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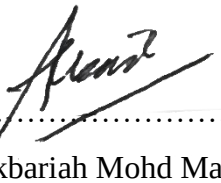

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
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MARCH 2017

I declare that this thesis entitled "*Marine Microbial Community Distribution in Malaysia Seawater Off-Terengganu Coast of South China Sea*" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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"*Blessed is He in Whose Hand is the dominion, and He is able to do all things, Who has created death and life, that He may test you which of you is best in deed, and He is the All-Mighty, the Oft-Forgiving*."

[Al-Mulk 67:1-2]

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ABSTRACT

Marine bacteria play a vital role in regulating global biochemical cycle for billions of years, and their function has been widely explored for the past fifty years. Marine bacteria exploration is considered as difficult and precarious, but every finding is fruitful in providing information to generate a better understanding of its purpose in the seawater. Marine bacteria exploration in Malaysia coastline is considered as new with no impactful data to represent the bacteria distribution in Malaysia's coastline, specifically heading towards the South China Sea. The purpose of this study is to assess bacteria diversity off-Terengganu coast as the foremost marine bacteria abundance screening in these areas. In this study, surface sea sediment that contains a variety of bacteria cells is collected in three random locations with three different depths. The DNA obtained from the cell extraction was identified with Next Generation Sequence method, which specifically targeted 16SrDNA V3-V4 properties to obtain the overall bacterial metagenomic profile. Results showed that off-Terengganu coast, bacteria diversity consisted of 25518 amplicons of 3301 unique OTUs, which signify 27 phyla. The OTU abundance decreased gradually with depth of sediment in the sea. The metagenomic profile revealed two sulphur-degrading bacteria were dominant in the surveyed area. *Sulfurovum* genus dominate overall bacteria community in two locations situated in the northeast area of sampling stations. Conversely, *Pseudoalteromonas* dominated the overall bacterial community in the southeast coastline. The Physical-geochemical analysis revealed that all surveyed areas contained sulphur, oil, grease, gasoline, diesel, and mineral oil, which perhaps are influencing sulphur-degraded bacteria community growth in the surveyed area. There is no concrete evidence to link *Sulfurovum* and *Pseudoalteromonas* as pathogenic bacteria that causes illness to the human. However, there are possibility that the surveyed areas are anthropogenically polluted and further physical-geochemical analysis is required. In conclusion, the research findings suggested the necessity to conduct a broader bacteria diversity research, such as bacterial dispersion scale, and community variation in order to measure an inordinate extent of environmental pollution in the surveyed areas.

ABSTRAK

Bakteria marin memainkan peranan penting dalam mengawal selia kitaran biokimia global sejak berbilion-billion tahun dan fungsi ini telah diterokai secara meluas lima puluh tahun yang lepas. Penerokaan bakteria marin dianggap sukar dan merbahaya, tetapi hasil kajian amat berhasil dalam menyediakan maklumat bagi menjana pemahaman yang lebih baik terhadap fungsi bakteria marin di dalam air laut. Penerokaan bakteria marin di persisiran pantai Malaysia dianggap sebagai baru dan tanpa data yang berkesan untuk menerangkan taburan bakteria di perairan Malaysia, khususnya yang menghala ke Laut China Selatan. Tujuan kajian ini adalah untuk menilai kepelbagaian bakteria di perairan luar Terengganu bagi menjana maklumat awal mengenai kepelbagaian bakteria marin di persisiran pantai. Dalam kajian ini, sedimen di permukaan laut yang mengandungi sel bakteria telah diambil dari tiga lokasi rawak dengan mengambil kira kedalaman paras air yang berbeza. DNA yang diperoleh melalui proses pengekstrakan sel bakteria dikenalpasti melalui kaedah *Next Generation Sequence*, dengan mensasarkan sifat 16SrDNA V3-V4 khususnya untuk menjana keseluruhan profil metagenomik bakteria. Hasil kajian menunjukkan kepelbagaian bakteria di perairan luar Terengganu terdiri daripada 25518 amplikon daripada 3301 OTU yang unik, yang menandakan 27 filum. Kekuatan OTU semakin berkurangan dengan kedalaman sedimen di dalam laut. Profil metagenomik menunjukkan dua genus bakteria pendegradasi sulfur adalah dominan di kawasan kajian. Genus *Sulfurovum* mendominasi keseluruhan komuniti bakteria di dua lokasi yang terletak di kawasan timur laut dari stesen pensampelan. Sebaliknya, genus *Pseudoalteromonas* mendominasi komuniti bakteria di kawasan tenggara persisiran pantai. Analisis fisio-geokimia mendedahkan bahawa semua kawasan kajian mengandungi sulfur, minyak dan gris, gasolin, diesel dan minyak mineral, yang mungkin mempengaruhi pertumbuhan komuniti bakteria pendegradasi sulfur di kawasan kajian. Tidak ada bukti kukuh untuk mengaitkan *Sulfurovum* dan *Pseudoalteromonas* sebagai bakteria penyebab penyakit kepada manusia. Akan tetapi, ada kemungkinan kawasan-kawasan yang dikaji telah tercemar akibat perbuatan manusia dan analisis fisiko-geokimia lanjutan amat diperlukan. Kesimpulannya, hasil penyelidikan ini mencadangkan keperluan untuk menjalankan penyelidikan kepelbagaian bakteria yang lebih meluas, seperti skala penyebaran bakteria dan variasi komuniti bakteria untuk mengukur kadar pencemaran alam di dalam kawasan kajian.

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LIST OF ABBREVIATIONS

COD	-	Chemical Oxygen Demand
DNA	-	Deoxyribonucleic acid
DO	-	Dissolved Oxygen
DOM	-	High-Molecular Weight Dissolved Organic Matter (DOM)
HAB	-	Harmful Algal Bloom
HEM	-	Hexane Extraction Method
MEOR	-	Microbial Enhanced Oil Recovery
NGS	-	Next Generation Sequencer
NSCS	-	Northern South China Sea
NTU	-	Nephelometric Turbidity Unit
O&G	-	Oil and Grease
OTU	-	Operational Taxonomy Unit
PCR	-	Polymerase Chain Reaction
POM	-	Particulate Organic Matter (POM)
RDP	-	Ribosomal Database Project
ROS	-	Reactive Oxygen Species
TDS	-	Total Dissolved Solids
TOC	-	Total Organic Carbon
TPH	-	Total Petroleum Hydrocarbon
TSD	-	Terengganu Sediment
TSS	-	Total Suspended Solids
QC	-	Quality check
RDP	-	Ribosomal Database Project
SCS	-	South China Sea
SSCS	-	Southern South China Sea

LIST OF SYMBOLS

10^x cells ml^{-1}	-	*(10^x) is order of magnitude in Most Probable Number (MPN) method*
16S rDNA	-	*16 Svedberg ribosomal DNA*
bp	-	*DNA basepair*
km	-	*kilometre*
m^2	-	*square metre*
m^3	-	*cubic metre*
mg/l	-	*miligram per litre*
S	-	*Svedberg / sedimentation rate*
µm	-	*micro metre*
°	-	*degree*

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Introduction

This chapter describes research background, problem statements, research aims, research scopes, hypothesis, conceptual framework, and research limitations. The research background consists of short and brief information regarding the marine bacteria, information of the surveyed area, and few explanation on the necessity to conduct marine bacterial community study in the seawater, and in the proposed sampling station. Subsequently, a conceptual framework is introduced before addressing the research objectives, scopes, hypothesis, and limitation. Several critical information that requires further explanation in a different chapter are carefully mentioned (e.g. in Literature review and Methodology).

1.2 Research Background

The water interconnected body covers 70 percent of the Earth's surface where it consists of diverse marine life. The marine ecosystem in the ocean is been existed for about 3.5 billion years, where two-thirds of its community are the marine microbes (Munn, 2011). Although microbiology diversity study in the seawater is widely studied, there is no detailed conclusion to determine marine microbial roles in the seawater because, these kinds of research are difficult construe as it involves complexity of biological affiliation issue in the seawater. Therefore, the marine

microbe exploration progress brings a major hindrance to the microbiologist. For instance, cultivation of a live marine microbe outside its natural habitat is expensive and scientifically unstable. Most of the research outcomes are vacillating and it requires more cognitive approach to identify the unknown bacterium (Munn, 2011).

To date, several studies have confirmed that most of marine bacteria are a dynamic key player in the oceanic ecological system – where it regulates the biogeochemical cycle to support ecological sustainability (Hanson *et al.*, 2011; Worden *et al.*, 2015). The marine bacteria are microscopic in size and requires a selective nutrient to support their growth (Inagaki *et al.*, 2004; Takai *et al.*, 2004). There is one research has speculated that all marine bacteria consume the same nutrient compound for its energy resources (Dinsdale *et al.*, 2008). It is believed that local seawater physical-geochemical parameters may reflect a local microbial community such as: - pressure, salinity, oxygen concentration, temperature, and carbon source (Dinsdale *et al.*, 2008b). There is no concrete evidence that supports an equal marine bacteria diversity amount in a different marine environment (Munn, 2011).

Several findings show that a marine bacterium able to generate its own molecular signal, to observe its local environment. This unique and complex biological function is a useful for the marine bacterial "communication" because it regularly needs to transmit itself elsewhere: To surge its predatory skills, and permit cell modifications to protect itself in an extreme environment (Whitehead *et al.*, 2004; Gómez-Consarnau *et al.*, 2010). Investigation on local marine bacteria interaction is an ongoing process, with a purpose to improve a better deviation process; parallel to the global environmental alteration pattern (Van der Gucht *et al.*, 2007; Wang *et al.*, 2015). It is worth to mention that, a continuous research on marine microbial deviation process does illustrate a sturdier and gradual improvement: Such as, dispersion bio-geography model in various environments (Lindström & Lagender 2012; Bokulich *et al.*, 2014; Wang *et al.*, 2015).

The South China Sea (SCS): as illustrated in Figure 1.1, is a marginal sea with an average bathymetry depth of 1200m (Hogan, 2013). The SCS is considered as the golden waterway for the Eurasia with the Americas, because it provides a safe nautical

route. This sea serves as a terminal for the busiest container seaports traffics in the world, where it mainly located in China, Singapore, Taiwan, and Malaysia (Fan *et al.*, 2015). The SCS shallow water contains a valuable oil and gas reserves (Ismail *et al.*, 2015), a diverse marine life (Cao *et al.*, 2007), and a rich coral reef zone (Arai, 2015).

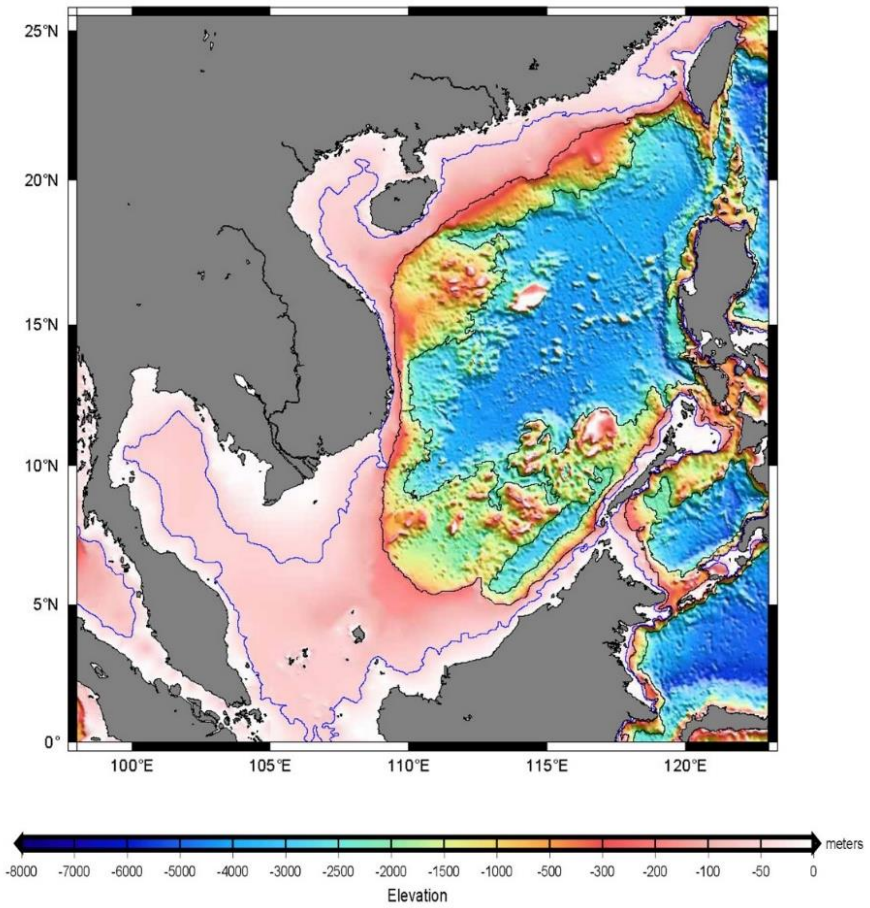


Figure 1.1 Illustration of the South China Sea bathymetry

(Image courtesy of Liu and Dittert, 2010)

Unfortunately, the SCS is notable for its dreadful cases of water pollution in several of its coastline (Rosenberg, 2009), where it is believed that mariculture activity contributes to the coastline pollution the most (Cao *et al.*, 2007). For example, several coastlines in the North SCS were badly affected due to mariculture management negligence; specifically, disposing the mariculture waste. In general, mariculture waste that is discarded into the seawater will increased the COD, active phosphorous, and ammonium values; eventually, transformed a hearty coastline ecology into a “dead

sea" (Feng, 1996; Cao *et al.*, 2007). A further discussion about anthropogenic pollution in the SCS can be referred in sections 2.3.2.

Prior to mariculture pollutant cases reported in the SCS coastline, the affected nations have reported several seafood poisoning cases that are mainly linked up to marine bacterial invasions such as: - Vibriosis, Pseudomonas invasion and Shewanella septic shock. Information on these diseases can be referred in sections 2.6. Before this research was conducted, numerous report that is being associated with marine bacterial infections in the affected SCS coastline was reviewed, where the result of this review is revealed in section 2.7.

However, this review was conducted with little information of physical-geochemical information available. Therefore, microbial community identification in both pristine and polluted coastlines is still difficult to predict. In this study, a comprehensive phylogenetic sequencing technology, namely Next Generation Sequencer (NGS) was utilized to describe a local bacterial community profile in three sampling points. The overcomes of this study may provide valuable information on microbial ability survivals in both normal and deprived regions.

In this study, the sampling area represents the SCS coastline, with no or minimum water intrusion occurs from the other sea region. In the Malaysian water, there are three coastlines that suitably signify the SCS coastline, which is: - Off – Terengganu coastline in Terengganu, Kota Kinabalu coastline in Sabah, and Bintulu coastline in Sarawak. The Off-Terengganu coastline are chosen as the sampling station because it is the nearest location for this study, and it is well positioned with no visible water flux influence expected to occur from the Gulf of Thailand.

The Off-Terengganu coastline is conjoined with the Kuala Terengganu river estuary, three small islands and several piers that are situated approximately two kilometres inside a curvaceous concrete breakwater. Based on a personal survey and visual information as depicted in Figure 1.2 and Figure 1.3, the Off-Terengganu coastline accommodate a moderate fishing vessel and speedboats traffics in daily basis. In addition, several water drainages are spotted in this area, where the effluent are

mainly influenced by a high-density fisherman's village, restaurant, mariculture, and hotels. Recent findings suggested that Off-Terengganu is vulnerable against anthropogenic pollutant with a notable amount of BOD, COD, TSS and, AN were reported (Suratman *et al.*, 2015; Kamaruddin *et al.*, 2016).

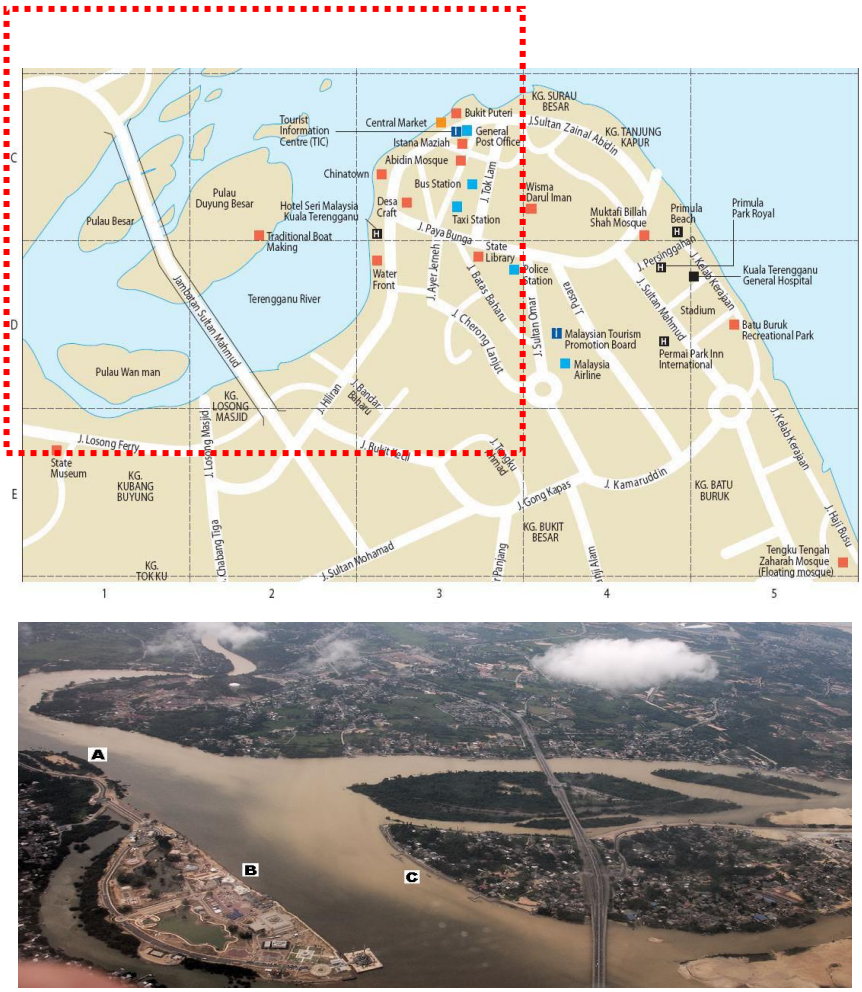


Figure 1.2 Illustration of the Pulau Duyong Besar Island (C), the Kuala Terengganu river (A), Pulau Wan Man, Pulau Besar, several hotels (B), fishing villages, and restaurants.

Given the context of possible sediment amiability towards the anthropogenic pollutant compound in the surveyed areas, the bacterial phylogeny profile in Off-Terengganu might not represent a spot-on native marine bacterial community description. Perhaps, it may illustrate a unique bacterial community that comprises several species that has its own metabolically readiness to utilize inorganic compound

substrate such as: Sulfate, Carbon, and Silica. Furthermore, this research might identify a waterborne bacterium that caused infection threat to other marine community and humans (Marziah *et al.*, 2016).

