the mangrove species, bacteria in the order Rhizobiales, Campylobacterales, Methylcoccales and Vibrionales are also found abundantly in samples from the rhizosphere layer (Gomes et al., 2011).

The importance of the mangrove ecosystem is not limited to ecological functions, but it also plays a major role as an income resource to local communities (Salampessy et al., 2015). Besides the multitude of uses of mangroves species, fisheries activities such as artisanal fishing, aquaculture and eco-tourism are common activities that can be found in mangrove areas. In this regard, microorganisms especially bacteria play major roles in mangrove productivity (Holgiun et al., 2001) as outlined earlier, ensuring the fitness and survival of mangroves and also the overall ecosystem. The latter also includes other co-inhabiting organisms that inhabit the ecosystem. Unveiling the diversity and structure of microbial communities in the mangrove environment and organisms of economic importance it supports, is thus the
first step towards a better understanding of bacterial communities and their role in ecosystem functioning, host interaction and ultimately its conservation and protection.

2.5 Merbok River of the Sungai Merbok Permanent Forest Reserve (PFR)

The sampling site for this study is the 35 km Merbok River which runs through the Sungai Merbok Permanent Forest Reserve (PFR). With an area of 3,881.90 hectares when it was first declared a forest reserve in 1951 (Laporan Ketua Audit Negara, 2010), the Sungai Merbok PFR is the largest mangrove forest in the northwest Peninsular state of Kedah. Other mangrove area in this region are located in Langkawi Island with 3,116 hectares which comprised of Kampong Kuala Isap-Gua Cerita mangroves, the Sungai Ayer Hangat-Kubang Badak mangroves and the Pulau Dayang Bunting-Pulau Tuba mangroves. Malaysia has a total of 105,537 ha of mangrove forests (Latiff, 2012) which cover less than 2% of the total land area (Shukor, 2004). However, the mangrove areas have shrunk over the years and Sungai Merbok PFR also lost 975.79 hectares from its initial size due to conversion to aquaculture ponds, small industries and other development projects (Jusoff and Taha, 2008).

Similar to other mangrove habitats, the Sungai Merbok PFR serves as an important ecological and socio-economic ecosystem for the inhabitants living in its surroundings. It is home to numerous marine and brackish (as well freshwater, further upstream) species that utilise the area for food, shelter and protection. It also serves as a nursery area to a plethora of marine, brackish and freshwater life including crustaceans that spawn among the roots of the mangrove (Laegdsgaard and Johnson, 1995, Mansor et al., 2012, Boulton et al., 2016). The falling mangrove leaves is an
important food source for the larvae nursing in the area. Thus, many species at the larvae stage can be found at the riverbanks among the roots of the mangrove tree.

With its high aquatic biodiversity, the Merbok River has supported the livelihood of the local community (Figure 2.2). Artisanal fishing activities can be seen along the river. Furthermore, the location and conditions of the Merbok River makes it suitable for fish and shrimp aquaculture activities. These aquaculture farms are owned by both small-scale farmers as well commercial companies. While this has brought about economic benefits to the community, it could lead to detrimental ecological effects on the ecosystem as a consequence of the activities. Knowledge on the microbiome of the host wild shrimp could provide an indicator of the health status of the shrimp based on the occurrence and abundance between the “bad and “good” microbes.

The study could also act as a model not only to survey the health status of the target group but also the surrounding environment in other mangrove sites. Thus, the data could facilitate planning of strategic plans for the management of the shrimp populations and therefore their sustainable utilisation for the benefit of the local community and investors. Due to its uniqueness and conservation value and socio-economic importance, efforts are underway to apply for the designation of the Sungai Merbok PFR and its surroundings as a UNESCO Biosphere Reserve.
Physicochemical parameters; rainfall, water depth, salinity, turbidity, temperature, conductivity and pH are believed to affect fish assemblages in the river (Mansor et al., 2012). Mansor et al. (2012) stated that physicochemical parameters are correlated with aquatic species occurrence and suggested the importance of the parameters in determining fish distribution, abundance and assemblage. In a detailed ecological study comparing two seasons (dry and wet seasons) of the Merbok River, Fatema et al. (2014) showed that some sectors of the river were at unhealthy levels based on higher dissolved oxygen (DO), nitrate, nitrite and ammonia concentrations. Rainfall during the wet season is also known to influence water quality, parameters such as salinity, conductivity, DO (Fatema et al., 2014).
2.6 Microscopic and cultural methods in bacterial taxonomic and bacterial diversity studies

The first recorded discovery of microorganisms was made by Anton Van Leeuwenhoek and Robert Hooke during the 1660s to 1670s (Porter, 1976) using one of the earliest microscopes. In 1683 they documented accurate descriptions of various types of bacteria and found many microorganisms in various environments such as water, mud, saliva and the intestinal contents of healthy subjects (Kumar, 2012). After the development of pure culture technique and growth media for bacteria culturation by Robert Koch, this then new approach for bacteria identification became widely applied (Blevins and Bronze, 2010). The generation of pure culture during microorganism isolation is highly critical for the success of a culture-dependent approach. Microbiologists need to maintain optimal environment of the media for microorganisms to grow (Liu et al., 1997) and followed by physiological and biochemical tests to assess the presence of the targeted microbes. Nevertheless, culture-dependent approach has limitations due to several factors; not all microorganisms could be grown in laboratory conditions and only a small number of microbial species can be detected in a culture (Amann et al., 1995). Connon and Giovavannoni, (2002) noted that only half out of 40 known prokaryotic phyla could be detected by cultural methods.

The critical limitation of the culturing method was highlighted when Staley and Konopka. (1985) observed discrepancies in data obtained between microscopic and cultural techniques. This was widely referred to as “the great plate count anomaly”. This described the situation whereby the plate counts of microorganisms through the culture method were observed to be lower than those seen under the microscope. Thus, it was concluded that there were numerous unculturable
microorganisms that remained undetected in any studied sample. Accordingly, for many years, these uncultured microorganisms failed to be detected and therefore not documented (Su et al., 2012). As only a minor fraction of the microorganisms in the environment were identified, the microbial diversity assessments were incomplete (Amann et al., 1995). Furthermore, identification of complex microorganisms that were strictly anaerobic and new species discoveries using the culturing method was nearly impossible.

Although the cultural method is still being applied until today, particularly in less well-equipped laboratories, more advanced techniques such as molecular approaches have replaced its wide usage. The molecular approach for microbial diversity study of shrimp was first introduced in the late 1990s using denaturing gradient gel electrophoresis (DGGE) and 16S rDNA clone library analysis (Blackall et al., 1998, Liu et al., 2011). Recently, advances in next generation sequencing (NGS) allows for more detailed and precise studies in microbiota diversity (Lasken and McLean, 2014, Bikel et al., 2015) the detection is not only limited to culturable microbes but also non-culturable microbes such as thermophilic bacteria, Treponema spp. and Prochlorococcus spp. (Steward, 2012).

2.7 Molecular identification of bacteria through cultural-independent metagenomics approach

Studies in microbial communities gained further impetus after the development of direct amplification and analysis of small subunit (SSU) ribosomal RNA (rRNA) in the late 1980s (Su et al., 2012). This was developed from the pioneering phylogenetics studies of Carl Woese and George Fox based on the SSU rRNA sequencing (Woese and Fox, 1977). Following to this, the study of microorganisms was divided into two major approaches; culture-dependent and culture-independent approaches.
The term “Metagenomics” was first introduced by Jo Handelsman in 1998 that referred to any culture-independent analysis of microbial communities. However, nowadays metagenomics is generally defined as the direct genetic analysis of the genomes contained in environmental samples (Thomas et al., 2012). Pace and his team applied this knowledge to study microbial communities by using PCR-based molecular techniques and DNA sequencing techniques (Pace, 1997). They proposed cloning method direct from environmental DNA (Pace, 1997). Other methods came to be developed such as terminal restriction fragment length polymorphisms (RFLP) (Liu et al., 1997), single-stranded-conformation polymorphism (SSCP) (Lee et al., 1996), denaturing/temperature gradient gel electrophoresis (DGGE/TGGE) (Muyzer, 1999), and quantitative PCR (qPCR) (Takai and Horikoshi, 2000) which have been used widely for studying microbial communities. Alternatively, non-PCR-based molecular techniques such as microarray and fluorescence in situ hybridization (FISH) have also been applied to study microbial community (Cho and Tiedje, 2001, Kong et al., 2010, Nierychlo et al., 2016).

With advances in sequencing technologies, metagenomics has become a powerful tool in microbial diversity studies. The metagenomics approach is able to provide simultaneous information of whole bacteria community in any studied sample. By using next sequencing generation technology, a mixed sample can be sequenced and analysed simultaneously, and bacteria are identified without need of isolation (National Research Council, 2007). In the study of bacterial diversity, metagenomics can provide information on species richness and distribution besides the metabolic potential of a community (DeLong, 2005). With a wide coverage of OTU detection, metagenomics can provide deep understanding of bacteria-host interaction in the ecosystem (Suttle, 2007, Gianoulis et al., 2009). A balanced equilibrium between
bacterial diversity and host is useful to ensure a healthy host, and consequently food security. Table 2.1 shows comparison of advantages and disadvantages of the metagenomics approach.