Figure 1.3 Bird Eye's View of Several Piers, Drainage, and Hotels in Pulau Duyong Interconnected with Off-Terengganu Coastline

1.3 Problem Statement

Ever since marine microbe exploration was initiated fifty years ago, investigations on marine microbial diversity in the ocean, its part in ocean ecology, its interaction with other marine life and its benefits for human beings have risen greatly among microbiologists around the globe. Despite excellent pioneering on such investigation, understanding of marine bacterial diversity was somehow slow and remains indecisive (Munn, 2011).

In principal, this study aims to create a steadfast foundation about marine bacterial community in the SSCS region - specifically in Malaysia seawater. Findings that are attained from this study are critical, because it will represent the first impression of marine bacterial community in the Malaysians' water (Marziah *et al.*, 2016). A massive marine bacteria phylogenetic study was previously identified in the British channel (Gilbert *et al.*, 2012), and NSCS (Zhu *et al.*, 2013) as an effort to describe a practical bacteria community profile in its local environment. Subsequently,

the outcome data have expanded global bacteria diversity coverage (Klindworth *et al.*, 2012; Gilbert *et al.*, 2012).

Identification of marine bacteria by the phylogenetic approach irrefutably reduces discrepancy in colony enumeration and taxonomy richness (Kim *et al.*, 2011; Mizrahi-Man *et al.*, 2013). Furthermore, the phylogenetic approach has revealed numerous of conspiring factors that propel a marine microbiology subject to the forefront of "mainstream" sciences; and becomes an exciting, fast-moving marine diversity research (Gilbert *et al.*, 2012; Munn, 2011).

Marine microbial ecology in the seawater requires a radical rethinking; to comprehend the oceanic eccentric, and delivers an intriguing insight of symbiosis phenomenon, food webs, and pathogenicity (Munn, 2011). Therefore, a correct methodology combination such as: phylogenetic approach, remote sensing, and sea exploration is required, in order to improve countless of data gap in the microbial diversity research. For instance, addressing the data gap in: species coverage and bacterial cell interaction in various environment condition. Currently, global marine bacteria exploration has identified approximately 44 percent of effective marine bacteria species, where it is mainly retrieved from Europe, East Asia, Middle America, Arctic Region, and the Atlantic Ocean (Gibbons *et al.*, 2013). In the South China Sea, only a minimum amount of the local marine microbial diversity data (based on phylogenetic method) is accessible. Therefore, it is hampering any efforts to compare and contribute marine bacterial diversity information in Asia with the other regions. Interestingly, the marine microbe research in the Southeast Asia region is mainly conducted in responds to seafood-related poisoning cases (Cahill, 1990; Austin, 2006; Anwar & Choi, 2014). For instance, there are several pathogenic marine bacteria have infested the fisheries products, and accidentally instigate a severe infection / mortality in the public community of Southeast Asia such as: *Bacillus* sp., *Vibrio vulfinicus*, *Shewanella* sp., and, *Pseudoalteromonas* sp. Therefore, it is essential to investigate the marine microbe's interactions in its local environment and develop an effective mitigation plan that will inhibit future outbreak (Anwar & Choi, 2014).

Bacteria cultivation is very important in the microbiology mainstream research because a bacterium cell is adjustable for a steadfast research preference and must be microscopically visible for continuous monitoring. Therefore, a pure cell culture is mainly used in the microbe susceptibility study to determine its virulence factor towards several living cells such as: - skin (Natsuga *et al.*, 2016), liver (Yeh *et al.*, 2016), brain (Wang *et al.*, 2016), blood (Moore *et al.*, 2016) etc. In addition, microbial susceptibility study helps to investigate antibiotic potential (Torres-Barceló & Hochberg, 2016) or antibiotic resistance factor (Yu *et al.*, 2016; Longo *et al.*, 2016). In recent claims, bacteria cultivation has demonstrated microbial ability to degrades dissolve or non-dissolved organic compound for energy (Thomas *et al.*, 2016; Canuel & Hardison, 2016)

The greatest challenge in marine bacteria cultivation is, by what method to imitate its growth outside its natural environment. Generally, there are notable physical-geochemical differences in the seawater, such as: - local chemical constituent, temperature, and atmospheric pressure (Alain & Querellou, 2016). Nevertheless, the success rate of obtaining a functional bacteria cell is trifling: because it is generally incapable to acclimatise in abrupt physical-geochemical changes (Suzuki *et al.*, 1997; Schut *et al.*, 1997; Cannon *et al.*, 2002)

Therefore, the microbial DNA extraction method is introduced in this study because it can be obtained from both live and dead cells. This technique reduces contaminated cell occurrences in the sample, throughout sampling, DNA extraction, and amplification (Strong *et al.*, 2014). Subsequently, the amplified DNA sequences are customarily targeted, to meet the research objectives before conducting a sequence assessment through genome depository interfaces such as: the NCBI, SILVA, and Genbank (Cole *et al.*, 2009; Pak & Kasarkis 2015). However, it is anticipated that the unknown phylum may be identified. Consequently, the unknown DNA must undergo a difficult and meticulous annealing process, before the exact sequence could be configured.

1.4 Research Objectives

i. To evaluate bacterial abundance in a selected coastline surface sedimentary layer
ii. To identify bacterial species that are dominant in a selected coastline surface sedimentary layer
iii. To identify, among those dominant species, a potential waterborne bacterium that causes disease towards the human.

1.5 Research Scope

i. This research is mainly focused on identifying a shallow benthic bacterial community from the natural coastline.
ii. Sampling is conducted in three different locations of different depths, to analyze the overall bacterial diversity in its local community
iii. The dominant genus based on the phylogenetic report is then analyzed for its interaction in the sampling area, and addressed its metabolic capability to induce infection in humans and animals.

1.6 Conceptual Framework

Implementation of the conceptual framework is essential in order to build conceptual distinction and organize research ideas effectively. Implementation of conceptual framework helps science research to advance faster and ensure every researcher to work inside an explicit framework of concepts and theories (Scheiner 2010). Historically, Suppe (1977) indicates that a conceptual framework for science always exists but never theoretically. In recent years, Scheiner (2010) believes that Suppe (1977) indication is parallel with general biological research. Generally, biology based research has no obvious predominant conceptual framework and has few general theories (Scheiner, 2010).

The conceptual framework is important because it clarifies thinking and forces a modicum of formality onto data interpretation. Scheiner and Willig (2008) believe that biologist does acknowledge only one theory - Charles Darwin's Theory of Evolutions: where these theories comprehend cells, organisms, and genetics evolution. To construct theories that represent a general biology research, it must have a potential applied it to every species with no limitation set of species. Accordingly, a fundamental principle must apply to all or most of the constitutive theories within the domain of the general theory. Those principles should work as basic assumptions behind all the constitutive theories and models, generating a link between constitutive theories. Next, the first fundamental principle of a theory should encompass the basic object of interest, and all the theory components should serve either to explain a central observation or to explore its consequences (Scheiner, 2010).

In overall, the conceptual framework for this study is constructed based on Scheiner's (2010) Towards a Conceptual Framework for Biology review, to reform formality thinking onto data interpretation, and averts any scientific disputes. Nevertheless, establishment of the conceptual framework may reveal a hidden information on specific models, or experiments where it perhaps clarifies the central questions that are being addressed by a scientific community. In this research, strategies on conceptual framework development are deliberated in the Chapter 2, section 2.7.

1.7 Limitations of Study

i. Bacterial 16S rDNA phylogenetic report only covers V3 and V4 hyper-region, which perhaps, impeding the chances to obtain targeted genus identification.
ii. Bacterial species and strain identification are not included in this study, because it requires a complex, expensive, and lengthy sequencing outline to construct a coherent cloning.
iii. Only three (3) sampling locations are selected for this study due to financial, time restriction and safety concern.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

A several decades of marine microbial investigations disclose that: A marine bacterium is the agent that regulates the ocean biogeochemical cycle to sustain overall earth ecology for billions of years (Munn, 2011; Suttle, 2005; Furhman, 1999). This claim was made based on numerous of findings, based on: - phylogenetic sequences, physiological features, and physical-geochemical study. For instance, a global-based genome surveillance reveals that, 44 percent of the marine bacteria species have been identified thoroughly (Gibbons *et al.*, 2013), and it is mainly consists of the marine bacterial community in the epipelagic zone (shallow-water column). It is also reported that, genome surveillance has yielded a heap of the unidentified microbial phylum (Louise, 2013).

In the meantime, marine microbial diversity research in the hadopelagic zone (deep-water column) are difficult to steer due to natural-complexity of physical-geochemical features such as: immense atmospheric pressure, pitch black and frigid cold environment. In addition, this kind of research requires exorbitant financial sources, and must overcomes difficulty to create an appropriate artificial environment that supports marine bacteria cultivations in the laboratory (Suttle, 2005).

Based on Deng *et al.* (2012) findings, oceanic physical-geochemical simulations such as atmospheric pressure, physical-chemical parameter and water influx are difficult to duplicate in laboratory environments. In addition, microbial

inimitability skills to adapt itself in various aquatic environment and its relations with the available nutrients in the seawater remains indecisive (Gómez-Consarnau *et al.*, 2010).

In general, a marine bacterium has a distinctive organelle named Flagellum; that acts as a propeller to allow bacteria movement in short or long distances in the sea, live host attachment (Anwar & Choi 2014), and water from ship ballast (Liu *et al.*, 2014b). In contrast to other marine microorganism, a marine bacterium is ideal for phylogeny-based study because: it is generally abundant, and has a simple and easily obtainable DNA structure. These such features support DNA annotation to meet research preferences effortlessly (Aranson, 2013).

Within several decades of marine microbiology exploration, there are three statements were made to appraise overall marine bacterial diversity in the marine environment. First, the marine bacteria community is usually abundant and diverse in the shallow sea water, in contrast to the deep water (Furhman, 1999; Suttle, 2005). Second, marine bacterium abundance is gradually diminished with the ocean depth, due to physical-geochemical variances (Kirchman, 2016 & Suttle, 2005). Finally, the marine bacteria diversity in the its local environment, typically reflected by its local organic content (Jiang *et al.*, 2010; Dinsdale *et al.*, 2011; Wang *et al.*, 2015b).

This experiment is configured only to investigate bacteria community and its phylum diversity in a selected coastline region. Accordingly, a related physical-geochemical parameter in each sampling point is studied for its nutrient availability evidence. The research objectives and conceptual framework for this study are build based on knowledge of marine bacterial physiology feature and speculations of local seawater physico-geochemical. Therefore, this chapter elaborates information of a marine bacterium and understanding its physiological capability to modulate its survival mechanism corresponds to physical-geochemical influences in variant region.

Subsequently, this chapter introduces the research methodology proposal for this study. By opting molecular biology as the principal of marine bacterial identifications, discussion is made to gain a clear judgement on selecting a correct

DNA replications, hypervariable (V) regions and primer pairs. Finally, information on waterborne disease, challenges in marine bacteria investigations, and benefits of marine microbe towards mankind are included.

2.2 The Sea Coastline

According to the Merriam-Webster's dictionary, the coastline is an area where it lines a form of boundary between the land and the ocean or, a lake. Benoit (1983) describes that no precise boundary line was performed to illustrate a precise coastline shape due to the coastline paradox. The coastline is considered as a dynamic environment where its shape is constantly changing with the influenced of sea level, waves, and various climate phenomena. Latterly, coastline is constantly facing sand erosion, accretion, and flooding which then forming continental shelves (CCSP, 2008; USGCRP, 2009)

The coastline as illustrated in Figure 2.1, is a home to a diverse number of marine creatures and its habitats. Its regional areas provide countless of benefits towards human civilizations, and local ecosystems. The estuary is naturally conjoined with the seawater that consists of freshwater and salt water mixtures provide sundry nutrients for the marine life. The salt marshes and beaches naturally support plants, animals, and insect growth – which it is essential to the marine food chain. In general, high levels of biodiversity, produce a high level of biological activity (CCSP, 2008).

Figure 2.1 Illustration of the sea coastline and its common habitats such as beaches, rock, pools, estuaries, and mangrove.

(Image copyright - Australian Museum 2015)

The coastline, also referred as littoral, or neritic epipelagic zone is mainly shallow in depth, and received maximum sunlight penetration. Thus, it effectively stimulates photosynthesis cycle to produce phytoplankton and zooplankton – which is a natural food staple for fish. Therefore, a quality fresh food sources in the seawater attracts human civilization for thousands of years. To date, coastal and sea activities such as marine transportation of goods, offshore energy drilling, resource extraction, fish culture, recreation, and tourism are integral to the nation's economy (USGCRP, 2009).

2.2.1 Coastline Impacts from Sea Level Rise

Growing populations and development along the coasts, increase the vulnerability of coastal ecosystems to sea level rise. An urban development may change the quantity of sediment delivered to coastal areas, worsen erosion, and damage wetlands. For example, in recent decades, the Louisiana coastline endures a massive 1,900 square miles lost in its wetlands due to anthropoid alterations in the Mississippi River's sediment system. It is believed that the sediment alterations were specifically built for oil and water extraction. The affected wetlands are gradually sinking, where it gradually lost its sediment structure, where it is naturally preserved the wetlands contour. Eventually, the natural wetlands lost its buffer function to an overwhelmed flooding (CCSP, 2008).

Rising sea levels may increase the salinity value in the ground water and shove the saltwater to further upstream (e.g. Estuary). A saltier estuary makes the water undrinkable without proper desalination process. It also harms the aquatic plants / animals that are generally vulnerable to salt. (Nicholls *et al.*, 2007; USGCRP 2009)

2.2.2 Coastline Impacts from Climate Changes

Climate change might affect coastline in a various way. Coastlines are sensitive to sea level rise, deviations of storm frequency and intensity, increases in precipitation and, warmer ocean temperatures. In addition, rising atmospheric carbon dioxide (CO_2)

concentration cause the oceans to absorb more of these greenhouse gases (GHG) and stimulates the ocean water to become acidic. It is reported that acidity rise in the seawater creates a significant impact on the coastal, and marine ecosystems (USGCRP, 2009). The climate change impacts are likely to aggravate several complications that coastal areas have already endured, such as: - shoreline erosion, coastal flooding and, water pollution from fabricated infrastructure. Confronting the existing challenges is already a concern to many governments and environmentalist. However, to address the environmental stress that is triggered by a climate change may require new approaches to managing land, water, waste, and ecosystems (USGCRP, 2009).

2.2.3 Anthropogenic Threats in the Sea Coastline

For the past two decades, a wild fisheries life resource as illustrated in Figure 2.2, has been declined due to global warming impact (Pratchett *et al.*, 2015) and unwarranted trawling (Guggisberg, 2016). It is believed that no precise calculation made to display the fish stock amount needed to fulfil global demands (World Ocean Review, 2013). Due to perpetual overfishing, mariculture system was introduced to integrate the stock productions. Although these methods were adapted to fulfil the fresh fish demands for decades, it is proven that the numerous mariculture industry fails to identify and mitigate the malpractice issue such as seawater eutrophication from hazardous waste released (Caruso, 2014).

In several mariculture-prone coastlines, a high accumulation of hazardous pollutant induces an irreversible coastline destructions such as hypoxia, hyperoxia, seawater ozonisation, and reactive oxygen species (ROS) stimulation; where it has eradicated local marine habitation and its food resources (Livingstone, 2003). For example, China is a major mariculture producer/industries in the world has consumed 590,455 hectares of its region specifically for this industry alone (Cao *et al.*, 2007).

On the appalling side, the annual environmental report reveals that 43 billion tons of contaminated water in China are derived from mariculture spillages (Biao & Kaijin, 2007). It is reported that the largest shrimp farm in the Northern division of

SCS causing a “lifeless sea” condition, due to the disturbing COD and active Phosphorous values; specifically, 200 and 900 times higher respectively compares to normal levels. Furthermore, active phosphorus and ammonium levels are 7.8 and 2.4 times higher during shrimp’s “*grow-out*” phase (Feng, 1996; Cao *et al.*, 2007)

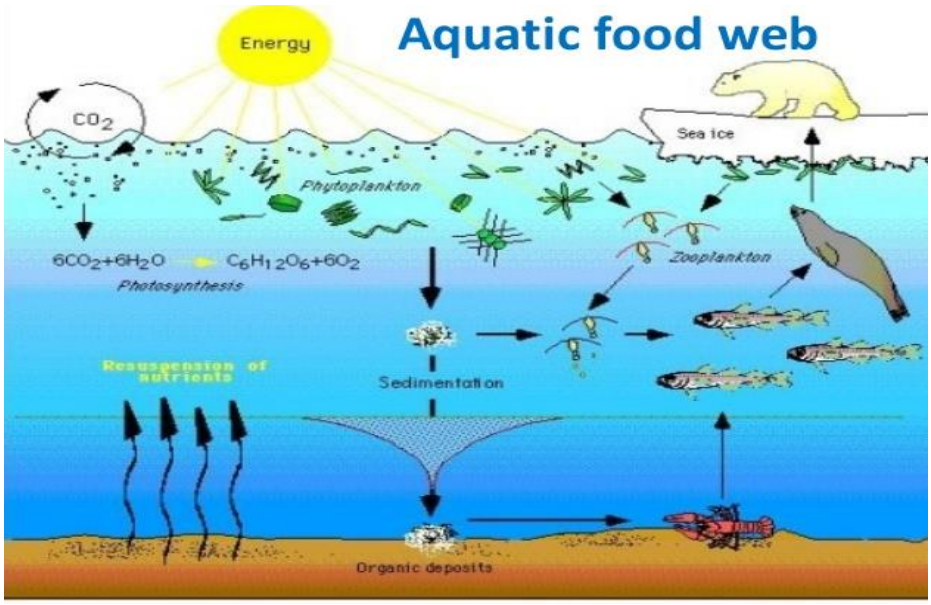


Figure 2.2 Illustration of typical aquatic food web

As illustrated in Figure 2.3, a small amount of toxicant input will dissipate quickly in the seawater. However, seawater is naturally incapable to dissipate a large toxic waste. Based on Hoyle & Richard (2014) claims, there is no exact mechanism was introduced to measure the sea capability to render noxious waste into a harmless concentration.

Based on Figure 2.4, it shows that a water pollution generally deteriorates photosynthesis cycle and aquatic lifespan. If no intervention plan conducted to reverse the current pollution impact, any damages that occur in the affected area is considered irreversible (Lin *et al.*, 2009).