Table 2.1 Comparison of advantages and disadvantages of metagenomics approach

<table>
<thead>
<tr>
<th>Advantages of metagenomics approach</th>
<th>Disadvantages of metagenomics approach</th>
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<tbody>
<tr>
<td>High-throughput sequencing approaches enable genomic analyses ideally of all microbes in a sample,</td>
<td>Metagenomics analysis requires large storage and good computational capacity for the hardware and established database besides skills in handling the programs for analysis (Thomas et al., 2012).</td>
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<tr>
<td>not just those that are amenable to cultivation (Quince et al., 2017).</td>
<td></td>
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<tr>
<td>A bulk sample collected from environment can be analysed simultaneously (Teeling and Glöckner, 2012).</td>
<td>16S rRNA-based techniques are known to be limited by the short-read lengths obtained, sequencing errors, differences arising from the different regions chosen, and difficulties in assessing operational taxonomic units (OTUs) (Poretsky et al., 2014).</td>
</tr>
<tr>
<td>Wide coverage of bacteria species with their functions can be studied (Wang and Qian, 2009).</td>
<td>Coverage of bacterial species depending on genome availability in the database (Teeling and Glöckner, 2012).</td>
</tr>
<tr>
<td>Distribution, species richness and evenness of bacteria can be analysed (Logares et al., 2014).</td>
<td>Biasness from PCR and amplicon sequencing may result in less precise output at species level identification (Poretsky et al., 2014).</td>
</tr>
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</table>
2.7.1 Sequence-based metagenomics

In the last decade, sequence-based approaches have become established as the method of choice to understand microbial communities and behaviour, when funds and facilities are available (Thomas et al., 2012). The method allows efficient identification of taxonomic groups from DNA fragments through sequencing. Sequence-based metagenomics involves collection of DNA from the environment (both internal such as host body and external such as habitat), its extraction, sequencing and analysis (Handelsman, 2004). The sequencing may involve whole genome or targeted genes based on gene-specific primer design. One of the latest techniques is the shotgun metagenomic sequencing which allows sequencing and detection of a high number of genes and species.

2.7.1(a) Amplicon sequencing

Amplicon sequencing is a targeted approach, focusing on a certain gene using a gene specific primer. The 16S rRNA gene is normally utilised and 16S rRNA amplicon sequencing has been used to study the microbial diversity in various macrohabitats and microhabitats; soils (Ligi et al., 2014), hot spring (Chan et al., 2015), temperate freshwater lake (Poretsky et al., 2014), deep sea (Sogin et al., 2006), mouse gut (Shin et al., 2016) and human gut (Penington et al., 2018, Laudadio et al., 2018). Amplicon sequencing is highly targeted and allows analyses of large data sets (Jovel et al., 2016). It is not restricted to investigation of a single gene. Studies can be conducted on sequence targets ranging from a few to hundreds of genes in a single run. This ultra-high multiplexed PCR approach expedites research by assessing multiple genes simultaneously (Li et al., 2019). Li et al. (2019) conducted a study to assess simultaneously numerous waterborne pathogenic genes in the stream water samples by using multiplexed PCR-NGS approach.
However, there are limitations to this technology that has not yet been addressed to date. For instance, the 16S rRNA sequencing is limited by the short-read length that are generated, sequencing error due to PCR bias and difficulties in assessing operational taxonomic units (OTUs) (Poretsky et al., 2014, Jovel et al., 2016) and is less precise at species level identification (Ranjan et al., 2016). It is also challenging to assess the diversity by using single marker gene (Poretsky et al., 2014).

However, 16S rRNA sequencing is still used in assessing microbial communities at higher hierarchical taxonomic levels. As an example, to assess the microbial communities in wastewater treatment plant, 16S rRNA gene amplicon sequencing was applied (Do et al., 2019). The study which was conducted in two wastewater treatment plant in Ireland detected high diversity of the phyla; Proteobacteria, Bacteroides, Actinobacteria, Firmicutes, Tenericutes and Verrucomicrobia phyla dominated the microbiomes 16S rRNA gene amplicon sequencing have also been used to study wild fish microbiome diversity. A study conducted by Nurul et al. (2019) on wild Bluestreak cleaner wrasse, *Labroides dimidiatus* from Karah Island, Terengganu, Malaysia showed the dominance of Proteobacteria, Bacteroides, Actinobacteria, Firmicutes, Fusobacteria and classified the samples to 36 different classes and 132 families.

### 2.7.1(b) Whole genome shotgun (WGS) sequencing

An advancement of the amplicon method that is finding wide popularity in recent years is the technique of whole genome shotgun (WGS). One of the earliest studies conducted using WGS for environmental DNA was that conducted by Venter et al. (2004). They found that WGS sequencing was more efficient in phylogenetic
diversity study compared to utilising single gene 16S rRNA amplicon. In WGS, the genome is first fragmented. These fragments are randomly sequenced to detect the diversity of microbial without need for a targeted gene. The main advantage of WGS sequencing is its precision in detection of diversity up to species level compared to 16S rRNA amplicon sequencing (Ranjan et al., 2016) with a higher probability to discover a new species if present (Jovel et al., 2016). This is due to the coverage (longer reads and depth) of sequencing. Besides that, detection is not limited to microbial presence but also fungi, viruses and eukaryotes (Jovel et al., 2016).

Unfortunately, results of WGS can be influenced by the complexity of the samples, DNA preparation method and sequencing platforms that chosen. Optimal selection of the platform is important for a successful outcome (Poretsky et al., 2014). A study conducted by Poretsky et al. (2014) compared between two metagenomics approaches; 16S rRNA gene amplicons only detected approximately 300 OTUs while shotgun metagenomics generated 1.5 times and, 10 times as many phyla and genera, respectively. However, WGS is also prone to several weaknesses; biasness caused by repetitive detection of some taxonomic groups while other taxonomic groups are undetected. In addition, WGS sequencing is also costly and require extensive data analysis.

2.8 Bioinformatics analysis in metagenomics

Due to the huge amount of sequencing output and highly complex bacterial community, analysis of the data output from these new technologies is very challenging. It requires large storage and good computational capacity for the
hardware. It also requires an established database and skills in handling the programs for analysis.

The enormous data generated from a microbiome study will need to undergo several computational and bioinformatics and genetic analyses before the final output is obtained. The short fragments generated are first assembled to form longer genomic contigs prior to bioinformatics analyses. Two strategies can be applied in metagenomics which are reference-based assembly (co-assembly) and de novo assembly. Co-assembly can be done by using the software such as Newbler (Roche), AMOS http://sourceforge.net/projects/amos/, or MIRA (Chevreux et al., 1999). However, it is limited to samples that are closely related to availability of reference genome. Otherwise the results obtained is inaccurate. De novo assembly are conducted in cases where there are no reference genomes. It is computationally challenging as it requires hundreds of gigabyte memory and several days to complete the run (Thomas et al., 2012). Common assembler used for shotgun metagenomic data are Meta-IDBA (Meta-Iterative De Bruijn graph de novo short read assembler) (Peng et al., 2011), MetaVelvet (Namiki et al., 2012) and MetAMOS (Treangen et al., 2013).

The next step in the process continues with binning that can be performed by using taxonomy-dependent and taxonomy-independent binning. This refers to the process of sorting and grouping the assembled genome into taxonomic classification (Sedlar et al., 2017). Taxonomy-dependent binning methods allow comparisons of the outputs with the available reference database such as BLAST (Altschul et al., 1990) and Pfam (Finn et al., 2015). However, very few genome data are available in the reference database and therefore, the binning process will often be through taxonomy-independent methods. In this approach, the assembled genome is assumed to have unique characteristics that can be grouped together by comparing their contents.
Methods utilised are sequence composition-based methods (e.g. of tools used are self-organizing maps (SOMs), MetaWatt, and LikelyBin), abundance-based methods (e.g. AbundanceBin, Canopy, and MBBC) and hybrid method which is a combination of composition-based methods and abundance-based methods (e.g. ComposBin, MetaCluster, and MaxBin) (Sedlar et al., 2017).

To simplify the process several common pipelines have been developed to facilitate the user. Some popular pipelines that are used include MG-RAST (Metagenomic-Rapid Annotations using Subsystems Technology) (Meyer et al., 2008), MEGAN (Huson et al., 2007), IMG/M (Integrated Microbial Genomes-Metagenomic) (Markowitz et al., 2008) and CAMERA (Community Cyberinfrastructure for Advance Marine Microbial Ecology Research and Analysis) (Seshadri et al., 2007).
CHAPTER 3
MATERIALS AND METHODS

3.1 Sampling activities

Sampling was done along the Merbok River, which is located in the district of Kuala Muda, Kedah at the northern part of Peninsular Malaysia encompassing 5°39’31.1”N 100°26’56.3”E. The Merbok River is 35 km long and varying in depth from 0.3 m to 15 m. Most part of the river is estuarine except for a few kilometres at the upper part which is freshwater (Ong et al., 1991). The upper part of the river starts from Lalang River and then drains into Pantai Merdeka (Merdeka Beach) and finally into the Strait of Malacca.

The samples were randomly collected at locations nearby to zones of various intensity of human activities, from more pristine habitats upstream to more intensively utilised habitats such as aquaculture, agriculture and residential areas, aimed to provide a snap-shot (as only a single sampling was conducted) of the influence of these activities to the bacterial community diversity and its relationship with the post larvae shrimp.

Sampling of post larvae wild shrimp was conducted at the riverbank, where shrimp larvae can be found abundantly. Sampling site was divided into four stations along the tributaries of the Merbok River that represent a wide coverage of the river and possible microhabitat, and ecological differences. This is because all the sampling sites have different activities that may influence the physicochemical conditions and microbial community diversity. The sampling sites were as follows; Station 1 (St 1) is nearby the Lalang River, Station 2 (St 2) in Semeling River, Station 3 (St 3) is nearby
the Keluang River and while Station 4 (St 4) in Terus River. The localities of the sampling sites were chosen due to availability of post larvae shrimp and nearby mangrove area and human activities such as residences and aquaculture farm. Figure 3.1 shows location of the four sampling sites along the Merbok River.

**Figure 3.1** Localities of post larvae wild shrimp sampling sites along Merbok River

**Table 3.1** Site localities and details of environment and human activities in the vicinity

<table>
<thead>
<tr>
<th>Localities</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Station 1 (St 1)</td>
<td>It is at the riverbank of the Bandar Laguna Merbok residences. The housing estate almost reaches the edge of the riverbank. On the opposite riverbank is a shrimp farm. Only a few mangrove trees can be observed in this area.</td>
</tr>
<tr>
<td>Station 2 (St 2)</td>
<td>The location of St 2 is at the riverbank of Pier Complex Sungai Merbok. The mangrove river cruise starts from here. There is a gallery and restaurant catering for tourists. Not so far from the Pier Complex Sungai Merbok is located an oyster farm. Mangroves trees can be observed in this area. A shrimp farm is located near the mangrove forest.</td>
</tr>
<tr>
<td>Station 3 (St 3)</td>
<td>The location of St 3 is surrounded with mangrove forest</td>
</tr>
<tr>
<td>Station 4 (St 4)</td>
<td>The location of St 4 is surrounded with mangrove forest and a shrimp farm located near Terus river</td>
</tr>
</tbody>
</table>
All sampling activities were completed within a month, in August 2016 to avoid influence of seasonal variations among sampling sites. This also coincided with the wet seasons and low tide where larvae are most abundant (Mansor et al., 2012). The post larvae shrimps were caught by using a scoop net with 500 μm mesh size. They were collected from the surface of the water where many larvae can be seen and located nearby human activities such as shrimp farms, plantations and residences. Sampling was conducted and completed during day time and for every sampling site, samples were scooped twice at the riverbank area and then pooled to minimise any bias.

Post larvae shrimp are distinguished by long and slender bodies, thin rostrum, fanned tail and long sixth abdominal segment. The rostrum with straight antenna was fully developed. The length of the post larvae shrimp from the beginning of rostrum to end of tail was approximately 0.4 cm to 1.3 cm. The post larvae were identified with the aid of the key by Williams (1959). Post larvae that has been caught as shown in Figure 3.2. Samples were kept in 250 mL sample bottles filled with water from the original habitats and preserved in an ice box during transportation to the laboratory in Universiti Sains Malaysia (Figure 3.3). No chemical preservative was added. The workflow of the sampling process is illustrated in Figure 3.4.
Figure 3.2 Post larvae wild shrimp samples

Figure 3.3 Post larvae shrimps were collected by using scoop net and keep in 250 mL bottles
3.2 Physicochemical parameters recorded

Environmental parameters such as water temperature, pH, salinity, dissolved oxygen (DO) and turbidity were recorded during the sampling to assess correlation of water quality with microbial diversity associated with the post larvae shrimp. YSI 550A (YSI incorporated, USA) was used to measure pH and dissolved oxygen (DO). The SCT Meter YSI Model 33 (YSI incorporated, USA) was used to measure water temperature and salinity while the secchi disk and tape meter were used for measuring the turbidity/transparency of the water.
3.3 DNA extraction for metagenome analysis

The 16S rRNA gene analysis was applied through the metagenome approach, on the samples collected to examine the microbial communities present in post larvae wild shrimp. The process began with the extraction of bacterial DNA in post larvae shrimps. Due to the small size of the post larvae (≈ 0.4 cm – 1.3 cm), whole body of pooled samples for each sampling site was homogenized by using mortar and pestle in preparation for the DNA extraction. At the initial stage genomic DNA extraction kit (Qiagen, USA) was used but the method failed to extract bacteria DNA in the post larvae wild shrimps. Thus, the extraction was conducted by using 2X hexadecyltrimethylammonium bromide (CTAB) protocol originally developed by Doyle and Doyle (1987) with slight modification for this study.

Process began with 25 mg homogenized samples were transferred into 1.5 mL microcentrifuge tube. A volume of 700 µL CTAB and 10 µL Proteinase K were added and heated at 55 °C in a heat block for 15 minutes, incubated at 60 °C and left overnight. On the next day, samples were taken out from incubator and was added with 700 µL chloroform isoamyl alcohol (CIA). The mixture was thoroughly shaken in the fume hood and centrifuged at 11000 rpm for 15 minutes. A double layer was formed and 500 µL of the supernatant was transferred into another 1.5 mL microcentrifuge. The solution was mixed well with 500 µL of cold absolute ethanol and incubated overnight at 20 °C.

This was followed by centrifugation at 11000 rpm for 15 minutes on the next day. The precipitated pellet formed was added with a volume of 600 µL of 70 % ethanol and 25 µL 3M NaCl, and recentrifuged at 13000 rpm for 15 minutes. After centrifugation, the solution was discarded, with only the pellet remaining. The pellet
was then air dried before dissolving in 40 µL milli Q water. The quality of the DNA was assessed by using NanoDrop® Quawell UV spectrophotometer. Finally, gel electrophoresis was run on a 1% of agarose gel and followed by staining with 1 µL Redsafe Nucleic Acid Gel Stain. After 20 minutes gel run, the banding patterns were observed by using Gene Flash, Syngene Bio Imaging, Synoptics Ltd, UK. A 1 µL volume of DNA was used to quantitate the concentration of DNA by using NanoDrop® Quawell UV spectrophotometer. The optical density (OD) values accepted for satisfactory concentration was in the range of 1.8-2. MilliQ water was used as blank solvent.

3.4 16S rRNA gene sequencing for metagenome analysis

Sequencing was carried out by Shanghai Majorbio Pharmaceutical Technology Co., Ltd. (Shanghai, China) using the Illumina MiSeq platform. After the sequencing, the raw data was first processed by merging the paired reads. Illumina Miseq sequencing can produce millions of short single-end reads of 75 to 300 bp in a single run in FASTQ format. However, when the target DNA fragments (16S rRNA gene) only produce half the length of the single-end reads, Illumina sequencing will generate paired-ends reads by sequencing the forward and reverse strands of each target 16S rRNA gene fragment. PEAR software was used to merge the 16S rRNA gene fragment to create overlapping region between them. This is important for correcting sequencing errors and potentially yield sequences of higher quality (Zhang et al., 2013). The pair-ended 16S rRNA gene at V1-V3 region was carried out using 27F and 907R primers (Mao et al., 2012) that possess 12 bp barcode tags. Then, the process was continued by trimming the sequences to remove the primers, barcode and adapter regions sequences by using an internally developed algorithm. The trimmed 16S rRNA sequences were then proceeded for clustering. UCLUST is a clustering
method that exploits USEARCH to assign sequences to clusters (Edgar, 2010). It was used to cluster the seed sequences and each cluster was then sorted by length and clustered with a 3% divergence cut-off to create centroid clusters. Clusters containing only < 2 sequences or < 100 bp in length were then removed. Seed sequences were again clustered at a 3% divergence level to confirm whether any additional clusters appeared. The of 16S rRNA read clustered into groups called Operational Taxonomic Units (OTUs) and typically at a 97% identity threshold. Consensus sequences from these clusters were then accurately obtained using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads were then analysed to remove chimaeras utilizing UCHIME in the de novo mode (Edgar et al., 2011). After chimaera removal, each consensus sequence and its centroid cluster were de-noised in UCHIME in which base position quality scores of >30 acted as the de-noising criterion. Sequence de-replication and OTU demarcation were further performed in USEARCH and UPARSE for constructing OTUs de novo from reads that achieved high accuracy in biological sequence recovery and improved richness estimates on bacterial communities. OTUs were then aligned using MUSCLE (Edgar, 2004) and FastTree (Price et al., 2010) that infer approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang et al., 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis et al., 2006).

3.5 Statistical analysis

To validate the metagenomics and bioinformatics data, the analysis of the bacterial diversity within samples (alpha diversity) and between samples (beta diversity) were applied. Moreover, it is important to assess the differential bacterial diversity composition for better understanding of the bacterial diversity. The relative
abundance of bacterial diversity in the four different localities was compared by using PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK), respectively, to conduct permutation multivariate analysis (Anderson et al., 2005). For this analysis, results of data collected from the MiSeq Illumina-based 16S rRNA gene sequencing were tabulated with the size bins combined across the samples, square root-transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients, Menhinick and Margalef indices. PERMANOVA was conducted using default settings with 9999 permutations. Multiple pairwise comparisons of alpha-diversity and beta-diversity were also performed to measure the abundance of the species between sampling sites (Whittaker 1960, Tuomisto and Ruokolainen, 2006, Barwell et al., 2015). The PERMANOVA-derived significance values were considered significant when $P < 0.05$. A summary of the overall workflow of the whole project is presented in Figure 3.5.