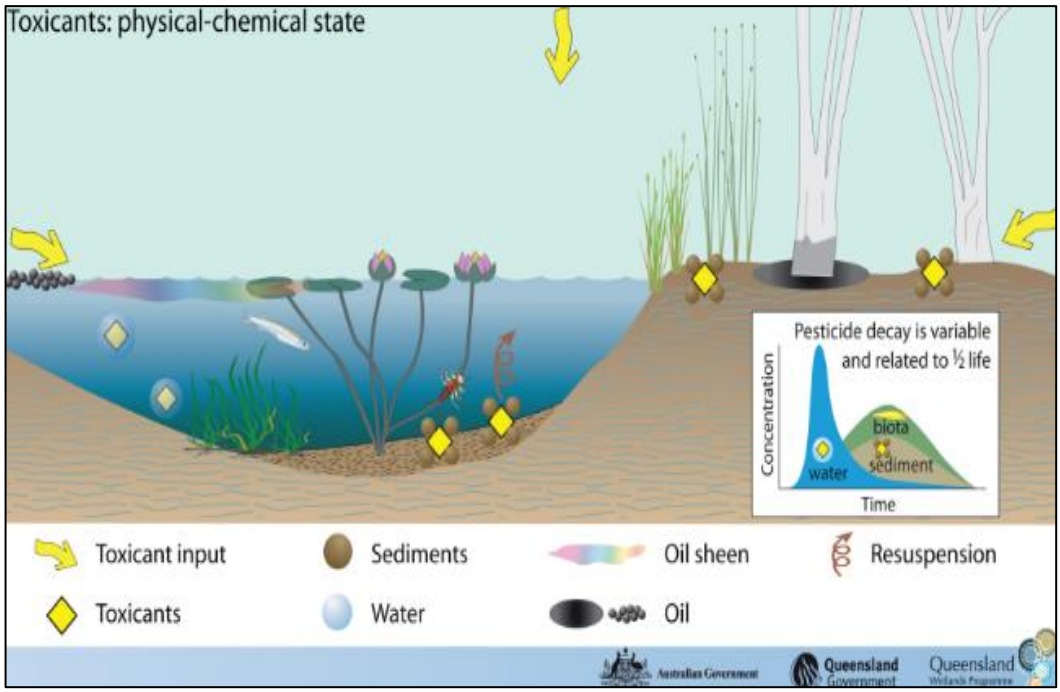


Figure 2.3 Illustration of Physical-chemical state from toxicant release

(Image courtesy of WetlandInfo, 2016).

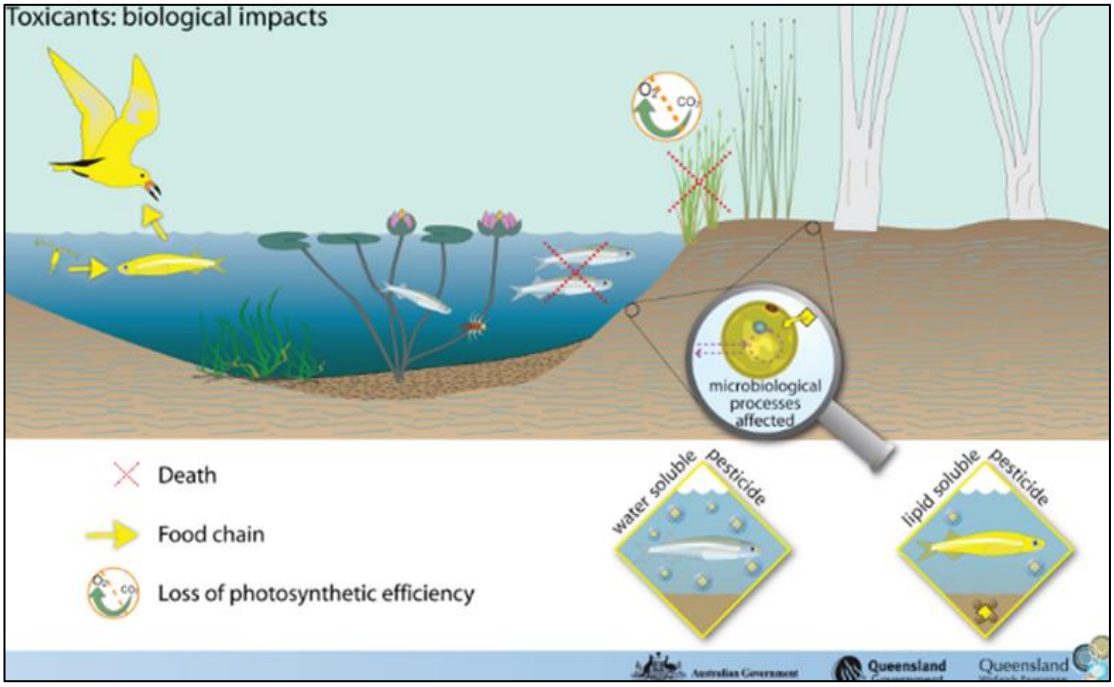


Figure 2.4 Illustration of biological impacts from toxicant release

(Image courtesy of WetlandInfo, 2016)

2.3 The South China Sea

According to Lee & Bong (2008), there are roughly 40 percent of the global ocean is consisted of the tropical sea. However, a detailed tropical water ecosystems description remains limited until today. Generally, the continental soil that is neighbouring to the tropical ocean are naturally rich in nutrition and has calmer waves on its coastline water. Therefore, these criteria are suitable for agriculture, mariculture, and transportations (Amberger, 2006).

Another unique feature that describes tropical water is, high coral reef diversity in the its coastline; where it indicates a healthy phytoplankton and seagrass vegetation. A healthy amount of vegetation in the seawater are beneficial for a marine animal that requires an endless food supply and protections from its natural predator. Indirectly, an abundant amount of marine life provides a fresh food sources for a human.

However, these stunning ecosystems are a vulnerable to a pollutant compound. Usually, the coral reef will be perished if it is exposed to a prolonged toxic effluent; originated from sea harbour, high-density municipal housing, mega factories, power plant, and mariculture industry (Morton & Blackmore, 2001; Rosenberg, 2009). Increasing amounts of greenhouse gasses (GHG) emission simulate the ocean to become warmer and acidic. A prolonged GHG effluent in the water will triggers coral reef decalcification. Eventually, if there is no intervention plan that is established to eradicate GHG's-based effluent in the environment, the "bleached" coral reef will be dissolved rapidly (Hoegh-Guldberg, 1999).

Marine pollution identification in the tropical seawater is inevitable because, tropical seawater regions that has an immense high coral reef abundance in the world. In this study, the South China Sea (SCS) is chosen as the region of interest because it needs to have an established microbial diversity data to support future marine ecology research. The SCS are mainly divided into two regions, the North-South China Sea (NSCS), and the South-South China Sea (SSCS). This research will focus on microbial diversity in the SSCS since it has lack of microbial diversity research in its region compared to the NSCS.

2.3.1 The Region of interest – the South-South China Sea

Geographically, the South-South China Sea (SSCS) (refer to Figure 2.5) has a shallow (±50m bathymetry depth) neritic epipelagic seabed and provides effective photosynthesis for plant vegetation processes. Therefore, it has a high coral distribution and diversity of its surroundings (Wang *et al.*, 2007; Morton & Blackmore 2001; Taylor & Hayes 1983). The SSCS has massive Tapis-grade crude oil reserves beneath its seabed (Ismail *et al.*, 2015), which it is signifying as the heart that connects Eurasia economy trade with the Americas via maritime route (Fan *et al.*, 2015).

In this study, the region of interest is located in the Off-Terengganu coastline (5°20 N, 103° 09 E) in State of Terengganu, East Malaysia. The Off-Terengganu was selected for bacteria diversity study because the location is strategically positioned in the SSCS region, close to two pristine island (The Perhentian Island and Kapas Island), acceptable marine life ecosystems (Arai, 2015), river estuaries, and Tapis-grade petroleum reserves (Ismail *et al.*, 2015).

There are several areas of the Off-Terengganu coastline has a few breakwater structures that are built to protect coastline piers, estuary, and mangrove from coastal erosion, flood, etc. In this study, a stern-curve Pulau Duyong breakwater - depicted in Figure 2.6 was constructed to protect three Pulau Duyong islands, Kuala Terengganu estuary and fisherman's village and tourist attraction's constructions. (Marziah *et al.*, 2016).

2.3.2 Type of Marine Pollution in the Coastline of the South China Sea

Recently, environmental deterioration issues have escalated on several SCS coastline water, where mainly involves by anthropogenic activity such as: - Illegal domestic waste dumping (Li *et al.*, 2015), heavy sea harbor activity (Blair & Lieberthal 2007), aquaculture waste (Anwar & Choi 2014), and power plant based effluent (Morton & Blackmore, 2001). In addition, an enormous human population density

watershed usually generates environmental pollution in its nearby coastline waters (Wu *et al.*, 2009; Wu *et al.*, 2010).

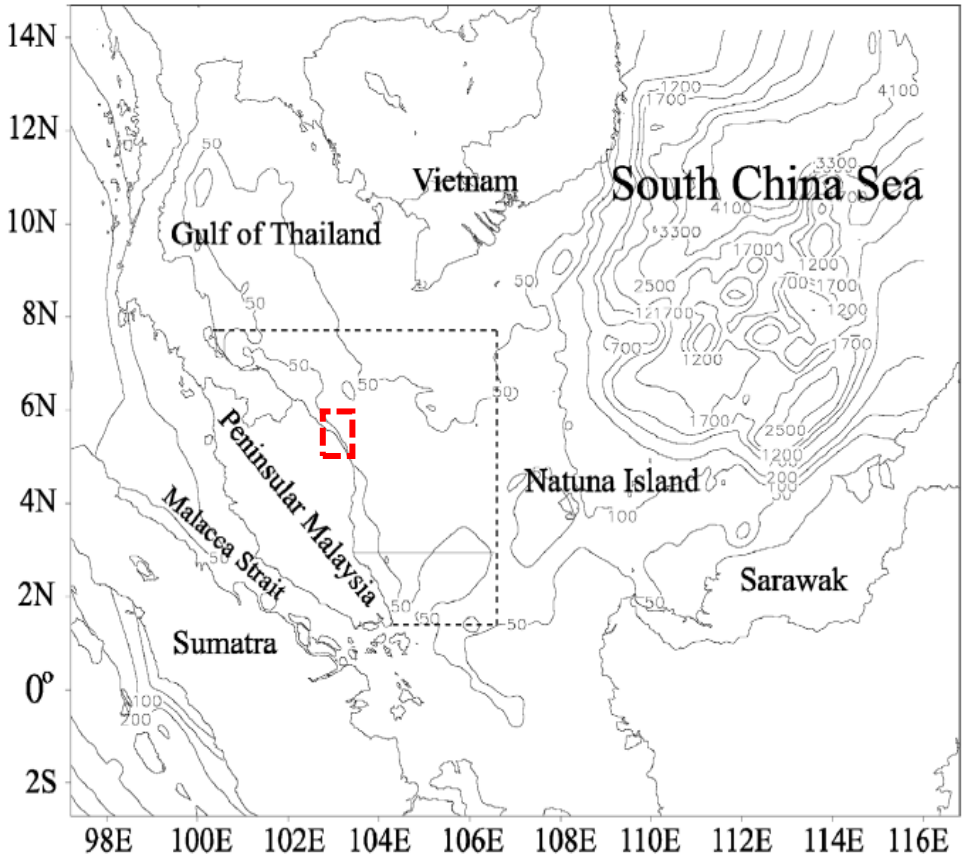


Figure 2.5 Depiction of South China Sea topography, where the region of interest[+] is bounded by the red dashes.

+Region of interest: The Off-Terengganu coastline in the Peninsular Malaysia (5°N 103°E) (Image adapts from from Daryabor *et al.*, 2014)

In principal, a xenobiotic compound generated from industrial waste was released into a runoff towards the nearby coastline, where it initiates a eutrophication phenomenon (Wu *et al.*, 2015). Based on illustration depicted in Figure 2.4, harmful nutrient fluxes that flow through runoff are gradually increased by years, and eventually triggering eutrophication in the affected coastline. The severity degree of eutrophication is evaluated based on the N: P: Si (Natrium: Potassium: Silicon) aggravation ratios (Wu *et al.*, 2015).

Figure 2.6 The aerial view of Kuala Terengganu breakwater

(Image courtesy of Zool's Studio)

The phenomenon of water eutrophication - specifically in the sea water, generally stimulates the Harmful Algal Blooms (HABs) proliferation; a condition that promotes algae overgrowth in the seawater from a high nitrogen and phosphorus stimulation (Tan *et al.*, 2015). The HABs algae produces a toxic, or non-toxic compound mainly to protect themselves. However, this toxicant substance is harmful towards several marine lives (Anderson, 2009). High HABs occurrence will initiate a severe threat towards mariculture industry (e.g. Mollusk and oyster cultivation) in the affected coastline. The HABs algae excretes a poisonous compound that caused disease in aquatic life and human; that consumed seafood intoxicated with HABs (Rosa *et al.*, 2014). Remarkably, the HABs manifestation is typically regional: - specifically, the tropic region. Previous research reports regularity of HABs manifestations in four SCS coastline: - (1) Pearl River Delta, China (Harrison *et al.*, 2008), Coast of Sanya, China (Wu *et al.*, 2015), western coast of Sabah, Malaysia (Anton *et al.*, 2007; Wang *et al.*, 2008; Adam *et al.*, 2011; Mohammad Noor *et al.*, 2012), Manila Bay, and the Mansinloc Bay in the Philippines (Wang *et al.*, 2008).

When a sea coastline is under anthropogenic stress, its local aquatic ecosystems might rapidly deteriorate. For instance, a climate change usually associated with coastline anthropogenic stress. A climate change stimulates water perturbations, such

as droughts, hurricanes, and floods; where it is frequently distress marine ecology in the estuary and coastline (Wu *et al.*, 2015). Until today, knowledge on complex marine community structure, its alteration's phase, and its function in distress conditions is difficult to interpret (Paerl *et al.*, 2002; Wang *et al.*, 2015).

An adequate understanding of anthropogenic and nature influences (e.g. monsoon-driven upwelling and mixing) provides an unswerving biodiversity understanding in the estuary and coastline ecosystem. Is it worth mentioning that, the effort of obtaining a data of water quality and fisheries habitat, are a difficult for marine biological research and management (Caruso, 2014; Livingstone 2003). In these sub-sections below, several anthropogenic issues that occur in the affected SCS coastlines are discoursed; to gather valuable information to generate the research hypothesis and expected outcomes.

2.3.2.1 Industrial Waste Pollution

China is the undisputed leader of industrial activity in the world. With the rapidity of the urbanization and tourism development in its maritime region, the anthropogenic impact is increased. Several coastlines are contaminated with agricultural, domestic, and industrial water discharge. In addition, nutrient enrichment and toxins are also derived from the cage mariculture. Based on type of noxious compound reported in Figure 2.7, it is widely speculated that China has the highest type of marine pollutant in the SCS region. It is assumed that China also has the highest pollutant coastline cases in the SCS region (Wang *et al*., 2005). Several areas in the NSCS coastline in China reports high concentrations of Chl-a, pH, Biochemical Oxygen Demand (BOD), Dissolved Oxygen (DO), Total Suspended Solids (TSS) (Wang *et al.*, 2006, Wu *et al.*, 2009; Wu *et al.*, 2015), and several inorganic contaminants such as: As, Cd, Cr, Pb, Cu, and Zn (Du *et al.*, 2008; Wu *et al.*, 2015; Li *et al.*, 2015). Furthermore, these areas face ecological degradation due to organic pollutant such as: - DDT, PCB (Wu *et al.*, 2009), PAH (Wurl & Obbard, 2015; Li *et al.*, 2015b), APEs, NPEs, OPEs (Chen *et al.*, 2006), PAEs (Liu *et al.*, 2014).

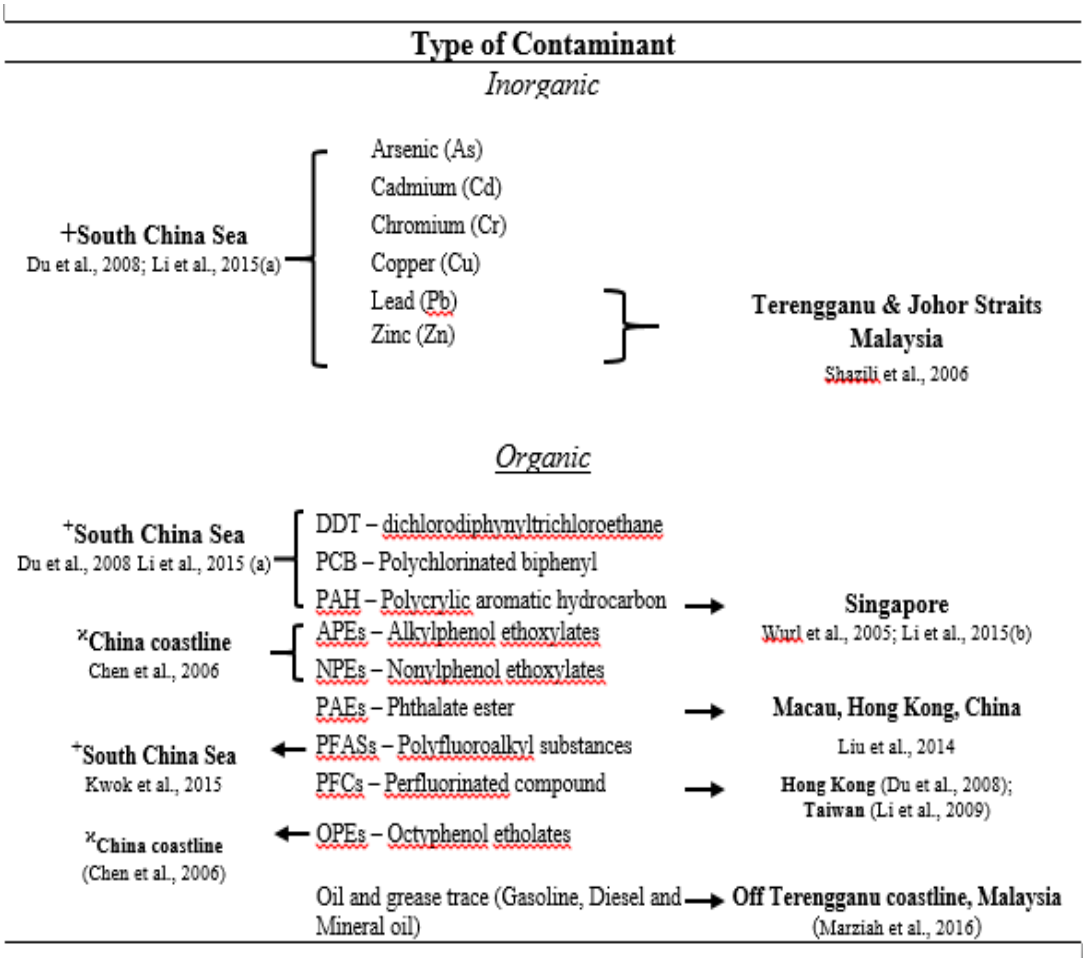


Figure 2.7 Diagram of organic and inorganic waste occurrences in several countries located in the South China Sea

Next, the PFASs (Kwok *et al.*, 2015) and PFCs (So *et al.*, 2004; Lin *et al.*, 2009) compound was reported in several unspecific regions in the SCS coastline.

In the Southern SCS (SSCS) region, an immense concentration of heavy metal and anthropogenic pollutant are detected in the Malaysia coastline that is conjoined with SSCS (Ong & Kamaruzzaman, 2009; Suratman *et al.*, 2016). Based on several findings, the anthropological-based effluent that affects Kuala Terengganu estuary mainly derives from a nearby municipal waste, agricultural runoff, organic pollution, and storm runoff (Kamaruddin *et al.*, 2016). High ammoniacal nitrogen, BOD, chemical oxygen demand (COD), TSS, Pb, and Cu value are also reported within Kuala Terengganu coastline (Suratman *et al*., 2016; Kamaruddin *et al*., 2016) and Johor Straits (Shazili *et al*., 2006). In Singapore, marine pollution mainly occurs due to land reclamation and shipping dredge activity (Dikou & Van Woesik, 2006).

2.3.2.2 Mariculture Waste Pollution

Mariculture is a fast-growing industry that is essential to accommodate feasible protein sources throughout the world (Caruso, 2014). Mariculture grows rapidly compared to any other segment of animal culture industry (Cao *et al.*, 2007). However, this industry is being heavily criticized for triggering biological deterioration in the seawater based on several mismanagement factors cited: - marine species, culture method, stocking solidity, feeding type, hydrographic of the site and breeding practices (Wu, 1995). In addition, mariculture activity usually induced water eutrophication in the affected coastline from constant organic influx discharge; originated from fish hydrolysate and manure. Eventually, it stimulates organic enrichment, turbidity, oxygen intakes and decomposition process in the water (Caruso, 2014).

China leads the mariculture industries in the world by consuming an impressive 590,455 hectares of the area for these industries alone (Cao *et al.*, 2007). On the appalling side, it is reported that 43 billion tons of polluted water come from mariculture waste spillage every year in China (Biao & Kaijin, 2007). The largest shrimp farm in the Northern part of SCS resulted in "lifeless sea" with the COD and active Phosphorous levels are 200 and 900 times higher respectively compares to normal levels. Active phosphorus and ammonium levels are reported 7.8 and 2.4 times higher in the shrimp grow-out phase (Feng, 1996; Cao *et al.*, 2007).

For the past decades, Philippines mariculture industries have sprawled severely due to seafood poisoning. Fisheries production and trading were declined due to stocking solidity that induces a high organic influx in the seawater (Reichardt *et al.*, 2007). In 2002, a mariculture centre located in Pangasinan distinct, has lost an overwhelming 110,000mt of milkfish worth US$16 million due to seawater eutrophication; stimulated from an excessive mariculture waste. Eventually, it reduces dissolved oxygen (DO) level in the affected coastline (Holmer *et al.*, 2002).

2.3.2.3 Microbial Pollution

Other than inorganic and an organic compound, the seawater carries a pathogenic marine bacterium that is capable to elicit diseases in the aquatic life. In

general, sewage effluent is considered as an organic compound; therefore, it is subjected to bacterial decay (Islam & Tanaka, 2004). Plenty of domestic sewage discharge contains a mixture of non-pathogenic and pathogenic bacteria such as *Salmonella* spp., *Escherichia coli*, *Streptococcus* sp., *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In the aquatic life, bacteria will naturally infect other living organism for food, protection and populating themselves (Janssens & Stoks, 2014). Apart from bacteria, a few viruses that are transmitted into the aquatic ecosystem appears as zoonotic especially influenza, herpes, cytomegalovirus, and measles disease (Islam & Tanaka, 2004). Illustration of pathogenic bacteria sources can be referred in Figure 2.8.

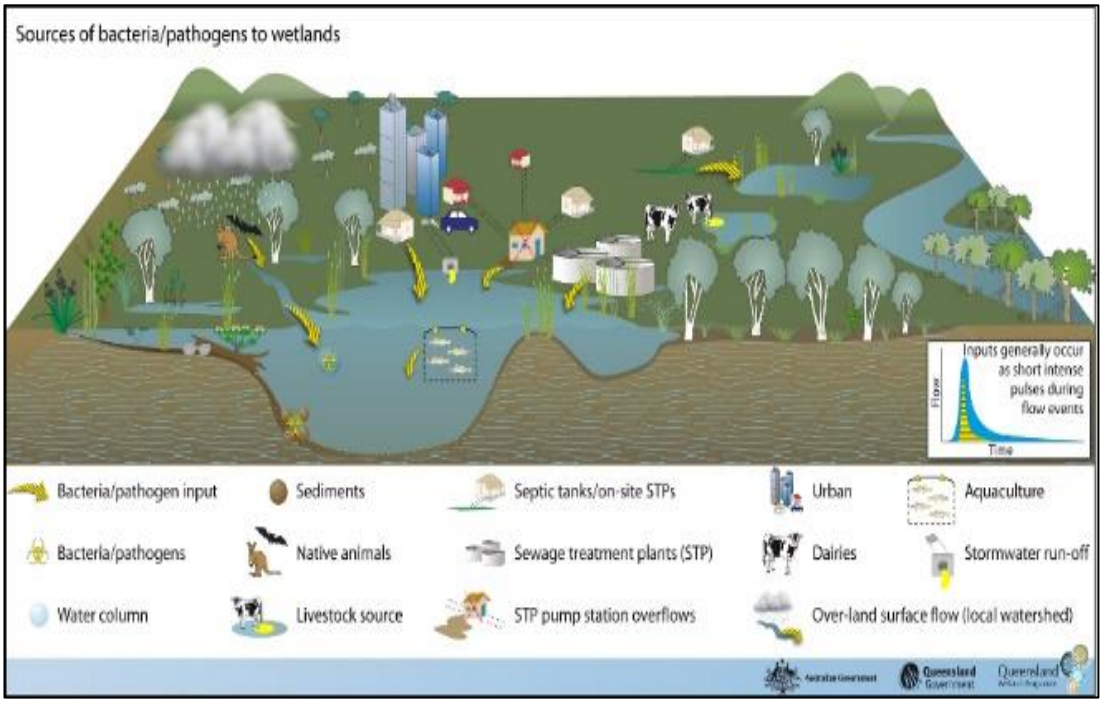


Figure 2.8 Illustration of pathogenic bacteria sources to wetlands

(Image courtesy of WetlandInfo 2016)

Sea currents act as freeways for microbes to transmit to another water column (Ruiz *et al.*, 2000). For example, *Vibrio* sp., a motile marine bacterium is commonly transmitted into the ballast water tank from some oceangoing vessels (Ruiz *et al.*, 2000). A ballast water that is taken from the seawater are pumped into the hull of a ship to stabilize the vessel against the rough condition of an ocean wave. When the

ship reaches the harbour, millions of litres of ballast water are discharged and indirectly release microbes into a new environment (Ruiz *et al.*, 2000). High cell number and motility features allow the marine bacteria to re-colonize in the new position. In fact, it may act as one of the primary causes of harmful algal blooms (HABs) phenomenon (Song *et al.*, 2009; Tan & Ransangan, 2015). Therefore, understanding microbial physiology knowledge is vital to predict its complex ability to survive in the new environment.

2.3.3 Marine Pollution in Off-Terengganu

There are few conditions that might influence the outcomes of the bacterial phylogenetic profile in the Off-Terengganu coastline. A recent study shows a high value of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), and ammoniacal nitrogen (AN) in the Kuala Terengganu river, which is directly connected to the Off-Terengganu coastline (Suratman *et al.*, 2015).

Another study specifies that municipal waste, surface runoff, agricultural runoff, organic pollution, and urban storm runoff have polluted this location (Kamaruddin *et al.*, 2016). Given the context of possible sediment amiability towards the anthropogenic pollution potency in the surveyed areas, the bacterial phylogenetic profile might not represent an abundance of the native marine bacterial community. Instead, it may illustrate a unique bacterial community with the capability to utilize inorganic compounds such as sulfur as its food sources, or perhaps, a waterborne bacterium that poses a threat in causing the disease to the marine community and to humans (Marziah *et al.*, 2016).

Therefore, the aim of this study is mainly to create a steadfast foundation of the marine bacterial community in the SSCS region - specifically in Malaysian seawater - since no published phylogenetic profile has been conducted in the surveyed areas (Marziah *et al.*, 2016).

2.4 The Marine Bacteria

Marine bacteria, a single-celled organism with no nucleus cell lives in every part of the water column and sediment layers where it mainly utilized the carbon dioxide for food and survival. Some species thrive in the water column by consuming oxygen. It is small in sizes, with the range of 0.6 µm and 0.3 µm (Belkin & Colwell, 2006). Marine bacteria able infect and decaying other marine life such as: algae, fish, crustaceans, and coral for its protection. Indirectly, based on cell decay, it produces a protein resource for other marine species (Anwar & Choi 2014).

There a few findings indicate that a marine bacterium regulates the phosphate compounds in the coastline to reduce eutrophication. Thus, sustaining seagrass productivity (Jankowska *et al.*, 2015). Technically, marine bacteria are found everywhere in the ocean, however, it is not uniformly dispersed over depth, region, or time.

Krebs (1972) has succinctly stated his view of ecological research:

> "*Ecology is the scientific study of the interactions that determine the distribution and abundance of organisms. We are interested in where organisms are found, how many occur there, and why*".

For the last century, bacteria are considered as part of the marine plankton. A Marine bacteria study has followed the tradition of the microbiological pioneers, Pasteur and Koch - wherein cells are first isolated from nature and, then cultured in the laboratory on artificial media. It is also known as a species identification method. In recent decades, a different approach emphasizes the role of microbes in their natural habitats; or also known as process approach. In the marine studies, process approach led to a new tradition pioneered by Nanaimo by the oceanographers, Parsons & Strickland (1962), wherein the biological activity of the cells is assayed in situ. A flourish of new ideas and results has followed, which clearly point to the vital importance of bacteria in the ocean.

Marine bacteria play an essential role to regulate the ocean's ecosystem for millions of years by controlling geochemical processes (He *et al.*, 2009). In the pelagic realm, bacteria are indispensable for two major reasons: other organisms eat them, and they degrade organic matter. Bacteria are at both the start and, end of the food chain where they contribute to the first production of particulate foodstuff (by conversion of dissolved organic substrates). They are also responsible for the ultimate breakdown of organic matter that leads to the return of nutrients to the sea (Li & Dickie, 2003).

Bacteria may be the crucial link or sink between detritus, dissolved organic matter, and higher trophic levels. For these reasons, bacteria occupy a central role in two interconnected environmental issues of global concern, namely the sustenance of harvestable living resources and the mitigation of climate change by sequestration of carbon into the deep ocean (Li & Dickie, 2003).

2.4.1 Marine Bacteria Form

In the marine ecosystems, bacteria are the main carbon cycling and nutrient regeneration agents. They are converting a dissolved organic matter to a biomass, which naturally supports microbial food webs and transfers energy and carbon to higher trophic levels (Lovejoy *et al.*, 1996). Bacterioplankton frequently categorized as either free-living or attached to particles (Crump *et al.*, 1999; Simon *et al.*, 2002).

Attached bacteria may have very high local concentrations compared to free-living bacteria (Fernández-Gómez *et al.*, 2013) and provide nutrition for macroscopic filter feeders (Prieur *et al.*, 1990). However, free-living bacteria are much more abundant than particle-attached bacteria in diverse marine (Ghiglione *et al.*, 2007) as well as freshwater ecosystems (Grossart & Simon, 1998). Free-living and attached bacteria communities can differ both morphologically and physiologically, for example, attached bacteria are often larger (Acinas *et al.*, 1999) and are reported to have lower growth efficiency than free-living bacteria, with comparatively less bacterial biomass produced per quantity of organic substrate taken up (Grossart *et al.*, 2003).

Some studies report a higher per-cell metabolic activity for particle-attached communities, compared to free-living communities (Becquevort *et al.*, 1998; Grossart *et al.*, 2007), while other studies report the opposite (Alldredge, 1986; Martinez *et al.*, 1996). Interestingly, Ghiglione *et al.* (2007) have reported a diel change in bacterial activity, with the free-living fraction being more active during the day and the attached fraction more active at night, consistent with different functional capacities in the two communities, which may be reflected in the taxonomy. Such observations suggest that the two communities are favored under different conditions, and understanding the dynamics and diversity of bacterial communities is an important step in characterizing an ecosystem as well as developing indicators to study ecosystem health and function (Mohit *et al.*, 2014).

Taxonomic richness and diversity were greater in the attached than in the free-living community, increasing over the summer, especially within the least abundant bacterial phyla. The highest number of reads fell within the SAR 11 clad (Pelagibacter, Alphaproteobacteria), which dominated free-living communities. The attached communities had deeper phylum-level diversity than the free-living fraction (Mohit *et al.*, 2014).

In a marine ecosystem, bacteria are the main agents of carbon cycling and nutrient regeneration, converting dissolved organic matter to biomass, which fuels microbial food webs and transfers energy and carbon to higher trophic levels (Lovejoy *et al.*, 1996). Bacterioplankton frequently categorized as either free-living or attached to particles (Crump et. al., 1999; Simon *et al.*, 2002). Attached bacteria may have very high local concentrations compared to free-living bacteria (Fernández-Gómez *et al.*, 2013) and provide nutrition for macroscopic filter feeders (Prieur *et al.*, 1990). However, free-living bacteria are often more abundant than particle-attached bacteria in diverse marine (Ghiglione *et al.*, 2007) as well as freshwater ecosystems (Grossart &g Simon, 1998).

Marine bacteria that are in the free-living and attached state can differ in terms of morphologically and physiologically. For example, attached bacteria are often larger in size (Acinas *et al.*, 1999) and have a lower growth efficiency than free-living

state bacteria. On the other hand, free-living bacteria have a lesser biomass produced per quantity when taking up the organic substrate (Grossart *et al.*, 2003). Some studies report higher per-cell metabolic activity for particle-attached communities compared to free-living communities (Becquevort *et al.*, 1998; Grossart *et al.*, 2007), while other studies report the opposite (Alldredge, 1986; Martinez *et al.*, 1996).

One of the ample sources to obtain attached bacteria is the marine sediment since it contained rich sources of organic matter. Organic matter in sediment consists of carbon and nutrients in the form of carbohydrates, proteins, fats, and nucleic acids. For example, bacteria quickly engulf less resistant molecules, such as the nucleic acids and several proteins for food. Sediment organic matter mainly derived from plant and animal detritus, bacteria or *in situ* phytoplankton or obtained from natural and anthropogenic sources in catchments. Sewage and effluent from food-processing plants, pulp, paper mills, and mariculture are examples of organic-rich wastes derive from human origin (Logan & Longmore, 2015).

Generally, a greater availability of organic matter may increase attached bacteria volume. Availability of nutrient that surrounding free-living bacteria would lead to a faster reproduction rate. However, it does not change its volume and sizes – where it is demonstrated best in attached bacteria (Mohit *et al.*, 2014). In additions, attached bacteria is more locally concentrated rather than free-living bacteria (Fernández-Gómez *et al.*, 2013). Bacteria metabolic activities are much of active in warmer condition and will conserve its energy in cold environments (Mohit *et al.*, 2014; Irriberi *et al.*, 1987). Two characteristics that need attention before sampling activity which is: (i) The bacteria have more energy for food conversion and survive in a warmer environment and, (ii) bacteria's cellular activity or, an increase in the number of cells occurs more on the attached bacteria, respectively (Iriberri *et al.*, 1987).

2.4.2 Marine Bacteria Abundance in the Seawater

Although marine bacteria are abundant in the ocean, no conclusive study that able to measure bacteria proliferation in the fluctuated environment such as hot, cold, alkaline, and high in phosphorus or high in iron. In general, the distribution of bacteria at the regional scale is poorly understood. Roughly, marine bacterial abundant (cells ml^{-1}) are highly measured in eutrophic lagoons and estuaries (10^7), coastal zones (10^6), and open ocean (10^5). It was set by the magnitude of the flux of dissolved organic matter: a manifestation of the dominance of "bottom-up" (resource limitation) over "top-down" (grazing pressure) control factors at large time and space scales (Calvo-Díaz *et al.*, 2014; Ducklow & Carlson, 1992).

In temperate water, the annual cycle of bacterial abundance is mainly consistent. Generally, cells are most abundant in summer compared to winter. At the seasonal scale, temperature emerges as a dominant influence. For instance, an earlier study by Taguchi and Platt (1978) had shown that microzooplankton biomass in Bedford Basin (Canada's Atlantic coast) are depressed through the winter and increased from May to a peak in September, suggesting significant grazing pressure in the summer. The function of substrate supply to the bacteria is not investigated here, but it assumes plays an important role in the summer when metabolic rates increase with temperature (Taguchi & Platt, 1978).

2.4.2.1 Sea Depth Influence

Naturally, bacteria are abundant in the sunlit upper layer, and their numbers are decreasing with depth. Based on a study in the Labrador Sea (Labrador Peninsula – Greenland), bacteria are abundant in concentrations of 10^5 to 10^6 per milliliter in the top 100 meters and, approximately 10^4 to 10^5 per milliliter at greater depths (Danovaro *et al.*, 2002). Bacteria are mainly sustained by the flux of dissolved organic matter, which is consist of the phytoplankton and zooplankton. Therefore, the restriction of primary production to the sunlit layer is a noticeable determinant in the vertical distribution of bacteria. Bacteria persist deep into the aphotic zone where

phytoplankton is absent. In there, they are a dominant metabolic agent mediating the dynamics of organic material (Danovaro *et al.*, 2002).

For instance, a previous study has shown that microbes are bound to the sinking detritus snow, and conveyed into the sea floor (Proctor & Furhman, 1991). The sea floor sediments will attain a rich microbial that is attached with particle fluxes (Danovaro *et al.*, 2002). Another finding reveals that the soluble proteins and carbohydrate values are assumed to be the labile organic matter tracers. The organic matter input from the photic zone to the deep-sea floor were significantly higher at the higher microbe abundance regions (Danovaro *et al.*, 1998).

Currently, no conclusive finding to address the pelagic-benthic coupling relationship between microbe distribution and particle fluxes (Danovaro *et al.*, 2002). However, microbes in the deep-sea sediments might be dependent, upon complex interactions with abiotic factors (e.g. Pressure, physical disturbance, and redox conditions) and biotic factors, including bacterial metabolic state and virus supply from the water column. Further research is needed to elucidate the causes of the low viral density, calculating the actual marine virus's impact on benthic microbial function, and to assess potential implications for biogeochemical cycles (Danovaro *et al.*, 2002).

2.4.2.2 Local Nutrient Availability

There are several factors that may determine bacterial abundance in the seawater, such as: - hypersalinity, heavy metals, another organism prey, nutrient competition, and particulate matter adsorption. (Mitchell & Chamberlin, 1974; Enzinger & Cooper, 1976; Gerba & McLeod, 1976; Gilbert, 2009). A marine bacterium requires inorganic ions to support its growth, metabolism and maintaining cell integrity. For example, Natrium (Na+) are an essential for marine bacteria metabolism to transport substrate inside the cell organelle. In some species, it requires a combination of magnesium (Mg++) and Ca++ (Calcium) to construct the cell structures. The effect of salts in maintaining the integrity of the bacterial cells requires

a great capacity to interact directly with the cell envelopes or by osmotic function (MacLeod, 1965)

Bacteria pathological behavior is also influenced by global warming and any irreversible damages in ecosystems (Marten *et al.,* 2001). A constant organic and inorganic-based pollution in the seawater makes the bacteria a consistent subject to environment stimuli myriad. With high organic and inorganic effluent released in the seawater, it is assumed that heterotrophic bacteria obtained its food source from the accumulation of complex-dissolved-particulate substrates such as, cage mariculture (Caruso 2014).

Mariculture farm is reported to have high alkaline phosphates input to stimulate the mineralization process concerning their labile or refractory nature. Thus, it stimulates the bacteria metabolic process. This occurrence is independent in heterotrophic bacterial density (La Rosa *et al.,* 2002; Caruso 2014). To date, no sufficient data to correlate bacterial abundance with the industrial waste emissions.