**Figure 3.5** Schematic diagram of project workflow
Samples were collected from four locations along the Merbok River, labelled as follows; Lalang River (St 1), Semeling River (St 2), Keluang River (St 3), and, Terus River (St 4).

4.1 Water parameters

Water parameters namely water depth (WD), turbidity (TURB), salinity (SAL), pH, surface temperature (TEMP), and dissolved oxygen (DO) were measured at all the sampling sites. St 3 was the deepest among sampling sites at 124 cm water depth while St 1 was the shallowest at only 38.5 cm depth. St 3 was also the most turbid compared to other sampling sites. Salinity, temperature, and dissolved oxygen values were found to be inversely proportional with distance to open sea where the values increased towards the open sea. The lowest salinity recorded was in St 1 at 10 ppt and the highest in St 4 at 24 ppt.

Similarly, St 1 showed the lowest temperature at 27.3°C and St 4 showed the highest temperature at 31.1°C. Likewise, dissolved oxygen value was lowest in St 1 at 4.4 mg/L while St 4 recorded the highest at 7.77 mg/L. However, pH value showed slight variation from this trend. St 1 showed the highest pH value at 6.8 while St 2 showed the lowest with pH value of 5.8. The results of water parameters collected are shown in Table 4.1.
### Table 4.1 Data for water parameters collected at St 1, St 2, St 3 and St 4.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WD (cm)</td>
</tr>
<tr>
<td>Lalang River (St 1)</td>
<td>38.5</td>
</tr>
<tr>
<td>Semeling River (St 2)</td>
<td>65.3</td>
</tr>
<tr>
<td>Keluang River (St 3)</td>
<td>124.0</td>
</tr>
<tr>
<td>Terus River (St 4)</td>
<td>113.5</td>
</tr>
</tbody>
</table>

#### 4.2 Bacterial community diversity based on the metagenomic approach

#### 4.2.1 Total number of bacterial compositions generated from each site

The NGS data provided an overall assessment of the lifeform diversity at the sampling sites. Based on the sequences generated, 93% of the operational taxonomic unit (OTUs) was assigned to the bacteria domain with only to 7% eukaryota and 0.1% archaea (Figure 4.1). The greatest number of OTUs generated were from St 2 (61442) followed by St 1 (44891), St 3 (38981) and St 4 (26408) (Table 4.2).
Figure 4.1 Bacterial compositions at domain level generated from 16S rRNA gene amplicon sequencing in total samples; St 1, St 2, St 3 and St 4.

Table 4.2 Total number of OTUs generated from each sample.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of OTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>St 1</td>
<td>44891</td>
</tr>
<tr>
<td>St 2</td>
<td>61442</td>
</tr>
<tr>
<td>St 3</td>
<td>38981</td>
</tr>
<tr>
<td>St 4</td>
<td>26408</td>
</tr>
</tbody>
</table>

4.2.2 Identification Relative abundance of bacterial phyla interacting with post larvae wild shrimps in each site

4.2.2(a) Relative abundance of bacterial phyla interacting with post larvae wild shrimps in St 1

The bacterial diversity detected in St 1 was dominated by phyla Proteobacteria (55.8%), Actinobacteria (23.2%), Firmicutes (12.4%) and Bacteroidetes (6.6%). Within the phylum Proteobacteria, class Alphaproteobacteria (40.7%) was found at highest abundance compared to Gammaproteobacteria (39.4%) and Betaproteobacteria (18.0%). Within the phylum Actinobacteria, 99% of the bacteria detected belongs to class Actinobacteria. The third dominant phylum in St 1
was Firmicutes. Within this phylum, class Bacilli showed the highest abundance at 87.9% and class Clostridia a distant second at only 10.5%. Then, in phylum Bacteroidetes, class Flavobacteria was the highest detected (49.3%), followed by Cytophagia (15.1%), Sphingobacteriia (14.6%), and Chitinophagia (1.1%). Phyla Acidobacteria, Planctomycetes and Cyanobacteria were detected with < 1% abundance from the total bacterial diversity. The remaining 1.6% bacteria which could not be classified bacteria were grouped as “other bacteria”. (Figure 4.2).

![Bacterial community diversity of St 1](image)

**Figure 4.2** Bacterial community composition of St 1 determined by using Illumina Miseq 16S rRNA gene amplicon sequencing
4.2.2(b) Relative abundance of bacterial phyla interacting with post larvae wild shrimps in St 2

In St 2, the most abundant bacterial phylum was Proteobacteria (65.4%). This was followed by phyla Actinobacteria (21.5%), Firmicutes (6.5%) and Bacteroidetes (3.4%). Class Alphaproteobacteria showed the highest abundance (55.3%) in phylum Proteobacteria compared to Gammaproteobacteria (22.8%) and Betaproteobacteria (17%). The most abundant class of phylum Actinobacteria was class Actinobacteria (96.6%). Phylum Firmicutes was dominated by class Bacilli (64.1%), class Clostridia (33.8%) and class Negativicutes (1.8%). Class Thermolithobacteria and class Tissierellia were detected at < 1%. In phylum Bacteroidetes, class Sphingobacteria (32.4%) was in highest abundance followed by Flavobacteriia (16.9%), Bacteroidia (7.4%), Cytophagia (8.9%) and Chitinophagia (1.1%). Other phyla such as Planctomycetes, Gemmatimonadetes, Fusobacteria, Chloroflexi and Acidobacteria accounted for < 1% present. Of the total bacterial diversity in St 2, 2.3% could not be classified and therefore grouped as “other bacteria”. (Figure 4.3).
Relative abundance of bacterial phyla interacting with post larvae wild shrimps in St 3

In St 3, almost half of the bacterial diversity was found to belong to phylum Proteobacteria (49.7%). From this, 63% of the bacteria in the phylum Proteobacteria belonged to class Alphaproteobacteria followed by Betaproteobacteria (24.5%) and Gammaproteobacteria (11.8%). Phylum Actinobacteria was found to be the second highest in abundance at 38.6%. In addition, almost all the bacteria from phylum Actinobacteria belonged to class Actinobacteria (99.1%) while in combination class Coriobacteriia, class Rubrobacteria and class Thermoleophilia accounted for only...
0.9%. Phylum Firmicutes only made up 7.3% of the bacterial composition in St 3 with 98.8% belonging to the class Bacilli and 1.1% belonging to the class Clostridia. Phylum Bacteroidetes made up 2.5% of the bacterial diversity with percentage abundance according to class as follows; Sphingobacteriia (47.7%), Cytophagia (40.5%), Bacteroidia (2.9%), and Flavobacteriia (1.8%). Finally, 2% of the total bacterial diversity belonged to other phyla such as Acidobacteria and Cloroflexi. (Figure 4.4) classified as other bacteria.

![Bacterial community diversity of St 3](image)

**Figure 4.4** Bacterial community composition of St 4 determined by using Illumina Miseq 16S rRNA gene amplicon sequencing

4.2.2(d) **Relative abundance of bacterial phyla interacting with post larvae wild shrimps in St 4**

Phylum Proteobacteria was found to be the most abundant phylum in St 4, at 54.5% of the total bacterial diversity. From this, 51.9% of the Proteobacteria belonged to the class Alphaproteobacteria, 23.9% to the class Betaproteobacteria, 20.2% to class Gammaproteobacteria and the least abundant class Deltaproteobacteria.
at 3.8%. Phylum Actinobacteria represented almost 25.6% of the total abundance, and 97.2% was composed of the class Actinobacteria. Class Acidobacteria made up 2.1% within this phylum while the classes namely Coriobacteriia, Rubrobacteria and Thermoleophilia were detected at less than 1%. Phylum Firmicutes formed 9.5% of the diversity in St 4. Within this phylum Firmicutes, the Class Bacilli (65.8%), class Clostridia (27%) and class Negativicutes (6%) were the dominant taxa. Class Tissierellia and class Thermolithobacteria only made up 1% of the diversity. Phylum Bacteroidetes showed 7.7% abundance with class Sphingobacteria (30.5%) followed by Flavobacteriia (23.3%) and Cytophagia (12.1%) being the three most abundant. Class Bacteroidia and class Chitinophagia showed lower abundance at only 4.1% and 2.3%, respectively. The other phyla (2.7%) were found at less than 1% each and were classified as “other bacteria” in St 4. (Figure 4.5).

**Figure 4.5** Bacterial community composition of St 4 determined by using Illumina Miseq 16S rRNA gene amplicon sequencing
4.2.3 Overall relative abundance of bacterial phyla interacting with post
larvae wild shrimps in Merbok river

Accordingly, twenty-eight bacterial phyla were discovered interacting with wild post larvae shrimp along the Merbok River. All sites showed the same pattern of bacterial diversity dominated by bacterial phyla; Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Figure 4.6). The percentage abundance reads of phylum Proteobacteria ranged from 50.4% to 66.3%, which were the most dominant bacteria in each sample. This was followed by phylum Actinobacteria ranging from 21.9% to 39.2%, phylum Firmicutes between 6.6% to 12.6% and Bacteroidetes between 2.5% to 6.7%. The remaining bacterial phyla namely; Armatimonadetes, Deferribacteres, Aquificae, Nitrospirae, Synergistetes, Nitrospinae, Thermodesulfobacteria, Gemmatimonadetes, Verrucomicrobia, Elusimicrobia, Spirochaetes, Fibrobacteres, Alphaproteobacteraeota, Fusobacteria, Chloroflexi, Acidobacteria, Chlamydiae, Planctomycetes, Cyanobacteria, Calditrichaeota, Chrysiogenetes, Ignavibacteriae, Dictyoglomi and Tenericutes were detected is less than 1% for each site (data is not shown).

Figure 4.6 Comparative abundance among stations of dominant bacterial phyla interacting with post larvae wild shrimps in Merbok river
4.2.4 Differential occurrence of classes within phyla Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes for each site

Phyla Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were found to be the most dominant phyla in each site. A more detailed analysis of differential proportion of classes within these phyla for each sample was conducted. **Figures 4.7 - 4.10** and **Tables 4.3 - Table 4.6** illustrate the detailed distribution of the major classes within the most abundant phyla for each station; Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes.

Within the phylum Proteobacteria, class Alphaproteobacteria dominated and attributed to more than 40% of the samples while Betaproteobacteria and Gammaproteobacteria generally showed similar proportions in the combined study site. However, in St 1, class Gammaproteobacteria had higher occurrence compared to the class Betaproteobacteria. Class Deltaproteobacteria occurred at less than 5% in St 1, St 2 and St 4. However, it was not detected in St 3.

![Differential proportion of sequence assigned within the phylum Proteobacteria](image)

**Figure 4.7** Comparison of differential proportion of classes within the phylum Proteobacteria in St 1, St 2, St 3, and St 4 samples
Within the phylum Actinobacteria, the differential proportion analysis estimated class Actinobacteria at more than 96% of the phylum Actinobacteria found. St 1 and St 3 had > 99% class Actinobacteria in phylum Actinobacteria. Only 2% of class Acidobacteria were found in St 2 and St 4 while the other classes were found at less than 1% in all samples. (Figure 4.8).

### Table 4.3 Percentage of differential proportion within the phylum Proteobacteria for each station

<table>
<thead>
<tr>
<th>Classes of Proteobacteria</th>
<th>Percentage of differential proportion within the phylum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St 1</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>40.7</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>18.0</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>39.4</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>1.6</td>
</tr>
</tbody>
</table>

### Figure 4.8 Comparison of differential proportion within the phylum Actinobacteria in St 1, St 2, St 3, and St 4 samples
Table 4.4 shows the percentage of differential proportion of bacterial diversity within the phylum Actinobacteria for each station.

**Table 4.4 Percentage of differential proportion within the phylum Actinobacteria for each station**

<table>
<thead>
<tr>
<th>Classes of Actinobacteria</th>
<th>Percentage of differential proportion within the phylum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St 1</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>99</td>
</tr>
<tr>
<td>Acidobacteriia</td>
<td>0.3</td>
</tr>
<tr>
<td>Coriobacteriia</td>
<td>0</td>
</tr>
<tr>
<td>Rubrobacteria</td>
<td>0.1</td>
</tr>
<tr>
<td>Thermoleophilia</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Within phylum Firmicutes, class Bacilli was found to be most dominant. It was detected with a frequency of more than 64.1% in St 2, and close to 98.8% in St 3. The phylum Firmicutes dominated in St 3. The class Clostridia were detected in all samples as the second highest in abundance of phylum Firmicutes. However, the percentage of class Clostridia varied with ≈ 33.3% at St 2 only 1.1% were detected. Class Negativicutes was detected in St 1 (0.9%), St 2 (1.8%) and St 4 (6%) but not in St 3 while, Class Tissierellia and Thermolithobacteria were detected at less than 1% at each site (**Figure 4.9**). Percentage values of bacterial diversity within the phylum Firmicutes are shown in **Table 4.5**.
Figure 4.9 Comparison of differential proportion within the phylum Firmicutes in St 1, St 2, St 3, and St 4 samples

Table 4.5 Percentage of differential proportion of bacterial diversity within the phylum Firmicutes for each station.

| Classes of Firmicutes | Percentage of differential proportion within the phylum (%)
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>St 1</td>
</tr>
<tr>
<td>Bacilli</td>
<td>87.9</td>
</tr>
<tr>
<td>Clostridia</td>
<td>10.5</td>
</tr>
<tr>
<td>Thermolithobacteria</td>
<td>0</td>
</tr>
<tr>
<td>Tissierellia</td>
<td>0.2</td>
</tr>
<tr>
<td>Negativicutes</td>
<td>0.9</td>
</tr>
</tbody>
</table>
Within phylum Bacteroidetes, class Sphingobacteriia dominated in St 2, St 3 and St 4. However, class Flavobacteriia was dominant in St 1. The dominant phylum in St 3 was mainly comprised of class Sphingobacteriia and Cytophagia with a low percentage of Flavobacteriia. Class Bacteroidia was present in less than 10% in each site (Figure 4.10). Table 4.6 shows the percentage of differential proportion of bacterial diversity within the phylum Bacteroidetes for each station.

![Differential proportion of sequences assigned within the phylum Bacteroidetes](image)

**Figure 4.10** Comparison of differential proportion within the phylum Bacteroidetes in St 1, St 2, St 3, and St 4 samples

**Table 4.6** Percentage of differential proportion of classes within the phylum Bacteroidetes for each station

| Classes of Bacteroidetes | Percentage of differential proportion within the phylum (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidia</td>
<td>St 1  1.3  St 2  7.4  St 3  2.9  St 4  4.1</td>
</tr>
<tr>
<td>Flavobacteriia</td>
<td>49.3  16.9  1.8  23.3</td>
</tr>
<tr>
<td>Sphingobacteriia</td>
<td>14.6  32.4  47.7  30.5</td>
</tr>
<tr>
<td>Cytophagia</td>
<td>15.1  8.9  40.5  12.1</td>
</tr>
</tbody>
</table>
4.2.5 Dominant bacteria according to class, order, family and genus interacting with post larvae wild shrimps in Merbok River

Eleven classes were detected as the most dominant bacteria associated with the wild post larvae shrimps. The dominant bacteria belonged to the classes Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Bacilli, Betaproteobacteria, Flavobacteria, Clostridia, Chitinophagia, and Sphingobacteriia, Deltaproteobacteria. Of these, the classes Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Bacilli were well distributed at all sites. However, the classes of Flavobacteriia, Clostridia, Chitinophagia, Sphingobacteriia and Deltaproteobacteria were each detected at a lower frequency than 5% (Figure 4.11).

![Comparative bacterial class abundance in St 1, St 2, St 3 and St 4 along Merbok River](image)

**Figure 4.11** Comparative bacterial class abundance in St 1, St 2, St 3 and St 4 along Merbok River
Twenty-four bacteria orders were detected in all four sites (as labelled in Figure 4.12). Of these, Rhizobiales, Bacillales, Sphingomonadales, Clostridiales and Streptomycetales were found to be the most abundant in each site. However, Chitinophagales, Cytophagales and Burkholderiales were also found to be abundant in St 3 and St 4. On the other hand, Propionibacteriales and Micromonosporales were abundant in St 1, but less abundant in other samples. Other orders were identified as less abundant but present >1% in each site. The orders were Sphingobacteriales, Flavobacteriales, Micrococcales, Saccharomycetales, Myxococcales, Rhodobacteriales, Corynebacteriales, Xanthomonadales, Enterobacteriales, Streptosporangiales, Rhodospirillales, Pseudonocardiales, Oceanospirillales, and Pseudomonadales. A summary of the orders present is presented in Figure 4.12.

Figure 4.12 Heatmap of bacterial order abundance in St 1, St 2, St 3 and St 4 along Merbok River
Eighteen families (as labelled in Figure 4.13) dominated the sampling sites. Streptomyces was the dominant bacterial family group associated with each sample. In addition to this, several families were also prominent at specific sites. For example, for St 1, Xanthomonadaceae, Bacillaceae, Microbacteriaceae, and Sphingomonadaceae was also abundant in addition to Streptomyces. St 2 was dominated by Pseudomonadaceae and Streptomyces while St 3 was dominated by Streptomyces and Burkholderiaceae. In St 4, Rhizobiaceae, Bradyrhizobiaceae, Pseudomonadaceae, Rhodobactericeae, Burkholderiaceae, Sphingomonadaceae in addition to Streptomyces were abundantly found. The results as presented by the heatmap (Figure 4.13) also include other families with > 1% abundance in addition to the dominant ones.