2.4.3 Marine Bacteria Physiology

Several previous studies indicate that marine bacteria from Proteobacteria clade are the most abundant phylum in the world since they have the locomotion advantage (Eilers *et al.,* 2000; Madigan & Martinko 2005; Kirchman *et al.,* 2010). The Proteobacteria versatility in global natural sources was scientifically addressed (Gibbons *et al.,* 2013). For example, Proteobacteria thrive in the cold sea region (Stibal *et al.,* 2015 & Sapp *et al.,* 2010), hydrothermal vent (López-García *et al.,* 2003; Zhu *et al.,* 2015), volcanic region (Giovannelli *et al.,* 2013; Wang *et al.,* 2015b), marine sediment (Wang *et al.,* 2015a; Wang *et al.,* 2015b; Zhu *et al.,* 2013), sponges (Schmitt *et al.,* 2012), and organic compound (Lin *et al.,* 2014; Kleinsteuber *et al.,* 2008).

The Proteobacteria, a gram-negative bacterium has the broadest variety of pathogenic species (E.g: *Escherichia coli, salmonella* sp., *Vibrio cholerae* etc.) (Madigan & Martinko 2005) and numerous free-living or nonparasitic bacteria (e.g.

Nitrogen fixing bacteria). Its phylogeny was divided into six parts, referred to by the Greek letter Alpha (α) through Zeta (ζ).

i. (Alpha) α-proteobacteria = the bacteria in this class are highly diverse and able to cultivate in a very low nutrient levels and has an unusual morphology such as stalks and buds. Many bacteria in this group are important for agricultural purposes as they capable of inducing nitrogen fixation in plant symbiosis (e.g. (*Wolbachia* sp. mainly infect arthropod such as crab and scorpions) (Gupta and Mok 2007)

ii. (Beta) β-proteobacteria = the bacteria in this class often utilize nutrient substance diffused from anaerobic decomposition of organic matter (e.g: hydrogen gas, ammonia, and methane) which also includes chemoautotrophs. (E.g: *Bordetella pertussis* is the bacteria that causes whooping cough) (Dang *et al.*, 2010)

iii. (Delta) δ-proteobacteria = the bacteria in this class are usually a predator to other bacteria. This class is an important sulfur cycle regulator. (E.g: *Desulfovibrio* sp. are found in anaerobic sediment and also fauna intestinal tracts) (He *at al.*, 2015 and Acosta-González and Marqués 2016)

iv. (Epsilon) ε-proteobacteria = Epsilonproteobacteria is a slender rod bacterium that looks helical or curved in shape. They are flagella-equipped bacteria, which makes them moves easily (Beepy 2015). They are also microaerophilic. (e.g: *Helicobacter* sp. is the most common cause of human peptic ulcer and stomach cancer) (Cravedi *et al.*, 2015)

v. (Gamma) γ-proteobacteria = the largest subgroup in Proteobacteria clad. It consists massive pathogenic bacterium towards the human. (E.g: *Pseudomonas* sp, *Escherichia coli*, *Salmonella* sp. and *Serratia marcescens*) (Schulz *et al.*, 2015)

vi. (Zeta) ζ-proteobacteria = Zetaproteobacteria is the most recently described class of the proteobacteria (Emerson *et al.*, 2007). Only one species identified in this class that is *Mariprofundus ferrooxydans*, an iron-oxidizing neutrophilic

chemolithoautotroph. It is believed that many more subgroups in Zetaproteobacteria have not yet been cultured (McAllister *et al.,* 2011)

Proteobacteria that has a selective ecological preference has demonstrated its domination and limitation in different sea regions. For example, Alphaproteobacteria are identified in the benthic region of the Atlantic Ocean with 55.7% (Zinger *et al.,* 2011) and in the East China Sea with 20.1% (Wang *et al.,* 2015a). Gammaproteobacteria dominates the benthic bacterial community in the NSCS with 53.4% (Zhu *et al.,* 2013).

Research in bacteria physiology is still new. Therefore, knowledge in bacteria physiology that facilitates its motility is limited. The previous finding indicates that Proteobacteria, a pro-motility phylum generally lives in a dense colony where each cell gap is close enough to generate a fluid flow interface which allows them to swim (Wolgemuth 2008). There are several species are classified as a non-motile bacterium, and generally depend on its "gliding" mechanism - a process whereby a bacterium can move under its own power by relying on sea current, water flux and hydrothermal plume such as - cyanobacteria and myxobacteria. The gliding mechanism for other phylum remains unknown (McBride, 2001).

A recent study reveals that many bacteria migrate *en masse* over a large distance in an organized dense group called "swarming formation" (Aranson, 2013; Dunkel *et al.,* 2013). Bacteria swarming formation provides an advantage for colonization in new territories, gets more food, high chances to survive in the harsh environment and, generates resistance against antibiotics (Butler *et al.,* 2010).

Implementation of live cell motility in the ecology research has been instigated for three decades. The bacterium was considered as the best candidates for motility analysis based on its simple movement pattern compares to another living organism. Furthermore, most the bacteria are self-propelled, easy to grow in large quantities and, effortlessly to control in any experiment. In principle, motility pattern of bacteria was investigated when the colonies are added into an ideal fluid (Aranson, 2013). Based

on several experiments, *Bacillus sublitis* are the preferred choice for motility research because this species demonstrate its distinctive motility pattern (Kearns & Losick 2003; Dombrowski *et al.*, 2004).

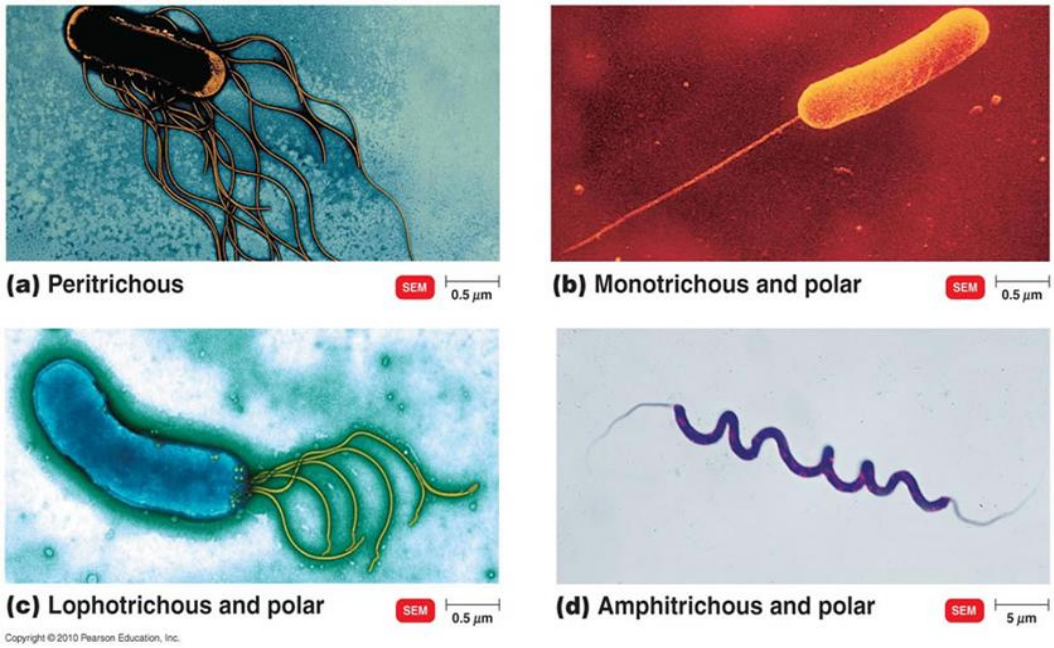


Figure 2.9 Bacterial flagella arrangement from Scanning Electron Microscope (SEM) view

(Image courtesy of Pearson Education Inc. 2010)

2.4.4 Bacteria Molecular Modulation

Generally, molecular modulation is a process on how the organism will respond towards fluctuated environment or if illness or mutations occur in the cell. Additionally, it acts as the first line of defence if the unfavourable condition affects its productivity and survival (Whitehead *et al.*, 2001). Although bacterial cell has a simple morphology, their molecular modulations are intriguing and complex, which perhaps, demonstrate their sustainability on the earth for millions of years. Understanding bacteria modulation is still an ongoing study, but it is already demonstrated several enthralling findings as discusses in subpoints below:

2.4.4.1 Bacteria Starvation Phase

When subjected to starvation, most bacteria cell will undergo modulation on its cell envelopes, sizes, and metabolic forms. However, the starvation phase only applicable experimentally by adjusting the pure culture densities in the range of 10^7 to 10^8 cells ml^{-1}. In the natural environment, the cells cannot be obtained with a great number such as pure culture (Belkin & Colwell, 2006). Other studies show that bacteria are able to mutate its original metabolic systems to adapt themselves towards environmental fluctuations (Whitehead *et al.*, 2001).

For example, a two-component signal transduction phosphorelay schemes allow bacteria to sense and respond; by activating and repressing specific target genes towards multiple environmental factors. *Vibrio* sp. is a species that contains proteorhodopsins, which is a photoprotein in a bacterial cell that acts as an energy supplier to enable cell survival in a harsh condition. Thus, it represents a novel mechanism for bacteria to endure frequent periods of resource deprivation at the ocean's surface (Gómez-Consarnau *et al.*, 2010).

In a molecular description, the expression of mixed sigma factor in response to various signals enables specific transcription inside the bacteria (Wbösten, 1998). Specifically, the transcription profile of an altered bacteria will change their DNA topology and it is protein-mediated (Atlung & Ingmer, 1997). It is believed that bacteria able to derive its signaling molecule into several chemical classes and it is divided into two main categories: (1) Gram-positive bacteria: utilized Amino acids and short peptides and (2) Gram-negative bacteria: utilized fatty acid derivatives (Visick & Fuqua, 2005).

2.4.4.2 Chemical Degradation

In some conditions, bacteria can degrade and utilize several organic compounds for its energy (Muyzer & Stams, 2008). The sea particles and aggregates were degraded and turn into a dissolved molecule that is beneficial for bacteria communities (Jørgensen & Marshall, 2016). Some of the bacteria degrade Particulate Organic Matter (POM) and High-Molecular-Weight Dissolved Organic Matter

(DOM) by excreting an ectoenzyme that will hydrolyze macromolecules into a smaller substrate for an easier transference and utilization (Arnosti, 2011; Benner & Amon, 2015). Product utilization from the enzymatic effect will support heterotrophic bacteria to incorporate carbon and associated elements from small labile molecules into cellular macromolecules (Benner & Amon, 2015). However, some elements are resistant to microbial utilization and they appear to derive from bacteria for a long time (Ogawa *et al.*, 2001).

2.5 Marine Bacteria Linked Disease

The effects of mariculture pollution towards the seawater aestheticism are noticeable (Zyranov, 2015). A recent study suggested that marine bacterial reacts more in the mariculture industries, based on the frequency of bacterial-caused illness in a fish culture (Anwar & Choi, 2014) rather than a non-mariculture based pollution. Illness in the mariculture mainly occurs in the Asian country, since most of its county contributes to world's fresh food production. It is reported that the *Vibrio* sp., *Pseudomonas* sp., *Aeromonas* sp., *Escherichia coli*, *Bacillus* sp., *Pseudoalteromonas* sp. and *Shewanella* sp. are the most abundant species found in the SCS coastline and commonly infects other marine life for food and survival (Anwar & Choi, 2014).

Predation / Infection

Nearly all marine bacteria are gram negative and a native species in the seawater. It can be a normal microflora or an opportunistic pathogen that elicit illness (Cahill, 1990; Austin, 2006; Anwar & Choi, 2014). A marine bacterium that is isolated from the skin may be transient, rather than a resident on the fish surface. Basically, low ambient temperatures may inhibit the anaerobes growth in the host such as a rainbow trout (Cahill, 1990). In a different perspective, a bacterium that lives in the intestinal tract is from the environment or host's diet where the nutrient helps the bacteria to live and reproduce within the host (Anwar & Choi, 2014). However, a precise relationship between aquatic and fish microflora remains unknown.

Several pathogenic bacteria such as *Pseudomonas*, *Aeromonas*, *Vibrio,* and *Cytophaga* are common genus isolated from a healthy fish. But only certain species strains, excretes a virulence compound to induce disease (Cahill, 1990; Anwar & Choi, 2014). For example, the main reservoirs for *Vibrio cholerae* are human and aquatic life in brackish water and estuaries. These strains are indirectly transmitted into the water from a contaminated fish, shellfish, leftover foods, feces, etc. They are also associated with copepods, zooplankton, and aquatic plants. It has both pathogenic and non-pathogenic strains that co-exist in aquatic environments, which allow multiple genetic varieties. Nevertheless, gene transfer amongst recombinant of different *V. cholera* genes can lead to new virulent strains (Faruque & Nair, 2002).

In general, Gram-negative (G^{-ve}) bacteria are the most abundant species reported in global. It has an exclusive molecular feature that allows utilization of fatty acid derivatives that is commonly found in microalgae (Sahu *et al.*, 2013). Anwar & Choi (2014) claim that the G^{-ve} bacteria might survive even in the harshest oceanic condition because it has an intricate cellular defenses named lipopolysaccharide (LPS). Other than providing cell integrity, the LPS triggers marine host immunity to stimulate cell inflammation, which may end in severe infection or death (Anwar & Choi, 2014).

2.5.1 *Vibrio* sp.

Vibrio infection is mainly classified into two groups: Vibrio cholera infection and non-Vibrio cholera infection. *Vibrio cholerae*, a heterotrophic bacterium induced Vibriosis illness in mariculture centre in Sabah, East Malaysia where it leads to massive 4 million USD annual losses (Shariff & Subasinghe, 1994). *Vibrio vulnificus*, a lethal opportunistic human pathogen was reported in Taiwanese raw seafood products where it caused a lethal fulminate systemic infection in a human (Jones, 2009).

Nearly 37 years ago, *Vibrio* sp. is being studied for its capability to sustain its morphology from the starvation phase. This bacterium responds by reducing their size/amount, and cellular response per surface and volume ratio (Novitsky & Morita,

1976) and control it cellular component utilization (Novitsky & Morita, 1977). The research proposes that their routine will only increase in a nutritive environment (Novitsky & Morita 1976; Caruso, 2014). Other than the nutritive environment or eutrophication, Vibrio genus such as *V. cholerae* is able to disperse in the ocean because they are able to acquire serological determinants to excrete toxin genes by a gene transfer (Jiang & Fu 2001; Huq *et al.*, 2005).

In a recent study, *Vibrio* sp. and some of the non-indigenous marine species were assumedly transmitted into Taiwanese coastline by vessel ballast water discharges (Liu *et al.*, 2014b). Because of *Vibrio* sp. has a specialty in gene-transfer, it makes them easier to re-populate in a new location (Ruiz *et al.*, 2000).

2.5.2 *Pseudomonas / Aeromonas*

Pseudomonas is a common bacterium in natural seawater. Most of the fish illness and mortality cases in the world are due to *Pseudomonas* invasion. For example, *P. Anguilliseptica* is being considered as an extreme pathogenic bacterium for fishes, where it triggers ulcer influenced infection such as ulcerative syndrome, bacterial hemorrhagic septicemia, tail and fin rot, gill rot and dropsy (Shayo *et al.*, 2012; Anwar & Choi, 2014). *Pseudomonas* and *Aeromonas* invade and attached to the host's tissue by excreting its virulent enzyme and toxins to escape host immune defenses (Shayo *et al.*, 2012).

2.5.3 *Escherichia coli*

Escherichia coli (*E. coli*) are notable species with the ability to survive in the unsterile seawater for a long time. *E. coli* - known as fecal coliform is a popular reference for water fecal concentration (Liang *et al.*, 2015) and drinking water quality indicator (Jallifier-Verne *et al.*, 2015). Profoundly, this genus is used as an indicator of potential bacterial pathogen risk in the local water. Its behavior in nature was widely investigated in order to assess its growth in both sterile and non-sterile seawater.

(Gerba & McLeod, 1976). Nutrients in the sterile seawater will easily elute from the sediment after autoclaving and less elution occurs when sediment is mixed with natural unsterile seawater. The longer *E. coli* survives in the sediment, the great content of organic matter present in the sediment than the seawater (Gerba & McLeod, 1976). *E. coli* was also tested for their survival in the seawater and the effects of sunlight on their growth. *E. coli* occurrence, mainly linked to fecal contamination in the food. Moreover, *E. coli* infection is the main causative of water contamination and/or unhygienic condition during the food handling process (Costa, 2013)

2.5.4 *Pseudoalteromonas* sp.

Pseudoalteromonas sp., a single polar flagellum is a diverse group of the pathogenic bacteria. It is a gram-negative, aerobic, non-fermentative and requires seawater for optimal growth (Anwar & Choi, 2014). Some of these species such as *Pseudoalteromonas atlantica* may cause shell disease syndrome in crabs (Ramos & Rowley 2004). Some of these species are beneficial for antimicrobial properties to against coral pathogen such as *Vibrio shiloi*. (Nissimov *et al.*, 2008).

2.5.5 *Shewanella* sp.

Shewanella genus has about twenty described strains with a wide ranges, habitat, and interaction mode (Anwar & Choi, 2014). They are symbiotic, free-living, and usually extracted from a variety of algae, fish, and seawater (Beleneva *et al.*, 2007). *S. putrefacients* and *S. algae* are widely known as a pathogenic strain that capable to cause bacteremia and septic shock in humans and marine life (Anwar & Choi, 2014).

2.6 Conceptual Framework Implementation

The conceptual framework is the system of concepts, assumptions, expectations, and beliefs. It generates a theory to support on how the research works; which is a key part the research design (Miles & Huberman, 1994; Robson, 2011). Miles & Huberman (1994) have defined the conceptual framework as a visual or written product, one that: -

> "*Explains, either graphically or in narrative form, the main things to be studied— the key factors, concepts, or variables—and the presumed relationships among them*" (p. 18)

Scheiner's (2010) review on conceptual framework development for biology indicates that this application is intermittent, but still reliable. He indicates that a correct conceptual framework design will improve the expected research outcomes. For this research, the conceptual framework was built based on Scheiner's (2010) Towards a Conceptual Framework for Biology review, where it makes up the necessity to force a modicum of formality thinking onto data interpretation, thereby refereeing scientific disputes. According to Scheiner (2010), the conceptual framework may reveal assumptions that are hidden in specific models or experiments where it finally clarifies the central questions being addressed by a scientific enterprise.

A conceptual framework for science biology bases on Scheiner (2010) relies on six fundamental aspects: -

i. Factor that supports life sustainability/persistence (Biology)
ii. Factor that cause of organismal transformation and diversity (Evolution)
iii. Factor that leads to offspring resemblance with their parents (Genetics)
iv. How the cell maintains its structure and functions? (Cells)
v. How does an individual maintain its integrity? (Organism)
vi. Factor that able to explain the distribution of organism (Ecology)

To understand the marine bacterial evolution, it is essential to recognize its theory of evolution. Table 2.1 describes intergenerational patterns of the characteristic of an organism (bacteria), including reasons and consequences (Scheiner, 2010). The theory of evolution should be familiar to all biologists and every fundamental principle w articulated in Darwin's *On the Origin of Species*, were further refined during the Modern Synthesis. However, it is still widely debatable (Scheiner, 2010; Kutschera & Niklas, 2004; Smocovitis, 1996).