Figure 4.13 Heatmap of bacterial family abundance in St 1, St 2, St 3 and St 4 along Merbok River
Streptomyces spp., Mesorhizobium spp., Rhizobium spp., Bacillus spp., and Pseudomonas spp., were the most dominant bacterial genera identified from the total population. Total bacteria genera detected were 2717 in St 1, 2384 in St 2, 1880 in St 3 and 2798 in St 4. However, most of the genera were found to occur at < 1%. Streptomyces spp. were found to be the most abundant in each sample. Its occurrence was estimated at approximately 5% in St 1, 4% in St 2. In addition, Streptomyces spp. were also dominant in St 3 (8%) and St 4 (8%). Bacillus spp. abundance was estimated at about 3% in St 1 and St 3, as well as approximately about 2% in St 4 and 1% in St 2. Abundance of Rhizobium spp. was estimated at around 2% in all samples except in St 3, which showed only 1%. However, Pseudomonas spp. were found < 1% in St 3, compared to St 1 (6%), St 2 (4%) and St 4 (2%) (Figure 4.1).

**Figure 4.14** Comparative bacterial genera abundance in St 1, St 2, St 3 and St 4 along Merbok River
4.2.6 Comparison of bacterial diversity based on alpha (α) diversity and beta (β) diversity analyses

The analyses were conducted using PRIMER6 and PERMANOVA+ software (version 7.0.13; PRIMER-E, Ivybridge, UK). Estimates of diversity index, species evenness and species richness were calculated to quantify the alpha diversity while Bray-Curtis dissimilarity was calculated to assess beta diversity (Table 4.7). Shannon index is widely applied to measure the diversity index. The diversity indices were high for each sample. St 1 recorded the highest value among the samples. However, the evenness and equitability tests showed that the bacterial species evenness was highest in St 3. The Menhinick and Margalef indices showed that St 1 had the highest species richness with most taxa present within the sample.

Table 4.7 Comparison of diversity index, species evenness, and species richness in St 1, St 2, St 3, and St 4 samples.

<table>
<thead>
<tr>
<th></th>
<th>St 1</th>
<th>St 2</th>
<th>St 3</th>
<th>St 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evenness_e^H/S</td>
<td>0.6751</td>
<td>0.6616</td>
<td>0.7882</td>
<td>0.6011</td>
</tr>
<tr>
<td>Equitability_J</td>
<td>0.961</td>
<td>0.9576</td>
<td>0.9747</td>
<td>0.9481</td>
</tr>
<tr>
<td>Menhinick</td>
<td>99.15</td>
<td>81.51</td>
<td>83.17</td>
<td>66.54</td>
</tr>
<tr>
<td>Margalef</td>
<td>2151</td>
<td>1580</td>
<td>1230</td>
<td>1610</td>
</tr>
</tbody>
</table>

To calculate the beta diversity, the Bray-Curtis dissimilarity was plotted in non-metric MDS. The beta diversity between St 2 and St 4 was found to have high similarity while St 3 showed high dissimilarity of bacterial diversity from other samples. St 1 had the highest dissimilarity index compared to St2, St 3 and St 4. (Figure 4.15).
Figure 4.15 (a) Bray-Curtis dissimilarity plotted by non-metrical multidimensional scaling (NMDS) for quantification of beta diversity (b) Bray-Curtis dissimilarity plotted by non-metrical multidimensional scaling (NMDS) in 3D view.
4.3 Bacterial population of opportunistic pathogenic bacteria

Results of this study showed that the number of opportunistic pathogenic bacteria were too low and may not be significant to cause a disease outbreak. The Vibrio spp. were found to be at frequencies of 0.06% (St 1), and 0.09% (St 4) and not detected in St 2 and St 3. The Aeromonas spp. was found at frequencies of 0.3% (St 1), 0.01% (St 2) and 0.04% (St 4) and not detected in St 3. Flavobacterium spp. was found at frequencies of 0.9% in St 1, 0.5% in St 2, 0.4% in St 4 and not detected in St 3 Micrococcus spp. were not detected in St 1 but found at frequencies of 0.01% in St 2, 0.1% in St 3, 0.05% in St 4. Other opportunistic pathogenic bacteria that can cause diseases in marine animals such as Aliivibrio spp. and Pasteurella spp. were not detected in this study. In addition, coliform bacteria was also found but in low numbers (Escherichia spp. 0.06% (St 1), and 0.05% (St 4) and not detected in St 2 and St 3. Enterobacter spp. were found at 0.2% in St 1, 0.01% in St 2, 0.06% in St 4 and not detected in St 3. Klebsiella spp. were found at 0.2% in St 1, 0.01% in St 2, 0.05% in St 4 and not detected in St 3. In addition, Citrobacter spp. were found at frequencies of 0.2% in St 1, 0.05% in St 4 but not present in St 3 indicating the river was not contaminated at any significant levels by human and animal wastes. Pseudomonas spp. was found abundantly as shown in Figure 4.14.
5.1 Water parameters in Merbok River

As an estuarine river ecosystem, the Merbok River experiences daily tidal changes. As a consequence, there will be daily fluctuations in the water parameters (Lee et al., 2014). Furthermore, environmental disturbances from human activities such as residences, agriculture, aquaculture and recreational activities can affect the physicochemical conditions of the river (Gasim et al., 2009, Al-Badaii et al., 2013, Chen et al., 2018). The Merbok River is categorized in class III (slightly polluted) in the latest update in by the Department of Environment (DoE) Malaysia (Department of Environment Malaysia, 2016). Thus, although is some degree of pollution which has been attributed to the domestic sewage, agriculture and other factors as earlier stated, it appears not to be at a critical level. This study reports on the physicochemical parameters such as water temperature, salinity, dissolved oxygen (DO), turbidity and water depth along a salinity gradient from freshwater upstream to brackish water downstream. The parameters were collected in association with post larvae wild shrimp sampling. All collections were conducted during rainy season where shrimps were most abundant.

5.2 The Merbok River as a suitable habitat for shrimp and its associated bacterial community

Based on a single sampling activity, all parameters were still at levels suitable to support high shrimp species diversity. The water depth recorded at the riverbank increased from 38.5 cm at St 1 (Lalang river) upstream to a depth of 113.5 cm at Sungai Terus St 4 (Terus River) downstream. At all sites, shrimp samples were found in abundance. According to the Food and Agriculture Organisation guidance (FAO,
post larvae shrimp should be kept in 2-3 m depth with minimal water depth at 30 cm. Although, the FAO Guide was targeted to shrimp hatchery production, it gives an indication of the range for optimal survival of post larvae shrimp which could be extended to natural waters. Thus, in terms of water depth the Merbok River is suitable for supporting shrimp.

The water surface temperature ranged from 27.3 ºC (St 1) in the upstream to 31.1 ºC (St 4) in the downstream. This surface water temperature is slightly higher than deeper waters as it is affected by atmospheric temperature (Fatema et al., 2015). Nevertheless, the surface water temperatures are still in a range for shrimp survival. Temperatures between 27ºC to 28ºC are the preferred range for the larval stage, *M. rosenbergii* and 26ºC to 31ºC for larval growth for *Penaeus* spp., *Acetes* spp., *Lucifer* spp., and *Mysis* spp. (Agard et al., 1999, Arshad et al., 2011). Similarly, for the larval stage banana prawn, *Fenneropenaeus merguiensis* the optimum temperature need is between 29ºC to 33ºC (Zacharia and Kakati, 2004). Therefore, the water temperatures of Merbok river could support a wide range of post larvae shrimp growth as reflected by the high abundance of shrimp growth.

The salinity readings recorded ranged from 10 to 24 ppt along the Merbok River. As expected, the water salinity was found highest in St 4 at the downstream compared to upstream. Akin et al. (2005) mentioned that the high salinity in the downstream of estuarine was influenced by the high amount of sea water flowing into the river. However, the amount of sea water decreases going upstream of the river (Mansor et al., 2012). Salinity gives a big impact to survival and growth of marine and freshwater shrimps (Agard et al., 1999, Anger, 2003, Pan et al., 2007, Valencia-Castañeda et al., 2018, Ponce-Palafox et al., 2019). The adult giant river prawn, *M. rosenbergii* can grow and thrive from 0 to 15 ppt but is intolerant of salinity up to 20
ppt and above. However, its larvae have a narrower optimal range from 10 to 15 ppt with optimal temperature. Marine species such as *Fenneropenaeus merguiensis* display an optimal temperature for larvae development at 30 to 35 ppt. However, they can adapt to as low as 25 ppt (Zacharia and Kakati, 2004) which would allow the species to inhabit this river. Presumably many shrimp species of the Merbok River, in common with other brackish water environment has adapted to a gradient of salinity along the water environment. On the other hand, the salinity specialists could also find its own niche area to inhabit. Thus, salinity may also influence the distribution of shrimp populations.

pH values ranged from 5.8 (St. 2) to 6.8 (St. 1) and was found to be fairly uniform along the sampling sites. This corroborated with a previous study conducted by Mansor et al. (2012) which showed no significant differences between sampling sites along the Merbok River. Additionally, Fatema et al. (2014) showed that the minimum pH value in Merbok River was between 6.2 to 6.9 during the wet season. For shrimp survival, Anh et al. (2010) observed that at pH 7.5 to 8.5 the black tiger shrimp, *P. monodon* could grow to its maximum grow, but at pH 5.5 growth would be reduced (Allan and Maguire, 1992). A study conducted in seagrass beds of Johor Straits found that the larval stage of *Penaeus* spp., *Acetes* spp., *Lucifer* spp., and *Mysis* spp. prefer pH at pH 7.61 to 8.30 (Arshad et al., 2011). Even though pH recorded in Merbok River is lower than the preferred for most post larvae shrimps, but values recorded were still acceptable for post larvae shrimp’s survival.

The amount of dissolved oxygen needed for optimal growth, varies between organisms. Type and abundancy of species can survive in an estuarine water are determined by the amount of dissolved oxygen (DO) present (https://oceanservice.noaa.gov). Bottom feeders, crabs, oysters and worms need
minimal amounts of oxygen (1-6 mg/L), while shallow water fish need higher levels (4-15 mg/L) (Affan et al., 2018). The DO concentrations varied from 4.40 mg/L (St 1) in upstream and increased to 7.77 mg/L (St 4) in downstream waters. These levels could support a wide taxonomic range of organisms. Values increased from St 1 to St 4 nearer to the sea. DO concentration are expected to be inversely proportional with temperature. However, from the results obtained, DO concentration increased with temperature and salinity increases. This may due to the DO concentration in St 1 being depleted when large amounts of sewage enters the estuary or when nutrient loading is too high. Bacteria and other decomposer organisms then use oxygen to break down the organic matter resulting in reduced DO concentration. It is suggested that high DO concentration may indicate high abundance of bacteria (Leong et al., 2018).

Other parameters that were recorded during sampling was turbidity. The turbidity recorded ranged from 38.5 cm (St 1) in to 124.0 cm (St 3) along the river. However, a previous study by Mansor et al. (2012) showed a comparatively much lower range of turbidity in this mangrove estuary of 2.5 to 25 cm and lowest recorded during dry seasons and highest are during wet seasons. The current observations could be attributed to the heavy rain during the sampling activity that increased the water movement (Young et al., 2017) and increase the turbidity.

Accordingly, like shrimps, the surrounding environment can influence the microbial diversity associated with the shrimp. Diversity of microbial including bacteria are influenced by water quality factors such as temperature, salinity and dissolved oxygen (Apple et al., 2008, Tamames et al., 2010, Qu et al., 2018). The effect of environment on bacterial diversity has been widely studied in free living bacteria in sediments and waters but limited documentation is available on the microbiome associated with host (Ismail et al., 2017, Priya et al., 2018, Pramanik et al., 2019).
Cardona et al. (2018) found that the number of bacteria genera *Kineococcus* spp. and *Brevibacterium* spp. associated with the skin, chuff, and rectum of dolphin in aquarium was significantly correlated with changes in water temperature. The diversity index showed that all localities have high species evenness and richness of bacteria in the post larvae wild shrimp. However, this water quality factors also included pathogenic bacteria to growth. Prayitno and Latchford (1995) mentioned that, pH and salinity parameter are major factors influencing infection related to *Photobacterium* sp. and *Vibrio* sp. outbreak in shrimp larvae. A pH value more than 6.0 was suggested as optimum for pathogenic bacteria such as *Photobacterium* spp. and *Vibrio* spp.

Akazawa and Eguchi (2017) reported that extreme pH led to stress and weakening of the immune system of *L. vannamei* while *Vibrio alginolyticus* thrived very well at these conditions. Previous studies have shown that *V. alginolyticus* is able to invade the shrimps and cause a disease outbreak such as vibriosis (Ahmed et al., 2016). Thus, based on results of this study, the *Vibrio* spp. and *Photobacterium* spp. were found to be in insufficient quantity to cause disease outbreak in the wild shrimps of the Merbok river estuary. Water surface temperature, pH, salinity and dissolved oxygen seemed to support the diversity of wild shrimps in this area.

Malaysia has national water quality standard to classify cleanness of Malaysia water. Several parameters such as dissolved oxygen, pH, salinity, temperature, turbidity and presence of the pathogenic coliform bacteria have been listed as indicators for classification. Fatema et al. (2014) classified the Merbok River as having poor water quality based on dissolved oxygen, nitrate, nitrite, and ammonia concentrations. But their study did not include the bacterial diversity that is also an important indicator of the health status of a river and its inhabitants. Although data for nitrate, nitrite, and ammonia concentrations were not collected because it is not within
the scope of the study to conduct a comprehensive ecological survey across the Merbok River, the variables studied offered a glimpse of the ecological health of Merbok River This study showed that human activities have not detrimentally impacted post larvae shrimps. Furthermore, although the current study was only based on a one-off sampling, its overall classification by DoE in 2016 of a class III river (slightly polluted) indicated that it could still support fisheries and more tolerant species.

Previous studies (Kenzaka et al., 2001, Eisakhani and Malakahmad, 2009, Ghaderpour et al., 2015) on Malaysian rivers have shown that polluted rivers are largely associated with *Klebsiella* spp., *Hafnia* spp., *Serratia* spp., *Enterobacter* spp., *Citrobacter* spp. and *Escherichia coli*. An example is the urban Kelang River which was recorded to have a high presence of *Bacteroides* spp. and coliform bacteria such as *Escherichia coli*. Kenzaka et al. (2001) and Ghaderpour et al. (2015) detected the presence of *Escherichia coli* in the water and sediment of Matang mangrove that may have originated from fish farms and residences at the upstream of the estuaries.

### 5.3 Bacterial communities of post larvae wild shrimps

Bacterial diversity associated with host shrimps has been extensively described in adults (Liu et al., 2011, Dabadé et al., 2016, Iehata et al., 2017, Alfiansah et al., 2018) but poorly understood in larval shrimps. A few studies conducted on post larvae shrimps included that by Rungrassamee et al. (2013) on *P. monodon* and Huang et al. (2016), Zheng et al. (2017), and Sadat et al. (2018) targeting bacterial associated with cultural *L. vannamei*. Hossain et al. (2017) studied the bacteria associated in cultured post larvae of *M. rosenbergii*. However, there is no literature on studies of bacteria associated with wild post larvae shrimps in Malaysia by using metagenomic approach.
Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were found to be the dominant phyla in the studied area. This is similar to studies conducted by Tzeng et al. (2015) where they found that Proteobacteria, Firmicutes and Actinobacteria were the major phyla in adult oriental river prawn, *Macrobrachium nipponense*. Interestingly, bacteria communities detected in shrimp paste was also dominated by the same phyla obtained (Qi et al., 2018). Previous studies by Huang et al. (2016) and Zheng et al. (2017) reported that bacterial diversity of shrimps is significantly difference at genus level but at phylum level, the dominant bacteria are similar even after going through processing. This may due to the common occurrence of Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes at all life stages and species of the shrimp. A study conducted by Liu et al. (2011) on the intestinal bacteria diversity of adult oriental shrimp, *Fenneropenaeus chinensis* found the dominant bacterial phyla to Firmicutes, Proteobacteria and Bacteroidetes while Dabadé et al. (2016) reported that Firmicutes and Proteobacteria were the major phyla found in fresh tropical shrimps, *P. notialis* that inhabits brackish water. Thus, in summary, at the post larvae stage, the dominant phyla were common; Proteobacteria, Bacteroides, Firmicutes and Actinobacteria (Rungrassamee et al., 2013, Huang et al., 2016, Zheng et al., 2017). However, relative abundance composition of bacterial lineages vary at the class and family levels.

The highest abundance classes detected in this study were Actinobacteria, Alphaproteobacteria, Gammaproteobacteria, Bacilli and Betaproteobacteria. This study corroborates the study by Zheng et al. (2017) which showed dominance of the classes Alphaproteobacteria, Gammaproteobacteria, and Actinobacteria in their study of cultured post larvae of *L. vannamei*. Furthermore, these are not only associated with hosts, but Actinobacteria is also widely distributed and commonly found in terrestrial
and aquatic ecosystems including in the mangrove river ecosystem (Ward and Bora, 2006, Gupta et al., 2009, Ruiz-González et al., 2015, Fan et al., 2019). It is popular group that produces natural metabolite which is useful as antibiotics. More detailed studies should be conducted on mangrove Actinobacteria to investigate its bioactive compounds that are believed to have the potential in drug discovery for antifungal, antibiotics, antimicrobials and anticancer treatments (Azman et al., 2015). *Streptomyces* spp. is a major species in Actinobacteria which is abundantly found in their natural habitat (Ventura et al., 2007). It could be hypothesised that this is one of the factors for post larvae wild shrimps in Merbok River to be almost free from diseases.

Genera that were abundantly found in post larvae wild shrimp inhabitants in the Merbok River at each site were *Streptomyces* spp. *Mesorhizobium* spp., *Bacillus* spp. and *Pseudomonas* spp.. Previous studies had reported that *Pseudomonas*, *Aeromonas* and *Bacillus* appeared to be the common genera associated with *M. rosenbergii* (Kennedy et al., 2006) but Hossain et al. (2017) reported that pathogenic bacteria such as *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Enterococcus casseliflavus*, were also found in high quantities in cultured *M. rosenbergii* in Bangladesh water opposite with bacterial diversity result detected in Merbok River whereas the pathogenic bacteria (*Enterobacter* spp., *Klebsiella* spp. and *Enterococcus* spp.) were less or not found in each site that is may due to influenced from the environment factors.