Table 2.1: Theory of evolution domain and fundamental principles

Domain
The inter-generational patterns of the characteristic of organisms, including causes and consequences
Principles
1. The characteristic of organisms change over generations
2. Species give rise to other species
3. All organism is linked through common descent
4. Evolution occurs through gradual processes
5. Variation among organism within species in their genotype and phenotypes necessary for evolutionary change
6. Natural selection primarily causes evolutionary change
7. Evolution depends on contingencies

The first three principles are descended with modifications, speciation, and single origin where all are about the facts of evolution per se. Scientist community, mainly accepts these theories in circa the 1860s (Ruse, 1999) and it is not seriously questioned among themselves since (Bowler, 2004). The other four fundamental principles describe gradualism, variation, natural selection and contingency where it is mainly about the mechanism of evolution. Over time, the mechanism of evolution remains vociferously debated (Scheiner, 2010).

Natural selection is the differential survival and reproduction of individuals due to differences in phenotype. It is a key mechanism of evolution; the change in the heritable traits of a population over time (Zimmer & Emlen, 2013). In the early nineteenth and early twentieth centuries, natural selections were not accepted as the primary mechanism (Scheiner, 2010). Conversely, an emergence of the Modern Synthesis opens up a clear ascendancy of natural selection as the primary mechanism (Scheiner, 2010; Smocovitis, 1996).

The Modern Synthesis process has to trim away some of the mechanism such as goal-directed process and refine another process such as cell genetics. Nevertheless, these processes argue over the relative importance of mutation vs. drift vs. natural selection.

For this study, a conceptual framework was designed to identify bacteria community in the different depth of marine sediment; even though information of its previous existence in the purpose area is very limited. It is estimated that marine bacteria abundance in its local community depends on the complex evolution, interaction characteristic and, its environmental surroundings.

Information on bacterial diversity requires an opulent understanding on how the bacteria cells interact with the environment. Since it is a first insight of the bacterial community in the off-Terengganu, no improper genetics evidence was successfully obtained. Nevertheless, there are no adequate data to foresee the physical-geochemical of the surveyed area.

Based on Scheiner (2010) Towards a Conceptual Framework for Biology review paper, conceptual frameworks for this study are designed based on four phases of theories as depicted in Figure 2.10.

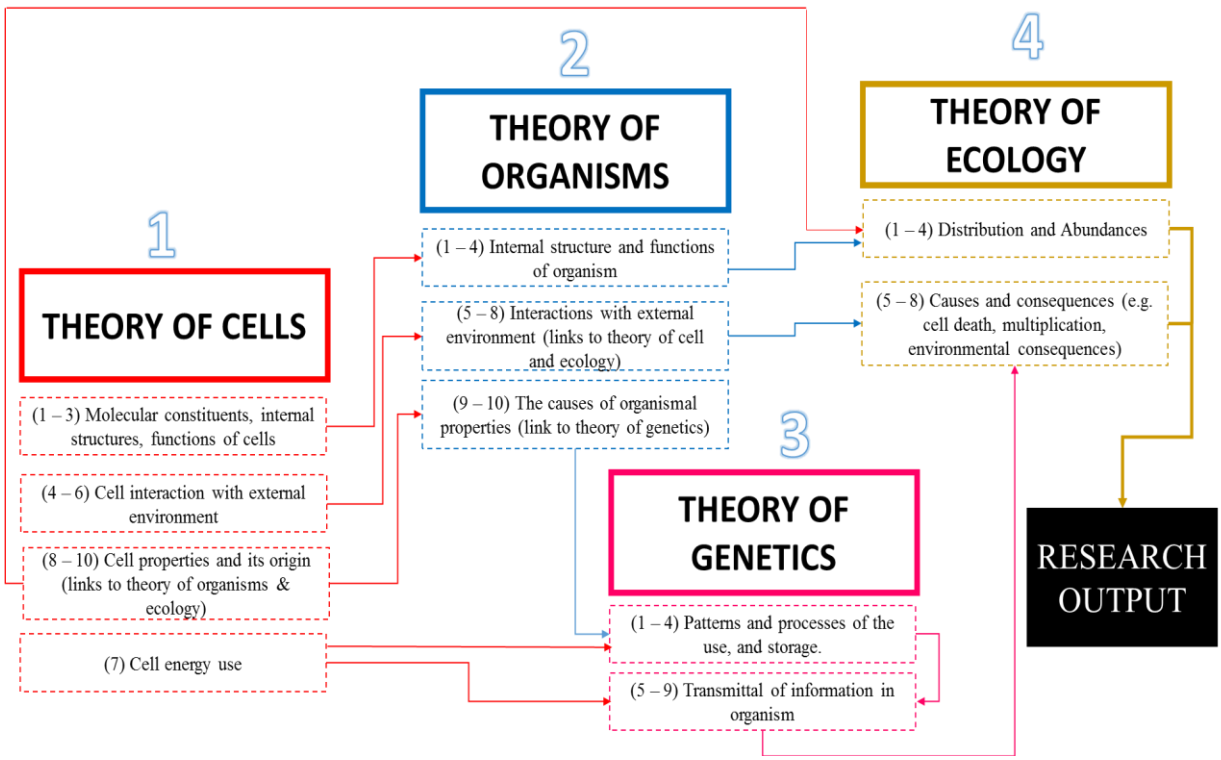


Figure 2.10 Conceptual framework and its phases

2.6.1 Phase One: Theory of cell

The first phase of the conceptual framework is designed to describe the living cell (e.g., marine bacteria). Theory of the cell (APPENDIX A) was encapsulated from Scheiner's (2010) ten fundamental principles of the theory of biology, where it describes diversity, and complexity of living systems, including causes and consequences. Therefore, it epitomizes the foundation of the conceptual framework.

The domain of the theory of cells is the properties and causes of the structure, function, and variation of cells. The first three principles describe the molecular constituent, internal structures and, functions of cells, and they provide links between biology theory and theory of genetics (Scheiner, 2010). The fourth to seventh principles describe the energy usage. The final three describe where the cells and their properties come from and, provide links with the theories of genetic and evolutionary.

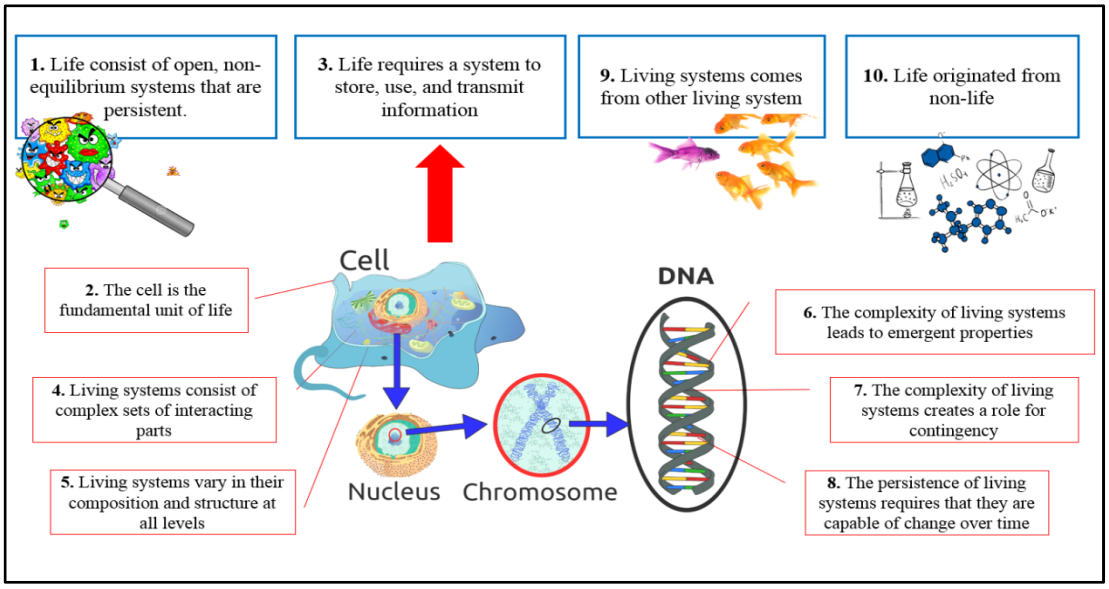


Figure 2.11 Theory of biology's ten fundamental principles

(Text adapted from Scheiner 2010)

Various surveys have shown that bacteria evolutions are similar to all fundamental principle in the Theory of Biology (refer to Figure 2.11). Firstly, the bacterium is part of living systems that are "open" (living systems take in and release matter of energy) and non-equilibrium (living systems consist of ordered structures in a universe that otherwise tends towards disorder and yet manage to persist in a lifetime (von Bertalanffy, 1950). For life to persist, the order must be actively maintained, Thus the persistence is surprising and in need of explanation (Scheiner, 2010). Evidence that supports the above statement can be referred in section 2.4: -The Marine Bacteria.

Secondly, bacteria cells able to maintain a pocket order in a disordered universe – where it holds together the complex machinery of life with the energy to power its systems (Scheiner, 2010). Evidence that supports this statement can be referred in subsection 2.4.1 (Marine Bacteria Abundance in the Seawater) and, 2.4.3 (Molecular Modulation).

Thirdly, although a bacterium is a unicellular, it does have a complex order that contains information. This is vital to ensure a bacterium could maintain itself by capturing and utilized the information contained in that order (Dancoff & Quastler,

1953). Evidence that supports this statement can be referred in section 2.4.2 (Marine Bacteria Physiology).

Fourthly, microbes such as bacteria, are varied in size, space, and times at all levels of biological hierarchy (Mayr, 1982). Information can be referred in section 2.4 - The Marine Bacteria.

Fifthly, the hallmark of bacteria is formed up of many kinds of parts, arranged in a complicated fashion and interacting with each other in many ways (Kolasa & Pickett, 1989). It is believed that interacting structure findings are non-additive and nonlinear (Lorenz, 1963). Other than that, cell complexity is a direct result of dynamic variation in its lifetime (von Betalanffy, 1951).

Sixth, based on the complexity of life system, there are emergent properties occurring at the certain level of organizations due to properties, structures, and processes that are unique to that level (e.g. locomotion consideration). Certain separates cell or species parts could not move on their own. Thus, the movement is an emergent property of the whole organism. For example, emergent properties such as protein depend on the sequences of amino acids and how the chain folded together into a precise three-dimensional (3D) shape. Cells are functioned by separating and concentrating molecules into a subdivision. Most the bacteria have an organelle (flagellum) that supports its unique movement - as described in section 2.4.2: - Marine Bacterial Physiology.

Seventh, life contingency is a combined effect of two processes: randomness and a sensitivity to initial conditions (Lorenz, 1963; Reason & Goodwin, 1999). One factor that allows randomness to play a role is due to the dynamic nature of living systems. Meanwhile, the complexity of cells creates the sensitivity to initial conditions. For the bacteria, it could utilize different sources to obtain food. Nevertheless, bacteria able to modulate its structure when living in an unfavorable environment – as discussed in section 2.4.4: - Bacteria Molecular Modulation.

Eighth, dynamic nature of bacteria is necessary and key for their persistence since each cell changes continuously for survival. Change is one part of the system creates stability in other parts (e.g. Section 2.4.3, Marine Bacteria Physiology - Starvation mode). It does not guarantee persistence, but the lack of cell change will guarantee extinction (von Bertalanffy, 1950).

Ninthly, several bacteria species evolutions occur from one generation to the next. Therefore, it enables continuity of living systems (Scheiner, 2010). That continuity embodies two principles – living systems come from other living systems and the completely new living systems are extremely similar to the ones that they are coming from. For example, a *Streptococcus pneumoniae* and *Streptococcus pyogenes* come from the same genus but a different species. Both have the same morphology; however, it opposed a different type of infections.

Lastly, the cell origin arose during the emergence of biology as a scientific discipline in the nineteenth century (Ruse 1999). The organic origin question was ardently disputed, with one extreme position, relying on the action of miracles and the other on processes governed by natural laws. The history of marine bacteria and its natural habitats can be referred in section 1.2 (Research Background), 2.1 (Introduction) and, 2.4 (The Marine bacteria).

Within the theory of biology are five general theories that span its domain: cells, organism, genetics, ecology, and evolution. Understanding the first four theories will implicate the fifth theory. In general, life exists only because it is possible to maintain highly ordered systems against the decay of entropy. The cell provides the wall between order and disorder. Therefore, cells are the foundation units of life which make an organism is the integrative units (Scheiner, 2010).

2.6.2 Phase Two: Theory of Organisms

Theory of organisms (APPENDIX B) mainly derived from the theory of cells. However, the domain of this theory of the organism is specifically described cell individuality and the causes of structure, function, and variation (Scheiner, 2010). The

first four principles describe the internal structure and function of the organism where it provides a theory of cells and genetics associations. The next four principles deal with interactions with the external environment and provide links to the theories on the causes of organismal properties, where it further connects to the theory of evolution.

2.6.3 Phase Three: Theory of Genetics

Theory of Genetics (APPENDIX C) is derived from the seventh principles of the theory of cells with the ninth and tenth principles of the theory of the organism. Domains of the theory of genetics are patterns and processes of the use, storage, and information transmittal in an organism - where has been described in nine fundamental principles (Scheiner, 2010). This phase is important to identify how organism utilized its energy to transmit information. For example, a bacterium molecular modulations characteristic, such as: - starvation phase, chemical degradation, and predation skills (refer to section 2.4.4)

2.6.4 Phase Four: Theory of Ecology

Theory of ecology, mainly derives from the eighth and ninth principles of the theory of cells; first until eight principles of the hypothesis of the organism; fifth to the ninth principle of the theory of genetics. The domain of the theory of ecology describes the spatial and temporal patterns of the distribution and abundance or organism, including causes and consequences (Scheiner, 2010). This theory is essential to identify how depth variation, nutrition, and physical-geochemical availability affect bacteria abundances. The outcomes from the theory of ecology may determine the consequences of living organism statuses such as mortality, increase, or paucity in proliferations frequency.

For this study, a conceptual framework is designed to predict bacteria interaction and deviation understanding based its local community (Van der Gucht *et al.*, 2007; Wang *et al.*, 2015a) where it may generate a significant microbial biogeography data in numerous environments (Lindström & Lagender, 2012; Bokulich *et al.*, 2014; Wang *et al.*, 2015a). Although there is no fixed conceptual

framework that incorporated with bacteria abundances research, one study suggested that the local bacteria derivation and spatial distributions are scales dependent (Martiny *et al.*, 2011; Wang *et al.*, 2015a). Based on Martiny *et al.*, (2011) bacteria dispersion prediction model, the bacteria spatial distribution scale it was determined in three conditions: -

i. **Global scale**: bacteria spatial separation tends to overwhelm the local environmental effect.
ii. **Small spatial scale**: environmental effects were frequently reported as the major determinant of microbial community composition and,
iii. **Intermediate scale** (ten to thousands of km), both environmental and spatial factors were important fractions in community variation (Martiny *et al.*, 2011; Wang *et al.*, 2015a).

The bacteria dispersion prediction model created by Martiny *et al.* (2011) has been applied in various studies such as: - Logares *et al.*, (2012), Lear *et al.*, (2014), and Wang *et al.*, (2015a), where it has demonstrated an improved bacteria dispersion analysis in terms of bacteria spatial variability among its community disparity, and in a local aquatic ecology.

Several findings suggested that each of marine bacteria is produced for a specific purpose (Dalton *et al.*, 1996; Dinsdale *et al.*, 2008; Deng & Wang 2016). To address these claimed, many researchers focused on bacteria stimulation by targeting physical (abiotic) and live (biotic) factor, such as: - Salinity (Lozupone & Knight, 2007; Mapelli *et al.*, 2015), temperature (Lindh *et al.*, 2013; Mapelli *et al.*, 2015), sea depth (Fortunato *et al.*, 2013), specific substrate (Deng & Wang, 2016), and ocean upwelling (Nelson *et al.*, 2014).

Until today, no exact conclusion to describe the bacteria stimulation in both local and global scales (Wang *et al.*, 2015a), and survival capability in nutrient deprivation state (Gómez-Consarnau *et al.*, 2010). To date, most marine bacteria carry multiple processes when it comes to carbon cycling: - A common natural element composition of the ocean that is beneficial for bacterial stimulation (Dinsdale *et al.*,

2008). A recent survey indicated that chemical complexity in certain substrates (e.g. Lignocellulosic biomass and glucose) might affect the way bacteria interacts in the seawater. Theoretically, synergistic interaction among bacteria is important to promote substrate degradation in the environment (Deng & Wang, 2016).

2.7 Reviews: Marine Bacteria Abundance in the South China Sea Coastline

This review was conducted as part of constructing a personal reference library to distinguish dominant bacteria abundance in a diverse SCS coastline topographies. In recent years, marine microbiology research is mainly conducted in response to a potential or confirmation of microbial infestation in the mariculture sites (Beleneva *et al.*, 2007; Manset *et al.*, 2013; Albert & Ransangan 2013), shipping harbour port and polluted estuaries (Liu *et al.*, 2014b; Jean *et al.*, 2006). Bio-remedial potential of bacteria (refer to point 2.9.2) that is isolated from seawater is currently wide studied (Rani *et al.*, 2010). Jiang *et al.* (2010) on the other hand compares pathogenic bacteria abundance in a different monsoon.

2.7.1 Background of Review

In this inspection, numerous data was extracted from various studies that unambiguously conducted in the SCS coastline region: - China, Philippines, Taiwan, Vietnam, and Malaysia (West Peninsular, Sabah, and Sarawak). The length of the sampling location is approximately 5-8 kilometers from the shore. The bacteria DNA was extracted from a shallow seawater, fish, coral and, molluscs are amplified based on 16S rRNA primers and subsequently cloned with Polymerase Chain Reaction (PCR) analysis. Bacterial diversity in the deep seawater/river/lakes and bacteria isolation from the retail fisheries are excluded from this study.

2.7.2 Results

Based on Appendix G, bacteria diversity report in several countries the SCS regional water, such as - China, Philippines, Taiwan, Vietnam, and Malaysia are included in this review. Results disclosed that Seventy-eight species of bacteria in the SCS coastal seawater are identified. Sixty-two species come from the open water and the rest is isolated from seawater cultivated marine life such as fish, coral, and mollusc.

Figure 2.14 shows that *Vibro* sp. from Proteobacteria phylum tops up the abundance list, where 18 of its genus strains are identified in the overall SCS coastline. Nine of *Vibrio* sp. strains are found in Taiwanese coastline alone (Chiu *et al.*, 2007). Subsequently, the lists are followed with *Shewanella* sp., *Pseudoalteromonas* sp., *Bacillus* sp., and *Pseudomonas* sp. genus where 4, 3, 4 and 6 species are identified respectively. Consequently, all bacterial genes obtained in this review were arranged per its phylum as listed in the Figure 2.13 - where it indicates that 64% of bacterial genes belongs to Proteobacteria phylum, followed by Bacteroides, Firmicutes, Actinobacteria and others with 12%, 9%, 6%, and 9% respectively. The Proteobacteria groups are classified as anaerobic, chemoautotrophs and heterotrophic.

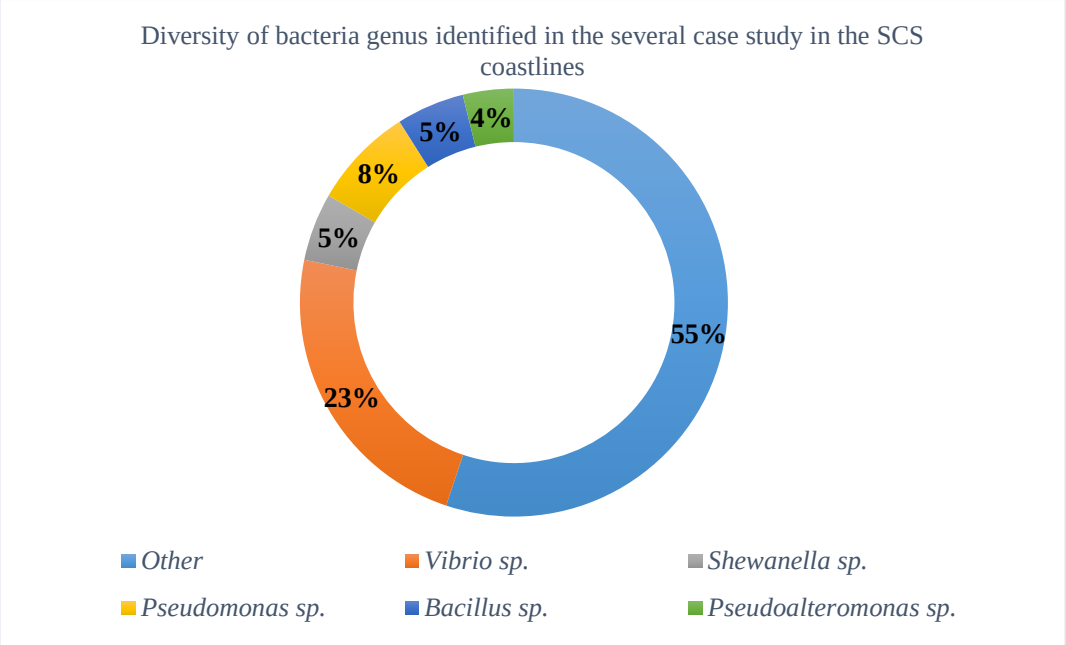


Figure 2.22 Bacterial genus diversity identified in several case studies in the SCS coastline

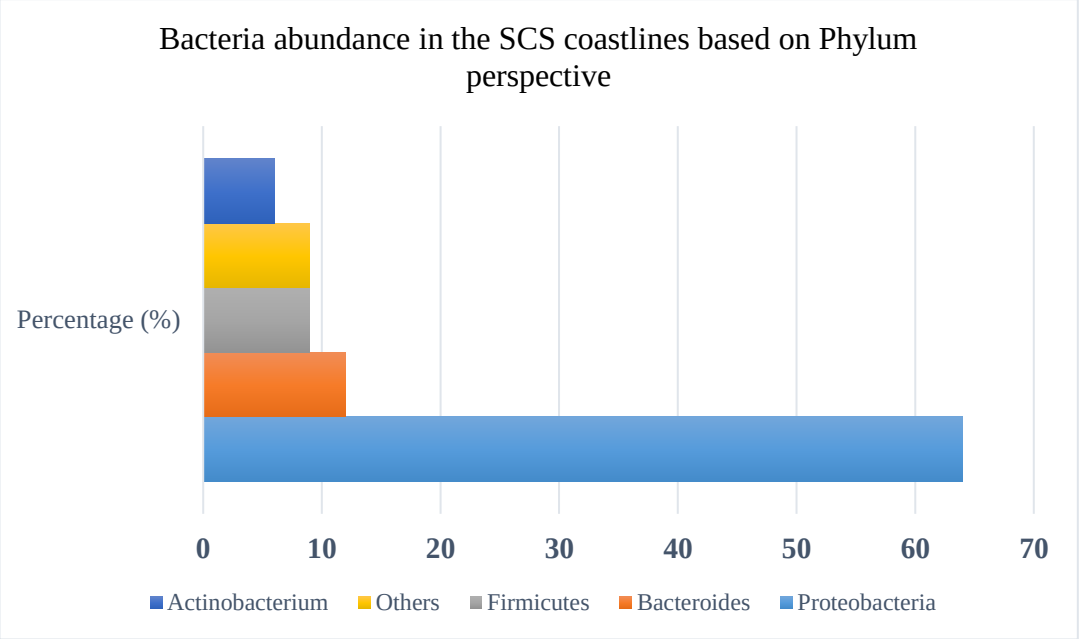


Figure 2.13 Bacteria abundance in the SCS coastlines based on phylum perspective

In easier explanations, Proteobacteria are capable to survive with no oxygen supply. This phylum mainly utilized inorganic substance, and rely on the organic carbon utilization to obtain beneficial nutrients for its growth. It is expected that bacteria that are identified in this case study are mainly reliant on its unique natural physiological ability as discussed in advance marine bacteria physiology reviews in point 2.5 based on several factors: -

i. The Proteobacteria group is mainly fortified with a flagellum (refer to point 2.4) where it permits the bacteria to transmit to other preferable location. Therefore, it diminishes the probabilities of species extinction.

ii. *Vibrio* sp. (Refer to point 2.6.1) physiological characteristic are deemed as a flexible and aggressive, which makes the genus can be identified in a variety of locations such as saltwater marine products (e.g. fish guts, mollusk, cockle etc.) (Jones 2009), ship ballast water (Liu *et al.*, 2014b; Ruiz *et al.*, 2000), human circulatory system (Jones 2009), human waste in the seawater (Jiang and Fu 2001; Huq *et al.*, 2005)

iii. Phosphate concentrations increased in the seawater (mainly contributed by caged mariculture waste) will stimulate bacterial growth in the near water column (Caruso 2014). Consequently, it will reduce food grazing in the seawater with eventual transience the ecological system in its surrounding (Caruso, 2014; Cao *et al.*, 2007).

2.7.3 Impacts of These Reviews

In overall, no adequate study was conducted to predict bacteria community in both natural and pristine coastlines. A pristine coastline signifies a remote island water feature: - which is cleared non-polluted water column. Previous marine bacteria research in the SCS was mainly conducted to investigate its pathogenicity in the human and animals, specifically, based on seafood poisoning occurrences and its abundances in polluted water (refer to section 2.5). This assessment reveals an alarming high anthropogenic pollution in the SCS coastline. Irrefutably, it represents as most polluted coastline region in the world.

This review was conducted mainly to forecast the severity of several marine bacteria genus that has devoured mariculture businesses and seawater quality. These conditions have developed a severe illness incidence in human. The seafood trading in the Southeast Asia was declined due to stocking solidity, negligent, and improper mariculture maintenance (Reichardt *et al.*, 2007). Several food poisoning cases and economic losses in mariculture industry were discussed in subsection 2.3.2.

A mariculture waste in the seawater induces a high organic influx in the seawater and stimulates eutrophication phenomenon (Janssens & Stoks 2014; Caruso 2014). Eutrophication (over-fertilization) in the seawater has a great connection with pathogenic bacteria abundance in the seawater (Smith & Schindler 2009; Caruso, 2014). Over-fertilization usually influence the microbial abundance, organic composition, and microbe's virulence in the aquatic ecosystems (Caruso, 2014). For example, an increase in nitrogen and phosphorus concentration in the seawater may

stimulate HABs growth, aquatic viruses replication rate (Wilson *et al.*, 1996) and, bacteria blooms on the water surfaces (Hofmann & Beaulieu 2006; Caruso 2014).

Identification of marine bacteria in a eutrophication region will portray a unique community distribution. This sort of research requires a crucial choice of analysis to obtain adequate DNA information for marine bacteria phylogeny analysis, since it is difficult to cultivate a live marine bacterium in the laboratory. Section 2.8 below, describes the proposed techniques that are used to sequence bacteria as part of microbial diversity study.

2.8 Next Generation Sequencing (NGS): The Future of Microbial Diversity Analysis

Originally proposed by Woese & Fox (1977), ribosomal RNA gene classification has been the gold standard for molecular diversity research (Pace, 1997; Woese & Fox, 1977). Historically, molecular phylogenetic analysis has been applied to characterize microbial subpopulations and communities in a diversity of environments (Gray & Herwig, 1996). Conventionally, cloning and sequencing of the ribosomal DNA gene (rDNA) using conserved broad-range PCR primers were commonly used to identify bacteria biodiversity (Klindworth *et al.*, 2012).

Analyzing environmental samples, DNA extraction and purification can be problematic due to a variety of factors (Lovell and Piceno 1994). To overcome this problem, some researchers have attempted to remove the microbial community from the environmental matrix (Atlas, 1993; Holben *et al.*, 1993; Steffan *et al.*, 1988; Tsushima *et al.*, 1995) and while others opt to lyse the cells in-situ (Atlas, 1993, Steffan *et al.*, 1988; Tsai & Olson, 1991). The primary concern of either approach is the efficiency of cell lysis as well as the integrity and purity of the extracted DNA. Generally, the *in-situ* approach produces more quantitative results; the lysis efficiencies can be more than one order of magnitude superior compared to cell removal techniques (Tsai & Olson, 1991). Several investigations have focused on the concerns as they apply to lysis procedures based on bead mill homogenization (Atas, 1993). Nevertheless, these approaches only manage to identify only 0.001 to 1%

cultivable bacteria. Furthermore, the scarcity of well-characterized microbes and the lack of a reliable prokaryotic taxonomy system often make it difficult to classify microbes to species or sub-species level will solely base on 16S rDNA gene sequences (Atlas, 1993).

16S ribosomal RNA gene (rDNA) amplicon analysis remains the standard approach for the cultivation-independent of microbial diversity (Klindworth *et al.*, 2012). In addition, 16S rDNA gene sequences able to provide more objective and reliable classification of microbes that phenotyping (Schloss & Handelsman; 2004). Therefore, efforts to improve molecular tools based on the PCR and bacterial ribosomal gene phylogenetic tree (16S rDNA / rRNA) are essential to expand global bacteria biodiversity coverage (Klindworth *et al.*, 2012) by minimizing difficulties in microbial taxonomy investigation (Kim *et al.*, 2011). Subsequently, it complements, or augments taxonomy data archive derived from culture-based procedures (Gray & Herwig, 1996).

2.8.1 Introduction of Pyrosequence / Phylogenetic Analysis

Lane *et al.*, (1985) were first described the use of 16S rDNA gene for identifying and classifying uncultured microbes in the environment. PCR amplification, cloning, and sequencing have been the primary technologies used in determining 16S rDNA gene sequences from various environments. In recent years, molecular phylogenetic analysis has been used to characterize microbial subpopulations and communities in a variety of environments (Amann *et al.*, 1995). Historically, only 0.001 to 1% of existing bacteria are cultivable by using a conventional method such as colony incubation (Ward *et al.*, 1990). Therefore, to complement the data that derive from culture-based procedures (Gray and Herwig 1996), researcher have adapted a modern molecular tool based on the PCR and phylogenetic of the 16S rRNA gene. (Gray & Hedwig 1996; Klindworth *et al.*, 2012; Mizrahi-Man *et al.*, 2013)

For the past two decades, more than 1.3 million of bacterial 16S rDNA gene sequences have been archived in the Ribosomal Database Project (RDP) (Cole *et al.*, 2009). These sequences are curated where it accounts 16S rDNA genes recovered from

both cultured and uncultured prokaryotes, later, configuring for most of the sequences. The 16S rDNA gene sequence in RDP has been classified into genera among 35 bacterial phyla, but many of these phyla are composed primarily or entirely of uncultured prokaryotes (Schloss & Handelsman, 2004).

The 16S rDNA gene sequences generated from microbial are typically clustered into the operation taxonomic unit (OTU) at few distance levels to determine species richness, diversity, composition and, community structure. Species, genus, family and, phylum are conventionally defined with distance values by 0.03, 0.05, 0.10 and 0.20 respectively, based on full-length (1540bp) of 16S rDNA gene sequences (Schloss & Handelsman 2004). However, 16S rDNA gene sequences produced in most studies are partial sequences of 700 bp (Sanger DNA sequencing) or shorter due to cost restraint or technology limitation (from NGS analysis).

Currently, RDP database has less than 44% of bacterial sequences that are longer than 1200 bp. Only a small percentage of the sequences on RDP reached nearly full length. Therefore, the most researchers used partial 16S rRNA gene sequences to make taxonomic assignments. This discordance may create vagueness in the taxonomic placement of OTUs due to following reasons:

1. Divergence among different 16S rRNA gene sequences is not distributed evenly along the 16S rRNA gene, but concentrated primarily in the nine hypervariable (V) regions (Stackerbrant & Goebel, 1994),

2. Some of the V regions are more variable than others (Youssef *et al.*, 2009; Yu & Morrison, 2004) and,

3. Some regions of the 16S rRNA genes produce more reliable taxonomic assignments than others (Liu *et al.*, 2007; Lie *et al.*, 2008; Wang *et al.*, 2007).

It is assumed that different V regions may produce different results with respect to estimated species richness, diversity and, microbial composition and structure. Furthermore, some partial sequence regions may be better suited for microbial analysis than others (Kim *et al.*, 2011).

In recent years, modern molecular microbiology technology such as NGS has been a major contributor for 44% of marine bacteria community identification globally (Gibbons *et al.*, 2013; Gilbert *et al.*, 2012). Various findings that are produced from NGS leads to titillating speculations: the same bacterial community may have identified in all global oceans. A team of marine microbiologist address these speculations in the Proceeding of the National Academy of Sciences back in the year 2012. The microbial ecologist team leads by Jack A. Gilbert has studied the bacterial communities in the Western English Channel (WEC) where it generates an approximately 10 million bacteria sequences from 16S rRNA-V6 pyrosequencing analysis. Consequently, it matches 356 data sets read from the International Census of Marine Microbes (ICoMM).

As the team deepens the sequencing depth, WEC bacteria phylogenetic data has overlapped the global ICoMM database from 31.7% to 66.2%. Perhaps, it is possible that 100 percent of the world's marine bacteria can be identified if 1.93×10^{11} sequences read were applied in the same experiment settings (Gibbons *et al.*, 2013). Although marine bacteria are abundant in the ocean, Gilbert, and his team states that they still need to conduct more experiments to distinguish the marine bacteria growth in a fluctuated environment such as: hot, cold, alkaline, high phosphorus level, or high iron level.

Based on preliminary study, different species have its own specific conditions for optimum growth. If the condition suits them, the bacteria will rapidly take the advantage to multiply (Gilbert *et al.*, 2012). These speculations are somehow similar to Louise (2013) claims:

> *Conventional theory said that these bacteria must migrate to where their favourite resources are. However, what this paper suggests is that the old theory of bacteria moving into an environment is wrong. All the species are always there, just in very small amounts*

2.8.2 Challenges in Marine Bacteria Identifications

A marine bacterium is a simple living cell, but it is packed with metabolically complex capabilities. Different species have its own specific conditions for optimum growth, which it rapidly takes the advantage to multiply (Gilbert *et al.*, 2012). Unlike clinical bacteria, marine bacteria associations in the ocean remain a mystery, especially its sustainability in a geochemical fluctuated circumstance: - hot, cold, brackish, acidic and, nutrient-rich zone such as phosphorus, iron, or sulphur compound. Additionally, no satisfactory data to correlate bacterial abundance in an inorganic and xenobiotic compound in the seawater since this kind of research was conducted only in the laboratory phase by using pure bacteria culture (Antoniou *et al.*, 2015).

Marine bacteria that has been isolated from its natural sources are difficult to cultured; due to atmosphere differences, and imitation effort to mimic its natural environment in a laboratory is costly and interminable. In general, the molecular identification approach is the most preferred method to study the marine bacterial diversity, since it does not require a live cell. Identification is done by cloning the targeted DNA fragment to reconstruct similar complete genetic sequences.

In recent years, the Next Generation Sequencing (NGS) analysis promptly introduced to support Polymerase Chain Reaction (PCR) and Denaturing gradient gel electrophoresis (DGGE) analysis as part of cell DNA research. NGS analysis is, efficient and ideal for biodiversity surveillance. The NGS operation cost is expensive but it does provide a fruitful data to support genome data depository (Pak & Kasarskis, 2015). Furthermore, based on the NGS result obtained, a meticulous plan is required to achieve the required research objective without causing financial constraint (Pak & Kasarskis, 2015).

2.8.3 DNA Replication and Selection of Primers

According to Alberts *et al.* (2012), DNA structures are double-stranded where both strands coiled together in a helix formation. Each of single strand consists of four types of nucleotides. The DNA nucleotides contain deoxyribose sugar, phosphate and,

nucleobase where it corresponds to the four nucleotides: - adenine (A), cytosine (C), guanine (G), and thymine (T). Adenine and guanine nucleotide are purine bases while cytosine and thymine nucleotide is pyrimidines. All A, C, G and, T nucleotide form a phosphodiester bond, creating the phosphate-deoxyribose backbone of the DNA double helix with the nucleobases pointing inward. Nucleotides are connected between strands through hydrogen bonds to form a base pair. The Adenine (A) nucleotide is paired up with thymine where it forms two hydrogen bonds and, the guanine paired up pairs with cytosine and create a stronger three hydrogen bonds (Alberts *et al.*, 2002).

Alberts *et al.* (2002) mentions that DNA strands consist of specific direction with two different ends for each single strand - “3' (three prime) end" and, the "5' (five prime) end". By convention, if the base sequence of a single strand of DNA is given, the left end of the sequence should be the 5' end, while the right end of the sequence positions with 3' end. The double helix strands are anti-parallel in a formation where one position is 5' to 3', and the opposite strand is otherwise: 3' to 5'.

These terms refer to the carbon atom in deoxyribose to which the next phosphate in the chain attaches. DNA direction has consequences in DNA synthesis because DNA polymerase able to synthesize DNA strands only in one direction by adding nucleotides to the 3' end of a DNA strand (Alberts *et al.*, 2002).

DNA polymerases are an important group of enzymes that carry out every DNA replication phase (Berg *et al.*, 2002). In general, DNA polymerases are unable to initiate synthesis of new strands. However, it is able to lengthen an obtainable DNA or RNA strands to pair with a new template strand. To initiate DNA synthesis, short fragments of RNA (primer) are created to pair up with the customized DNA template strand. Spiering & Benkovic (2013) stated that pairing DNA complementary bases through hydrogen bond indicates that the nucleotides (represent as information) that bond in each strand is redundant. The nucleotides that were constructed in a single strand formation able to reconstruct nucleotides on a newly synthesized pair strand to develop double strand formation (Spiering & Benkovic, 2013).

In general, DNA polymerases are very accurate, with an inherent error rate of less than one mistake for every 10^7 nucleotides added (McMulloch *et al.*, 2008). In

addition, some of DNA polymerases also have “proofreading” ability by naturally removing unequal nucleotides from the end of an emergent strand; in order to correct any mismatched bases. Finally, DNA error could be monitored and, repaired based on post-replication mismatch, which is based on a distinguishing report in the new synthesized DNA from the original strand formation. All these discrimination steps facilitate and strengthen DNA replication reliability with less than one mistake for every 10^9 nucleotides added (McMulloch *et al.*, 2008)

Historically, McCarthy (1976) believes that the DNA replication rate in the living cell is conceivable to enumerate. It has been demonstrated in T4 DNA phage elongation rate quantification in phage-infected E. *coli*. McCarthy (1976) has cited Drake (1970) experiments where it reveals that the life cell rate was 749 nucleotides per seconds, during the exponential DNA period increase at 37°C. In overall, Drake (1970) concludes that the mutation rate per base pair per replication during phage T4 DNA synthesis is 1.7 per 108 seconds.