Genera *Streptomyces* spp. and *Bacillus* spp. are known to produce antibiotics as secondary metabolites. Thus, they are used as probiotics in shrimp aquaculture (Ziaei-Nejad et al., 2006, Tan et al., 2016, Sadat et al., 2018). High abundance of “good” bacteria found associated with post larvae wild shrimps maintained the health
of the shrimp community in this area. It could be the reason why pathogenic bacteria such as *Vibrio* spp. was not detected in St2 and St 3 and only in low frequencies in St 1 and St 4.

5.4 Relationships between the post larvae wild shrimp bacterial diversity and environmental conditions

This study also showed the trend of human activity effects on the bacterial diversity associated with post larva shrimp inhabitants in Merbok River. Some species of bacteria for example, *Coliform, Escherichia coli, Streptococcus* sp., *Pseudomonas* sp., *Vibrio* sp., *Clostridium* sp., *Bifidobacterium pseudolongum, Arcobacter* sp., *Thiobacillus* sp., act as indicators of household waste (human and animal faeces, household wastes and others), heavy metal pollution and crude oils (Sumampouw and Risjani, 2014). From this list, only *Pseudomonas* spp. were abundantly found in St 1 (6%), St 2 (4 %) and St 4 (2%). However, in St 3, although genus *Pseudomonas* spp. was detected, but only at a low frequency of less than 1 %. Similarly, Clostridia was the second highest in abundance within phylum Firmicutes but was found at a frequency of less than 1 % present in St 3. Interestingly, bacteria diversity in St 3 was markedly different compared to other samples. While the dominant genera in the other three stations as mentioned above were low in frequencies at St3, class Bacilli occurred at almost 99% in St 3 compared to low frequencies at samples St 1, St 2, and St 4. Bacilli is a phosphate solubilizing bacterium that provides soluble phosphorus as a nutrient to the mangroves plant (Sahoo and Dhal, 2009).

This scenario could be attributed to St 3 being located in an undisturbed mangrove area and thus, Bacilli were abundantly found compared to other sampling sites that recorded less than 60 % occurrence. Other than that, Flavobacteria from
phyla Bacteroidetes are present only 1.8 % in St 3 and dominancy with Sphingobacteriia and Cytophagia. On the other hand, St 1 had almost 50% incidence from total abundance of Bacteroidetes. A more in-depth study is required to explain the factors that is driving this condition. Based on the microbiome evidence, despite being categorized as class III river and influenced by human activities that may disturb the inhabitants (hosts) and associated bacteria, the health of the Merbok River is still manageable and under control. However, all efforts should be made to ascertain its continual cleanliness and to ensure sustainability of this mangrove estuarine biodiverse hotspot.

5.5 Effectiveness of metagenomic approach to assess bacteria associated with post larvae wild shrimps

Metagenomics approach has revolutionized microbiology field in exploitation of microbial communities present in complex ecosystems. Metagenomics outcomes has improved our knowledge of morphological, physiological, and ecological features of bacterial taxa (Handelsman, 2004). To analyse bacterial diversity, 16S rRNA gene amplicon sequencing has allowed the investigations of microorganisms that cannot be cultivated by routine methods and have also been useful for phylogenetic studies (Pontes et al., 2007). Thus, exploration of bacterial communities and their functions is rapidly becoming a focus for further study.

The 16S rRNA gene has several characteristics that favour its use for molecular studies. Among these are its highly conserved regions among different species and ease of its manipulation (Baker et al., 2003, Rosselló-Mora and Amann, 2001). Today, it is believed that more than 53 divisions exist in the bacteria domain which has been identified based on 16S rRNA gene sequence and most of the bacteria described are not cultivable (Pontes et al., 2007).
Although the 16S rRNA gene sequences derived from Illumina-sequenced metagenomes could be prone to base-composition biases that may not randomly distributed (Aird et al., 2011, Nakamura et al., 2011), a number of protocols and base call algorithms have been developed to minimize such biases and improve the error rate of Illumina sequencing (Harismendy et al., 2009, Aird et al., 2011). In this study, PEAR, Usearch, UPARSE and UCHIME software were used to minimize biases and generate more meaningful sequence.

16S rRNA gene sequences requires the use of bioinformatics tools to efficiently and reproducibly process the large amount of data generated from amplicon sequencing to derive a taxonomic overview. This is the most challenging part and requires high computer skills. To process the large amount of data, high performance computers are also needed to support the analysis. The RDPipeline (Wang et al., 2007) and Greengenes pipelines (DeSantis et al., 2006) were used in this study to analyse 16S rRNA gene sequencing data. Besides, other various tools including QIIME (Caporaso et al., 2010), MEGAN (Huson et al., 2007) and MG-RAST (Meyer et al., 2008) also available to help biologists in bioinformatics data analysis. However, there is no standard pipeline for metagenomes analysis but the analysis commonly included algorithms for quality control, clustering of similar sequences, assigning taxonomy, calculating diversity measures and visualising results (Plummer et al., 2015).

Recently, the availability and reduced cost of high-throughput sequencing of the small ribosomal subunit 16S (16S rRNA) gene have facilitated more in-depth analyses of shrimp bacteria diversity (Cornejo-Granados et al., 2017). A great diversity of microbiome that could not be identified with traditional culture dependent methods was discovered. Diversity of the bacteria associated with shrimp species, such as *P. monodon* (black tiger shrimp) (Rungrassamee et al., 2014), *P. merguiensis* (banana
shrimp) (Oxley et al., 2002), and *L. vannamei* (Pacific whiteleg shrimp) (Huang et al., 2016), have been described under cultured conditions but bacteria diversity has only been studied in the intestine of *P. monodon* (Rungrassamee et al., 2014), *P. merguiensis* (banana shrimp) (Oxley et al., 2002) and *L. vannamei* (Cornejo-Granados et al., 2017). Unfortunately, these studies have focused on a single or only a few target species of commercial importance while the non-commercial ones which may have high ecological value have been given little or no attention.

To date, there has been no literature on studies related to bacterial diversity associated with wild shrimps in Malaysia and only gut microbiome diversity associated with *L. vannamei* using metagenomics in aquaculture setting have been reported (Zoqratt et al., 2018). However, the utilisation of the metagenomics approach is still challenging in the Malaysian research scenario. Even though cost of high throughput sequencing have reduced worldwide but it is still costly in Malaysia. Besides that, there is limited expertise and facilities in metagenomics studies in Malaysia.
CHAPTER 6
CONCLUSIONS

This study achieved to address the two objectives outlined in the Introduction, namely 1) identifying of microbial community diversity associated with the wild post larvae shrimps in Merbok Rivers and 2) to determine key major abundance of the wild post larvae shrimp microbial species and overall microbial community structure. As a conclusion, metagenomics approach 16S rRNA amplicon sequencing are able to reveal unculturable bacteria species and wide range of bacteria species also can be detected. Results from this study shown that, lowest OTUs generated were 26 408 OTUs in sample St 4 while highest with 61 442 OTUs in St 2 sample which then classified into twenty-eight phyla of bacteria. The abundance and diversity of bacteria associated with post larvae wild shrimps can be identify. Dominated phyla bacterial communities associated with post larvae shrimps detected through metagenomics approach were Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. From the total populations, *Streptomyces* spp., *Mesorhizobium* spp., *Rhizobium* spp., *Bacillus* spp., and *Pseudomonas* spp., were the most dominant bacterial genera identified. The abundance of these non-pathogenic bacteria in particular, *Streptomyces* spp., *Bacillus* spp. and *Pseudomonas* spp. versus low or absence of pathogenic bacteria such as *Vibrio* spp. and *Photobacterium* spp. indicated that the post larvae wild shrimps in Merbok River had a healthy status. The low frequencies of pathogenic lower the risks of infections. Thus, the human activities such as aquaculture, residences and plantations do not seem have any major adverse effects on the bacterial populations in wild post larvae shrimps. Therefore, at the present moment, despite being exposed to various human activities, the Merbok River and its mangrove surroundings can still serve as a good spawning and nursery sites of shrimps.
Though physicochemical parameter (dissolved oxygen, salinity, turbidity, pH and temperature) were collected during sampling, there is no sufficient data to indicate the health status of the river. Other parameter such as total nitrogen (TN), total phosphorus (TP) and metal concentrations should be collected together, and data should be collected periodically. Yet, result shown that high diversity of bacteria through the sites but low abundant of *E. coli, Salmonella* spp. and other indicator bacteria to pollutant were shown Merbok River water is safe and there is no contamination from human and animal waste.

For future works, it is recommended that more data is collected to conduct a comprehensive bacterial diversity investigation. It is suggested that bacterial diversity survey from the water samples or cultural organisms in Merbok River be conducted as comparisons to the bacterial diversity associated with wild host. Besides that, comparative studies between dry and wet seasons be done to observe differences in bacterial diversity. It is hoped that the current study will stimulate similar researches to be conducted in other species and ecosystems in Malaysia. The information obtained will be critical in ensuring ecosystem conservation and sustainability will move in tandem with development. It will also assist in understanding the patterns in microbial ecology across the biomes and habitats of our planet.
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