According to Saiki *et al.* (1988), DNA replication *in vitro* was eminently instigated by using the polymerase chain reaction (PCR) method. The PCR method uses a pair of specific synthesized primers in the span DNA target region. Consequently, it polymerizes DNA partner strands in each primer direction by using a thermostable DNA polymerase. These processes are reiterated through multiple amplification cycles within the targeted DNA region. During the initiation of each cycle, the mixture of DNA template and primers are carefully heated to isolate anew-synthesized molecule and template. When the mixture cools down, both components become DNA templates to anneal new primers and, activate the polymerase extension. Thus, the number of copies of the target region doubles each cycle and increasing exponentially (Saiki *et al.*, 1988).

Primer is a strand of short nucleic acid sequences (generally about 10bp) that serves as a starting point for DNA synthesis. To achieve an accurate DNA synthesis, selections of DNA primers are critical (Armougom & Raoult 2009; Schloss *et al.*, 2011; Klindworth *et al.*, 2012). DNA replications are essentially required by introducing a DNA polymerase enzyme to initiate the catalyst reaction to attach new

nucleotides into an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer and copies the opposite strand (Baker, 2003).

Unfortunately, utilization of sub-optimal primers may give poor interpretation (Baker, 2003). Furthermore, selections against single species or whole group are also equally important (Wang & Qian 2009; Hamady & Knight 2009) to prevent the doubtful biological conclusion (Hamady & Knight 2009; Andersson *et al.*, 2008).

The combination of forward and reverse primers may generate single primer bias. Therefore, it is essential to use synthesize primer with a similar overall coverage to minimize the overall bias (Klindworth *et al.*, 2012). A forward and reverse primer pair sequences (e.g. 16S rDNA) are generated based on a literature study, openly retrieved from the SILVA database (Quast *et al.*, 2013) or probeBase, a comprehensive online database of RNA-targets oligonucleotides. Only a set of primers with at least 75% of overall coverage above 75% set for either Bacteria or Archea identification were considered for DNA replication. (Loy *et al.*, 2008).

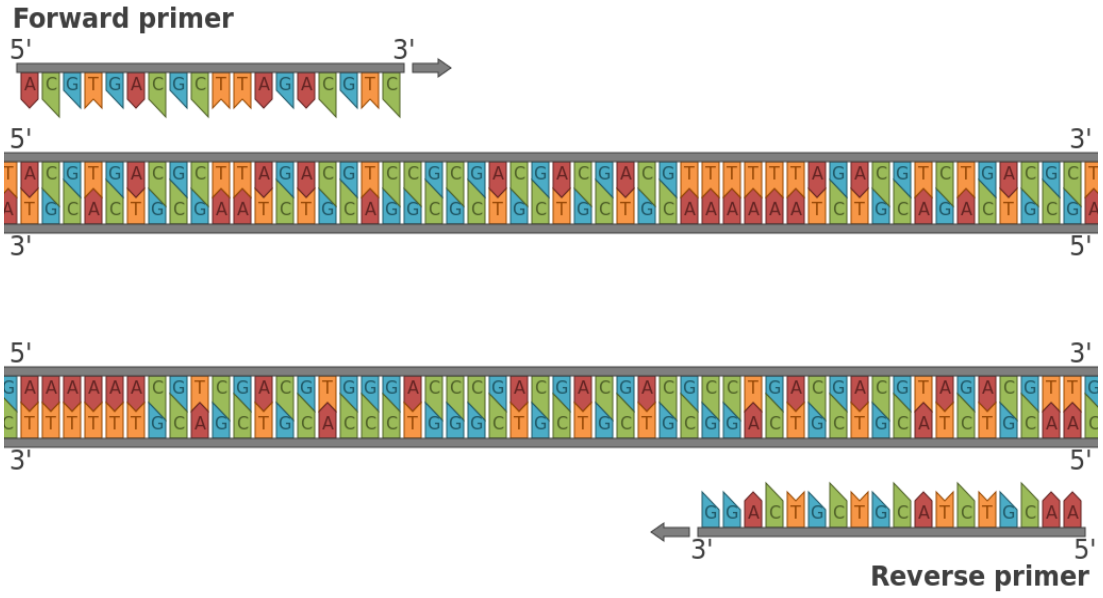


Figure 2.14 Diagrammatic representation of the primers for PCR, indicating the forward and reverse primers and the reverse complement sequence of the reverse primer

(Image credit to Richard Weeler +. https://en.wikipedia.org/wiki/User:Zephyris)

Other than selecting an accurate primer, a reliable DNA replications require an ideal PCR annealing temperature, amplicon length, and hypervariable region (refer to section 2.8.4). Because the PCR analysis anneals both reverse and forward primers simultaneously, a primer should have similar melting temperatures (T_m) as the targeted amplicons. Based on Kibble (2007) suggestions, if a primer T_m is higher than the existing amplicon annealing temperature, it may hybridize and extend at an incorrect location along the DNA sequence. If a T_m of primer is lower than the annealing temperature, the PCR cycle may fail to anneal and extend at all. OligoCalc provides a beneficial interface to calculate a correct annealing temperature (Kibbe, 2007). Primer pair with an annealing temperature difference of less than 5°C are generally accepted (Klindworth *et al.*, 2012).

Generally, the primer pairs that are selected for genomic sequencing must be structured accordingly to its ideal amplicon length. Wiesburg *et al.* (1991) have devised the most common primer pair referred to as 27F and 1492R (>1200bp length). However, for some sequencing applications such as 454 Roche Titanium requires a short amplicon (500bp); 27F-534R that mainly covers V1 to V3 hyper region (Mizrahi-Man *et al.*, 2013). Currently, 8F is used to regularly compare to 27F. Although 8F and 27F are almost identical, it has a different nucleic acid notation. Primer 27F has an M as ambiguous code for amino (AGAGTTTGATC**M**TGGCTCAG). C represents as an ambiguous code for Cytosine in primer 8F (AGAGTTTGA TC**C**TGGCTCAG). These nucleic acid notations are mostly applied to exploit DNA size, balance, and shape per research objectives (Mizrahi-Man *et al.*, 2013).

2.8.4 Selection of Hypervariable region (V)

Numerous of Bioinformatics studies have examined how to choose 16S rRNA gene region or hypervariable region (V) (Claesson *et al.*, 2011; Huse *et al.*, 2008; Soergel *et al.*, 2012; Wang *et al.*, 2007) in designated molecular microbiology research. In common knowledge, different V regions may produce different results with respect to estimates of species richness, diversity, microbial composition/structure and, some partial sequence regions may be better suited for microbial analysis than others. A different taxonomic cut-off value or distance level

may be required for a partial sequence region to give rise to similar results as nearly full-length sequences (Kim *et al.*, 2011).

Several of the research was conducted to compare the effectiveness of V region for biodiversity research. Some research has compared two single V regions and against a set of nearly full-length sequences in estimating OTU richness (Claesson *et al.*, 2009; Dethlefsen *et al.*, 2008; Huse *et al.*, 2008). There is one research has examined thousands of primers and read length combination, but it mainly focused on queries that have a close counterpart (97 percent identity) in the reference database (Soergel *et al.*, 2012). Results suggest that the choice of V regions has significantly affected OTU richness and diversity estimations.

One study showed that the V1 and V2 regions (approximately 350bp) and the V8 regions produced different OTU evenness from the same sample. Another study compared eight V regions in both singular and dual shows that the length of the partial sequence regions only ranged from 99 to 361bp (Youssef *et al.*, 2009).

The outcomes of this research indicate that no conclusive experimental design was established to perform on novel bacterial species, which is commonly encountered in environmental studies. Most of studies meets a differing conclusion to choose the most effective targeted hypervariable region. Generally, a bacterial diversity study is recommended to include one, or multiple hyper regions - Specifically, a combination of V2/V3/V4/V6 or V3/V4.

These results variations are possibly due to many factors, including specific primers examined, the environmental source of the reads, and classification method and the parameters chosen during analysis. This lack consensus is evident in recent literature, with most current studies focusing on either hypervariable region V3, V4 or V6 (Caporaso *et al.*, 2011; Hummelen *et al.*, 2011; Finkel *et al.*, 2011; Shepherd *et al.*, 2012) with no convergence on a single hypervariable region being chosen (Mizrahi-Man *et al.*, 2013)

In conclusion, the V1-V3 or the V1-V4 regions of 16S rRNA gene provides two advantages: - First, the V1-V3 or the V1-V4 regions are more divergent. Therefore, it offers a more phylogenetic resolution that other regions. A greater phylogenetic resolution is important for microbiomes analysis of specialized habitats such as - an intestinal tract of animal and humans, the lumens, anaerobic digesters, and biological wastewater treatment reactors - where great diversity exists at low taxa (Kim *et al.*, 2011).

Second, the RDP and other databases stored more partial sequences that correspond to the V1-V4 region than the downstream region. Inherently, a partial sequence corresponding to V1-V4 region will deliver more database sequences to compare and it facilitates a clearer phylogenetic analysis. The conclusions of this study were drawn from comparing short partial sequences recovered from one or few habitats. However, the conclusions derived from these studies may not be applied to broad environments (Kim *et al.*, 2011).

2.9 Marine Bacteria Contributions

In medical microbiology, the bacterium is generally described as the antagonist of health, because it mainly elicits a bountiful of disease towards human and animal. Most of the infestation is acute, and may lead to mortality if it does not treat. Several clinical-based bacterial species have also caused waterborne bacterial disease (e.g: *Salmonella* sp., *Vibrio cholera*, *Escherichia coli*, *Entamoeba histolytica* etc.) that has the prominent ability to survive in the aquatic environment, where it was transmitted to the mankind from contaminated water and seafood (Lebaron *et al.*, 2015).

However, no sceptic view was mentioned to questions a native marine bacterium's role to trigger infection in a human; because no notable outbreaks were reported (Young, 2016). In fact, marine bacteria are required to regulate anomaly in the seawater and provides food for other aquatic lives. The subsections bellows, describes how marine bacteria bring benefits to the mankind: -

2.9.1 Marine Pollution Monitoring

Generally, the organic pollutant is frequently occurring in the coastline. Organic pollutant is mainly derived from sea harbor, aquaculture, polluted estuaries, and industrial waste. Based on several literatures, the petroleum oil/hydrocarbon is a common organic pollutant found in the global coastline. It is discovered that a hydrocarbon molecule has an affinity to bind with marine sediment or remains suspended in the water column (Mistch, 2010; Suárez-Suárez *et al.*, 2011).

Numerous of comprehensive research are focused on evaluating pollutant constituent, and its severity towards marine ecosystem. However, every finding shows no exact prediction to describe pollutant that is associated with the seawater. It is assumed that seawater pollution is unpredictable, and oftenly ended up with rapid ecological perturbation (Suárez-Suárez *et al.*, 2011).

Generally, physical-geochemical value in the seawater plays an important role that determines marine bacteria abundances in the seawater, such as salinity, pressure, temperature, and nutrient availability. In addition, another microorganism such as, marine virus usually regulates marine bacteria blooms by killing it or “competing” for available nutrients (Gilbert, 2009). It is worth to mention that global warming and irreversible changes in ecosystems, has influenced bacterial pathological behavior. This conditions makes the marine bacteria as a consistent subject to a countless environment stimuli investigation (Marten *et al.*, 2001).

2.9.2 Bioremedial Properties

According to Environmental Inquiry (2009), "Remediate" means to solve a problem, and "bio-remediate" means to use biological organisms to solve an environmental problem such as contaminated land or groundwater. Bioremedial also stands for utilization of biological organisms to resolve environmental problems such as contaminated soil, groundwater, and seawater (Öztürk *et al.*, 2015). In a pristine environment, the microorganism is constantly degrading organic compound, while

another microorganism may die from the organic pollutant, others will engulf organic pollutant for survival.

Bioremediation works by providing these pollution-eating organisms with fertilizer, oxygen, and other condition to encourage their rapid growth. Bioremediation in a contaminated site works in two ways: (1) Relying the on pollution-eating microbes that are already abundant in the contaminated sites (Fulekar, 2009) and, (2) Adding specialized microbes to degrade the targeted contaminants (Jørgensen & Marshall, 2016).

Many cases of research suggest that bacteria possess the ability to degrade and utilize the pollutant compound for energy such as *Rhodococcus, Burkholderia xenovorans, Psedoalteromonuas, Sulfuricurvum sp., Sulfurovum* sp. etc. (Jørgensen & Marshall, 2016). The organic matter that binds to the seawater sedimentary consists heavy metals, hydrocarbon, pesticide etc. The impact of anthropological pollution in the marine ecosystem is depending on the history of environmental pollution itself. Consequently, a certain marine bacterium that was previously adapted in an oil spill pollution will occur faster, rather than in a pristine environment - as it has metabolically readiness to degrade a hydrocarbon compound (Païssé *et al.*, 2008).

Bioremediation offers a good mitigation plan for only certain types of pollution. However, some might not work at all. For example, bioremediation may not provide a feasible strategy at the sites with a high xenobiotic compound that is toxic towards bacteria, such as cadmium, lead, and sodium chloride (Rani *et al.*, 2010).

Depending on the sites and its contaminants, bioremediation may be safer and less expensive that alternative solutions such as incineration or landfilling of the contaminated material (Kumar *et al.*, 2011). It also has the advantage to treat the contamination in the place that does not require pumping out soil, sediment, or water out of the ground for treatment (Environmental Inquiry, 2009).

2.9.3 Antibiotic Properties

Generally, the antibiotic compound is created based from microbial synthesize to induce illness from microbial infection. For example, certain *Ascomycetous* fungi such as Penicillium were developed to overcome bacterial infection in the human body by producing a renowned antibiotic beneficial molecule: Penicillin (Nisa *et al.*, 2015). However, several microbial has developed its resistance against these antibiotic regimes, where some bacteria strain express its metabolically resistance to all known antibiotics. It is recently discovered that a notable microbial induced disease specifically, Methicillin-resistant Staphylococcus aureus (MRSA) is no longer curable (Deeny *et al.*, 2015). This condition occurs due to the undiscriminating use of heavy antibiotic doses in the health care industry, which it is fortifying the pathogenic bacteria defense system in responds to antibiotic attack (Deeny *et al.*, 2015).

To combat the alarming antibiotic resistance-based diseases, several efforts are prompted to develop a new antibiotic development. The aim of this new antibiotic development is to deliver an effective alternative before any bacteria are capable to modulate its resistance system towards the new antibiotic regime. The new antibiotic introduction must combine a brute-force screening and target a suitable genetic modification from a potential antibiotic-producing bacterium. It can be done by adjusting a structure wand from the potential bacterium (Hall, 2004). The search for new antibiotic is an ongoing process, because it expects challenges to reduce the probabilities of the antibiotic resistant bacterium occurrences from the new antibiotic generation. For example, a new generation antibiotic: Zyvox that is being introduced in 2000 is already had its first diagnosed Zyvox-resistant strain a year later (Hall, 2004; Eliopoulus, 2004).

A filamentous soil bacterium produces many known antibiotics: *Actinomycetes* (Streptomyces genus). These species are also identified in the seawater column. The *Actinomycetes* excretes its antibiotic compound via secondary metabolic pathways that are originated from small and simple precursors with consist of amino acids, small fatty acids, sugars, and nucleic acids. Several new antibiotics have been found by targeted or random inactivation of genes, leading to utilization of alternate biochemical

pathways (Zazo *et al.*, 2016). Investigation to identify mutant reaction from a novel and active antibiotics are inconclusive, but shows a good expectation. However, a long time is required before such antibiotic is consumed, as it needs a long clinical testing and must obtain a valid certification for each antibiotic (Raijmarkers *et al.*, 2002).

2.9.4 Role of Bacteria in Hydrocarbon Exploration

Microorganisms that has unique metabolic activities is being the key players in many environmental and technological processes. It has a fast metabolism and unique catalysed chemical reactions and physiology that helps it to synthesized variety of products. Microbial synthetizations allow researcher to customized microbiological processes course to suits their objectives (Turkiewicz, 2011). Specifically, in recent years, microbial involvement in hydrocarbon exploration has gained much interest and its practical application does give a prospect to oil and gas producing country such as Poland (Turkiewicz, 2011). However, some research faces uncontrolled and excessive microbe growth where it may lead to bacterial contamination. For example, incidences of drilling fluids biodegrading, microbiologically influenced corrosion and microbial contamination in the oil and stored gas (Turkiewicz, 2011).

Petroleum and gas institute specifically in Poland, use a hydrocarbon as a carbon source for bacteria isolation technique, that is to say, the Microbial Well Survey Technique (MWST) and the Surface Method Based (SMB). The MWST technique works by isolating the "indicator" microbes from oil and gas-bearing core zone in the geological deposits variation study. By using a specialized microbiological media, this technique is highly sensitive to detect the hydrocarbon amount in the surveyed areas. (Niewiadomska & Turkiewicz, 2000). The SMB method is designed to detect any microbial distribution anomalies in the soil samples. This method works well in the foundation that has detectable quantities of trapped hydrocarbon inside a subsurface oil reservoirs, where it moves erratically in upward direction. (Schumacher, 1999).

Major issues that are troubling the hydrocarbon industry is providing a strategy to recover a large amount of petroleum deposit in nature and virtually depleted oil

fields. In addition, the oil and gas field industry faces petroleum production lost due to difficulties to remove unwanted paraffin and asphaltene in the oil deposit. For years, the oil and gas company has introduced few tertiary techniques to attempt oil recoveries, specifically: - gas injection, water-flooding, and surfactant flooding. However, these techniques are not economically viable and oppose a numerous efficacy limits (Turkiewicz, 2011).

The microbial enhanced oil recovery (MEOR) technique seems attractive and environmentally friendly (Dietrich *et al.*, 1996; Jinfeng *et al.*, 2005). The MEOR technology is already used in Argentina, Canada, Venezuela, China, and the U.S.A. Outcomes obtained from hydrocarbon deposits from the North Sea, Mexico, Trinidad, and Australia have shown great potential for the MEOR application. Among the useful microorganism in MEOR is *Pseudomonas* sp., *Bacillus* sp., *Brevibacillus* sp., *Agrobacterium* sp., S*phingomonas* sp., *Rhizobium* sp., *Coprothermobacter* sp., *Thermolithobacter* sp. (Zhang *et al.*, 2010).

The selection of appropriate microorganisms with high potential will ensure a successful oil recovery by: -

1. Generating gasses that increase reservoir pressure and reduces oil viscosity
2. Generating acids that dissolve rock to improve absolute permeability.
3. Reducing permeability of channels
4. Producing bio-surfactants that reduce interfacial tension.
5. Reducing oil viscosity by degrading long-chain saturated hydrocarbons

(Zhang *et al.*, 2010; Turkiewicz, 2000)

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Introduction

Marine bacteria are widely known as a regulator of marine ecosystems. Understandings of their diversity are considered as a great mission for marine microbiologist (Klindworth *et al.*, 2012), because it is difficult to study its life cycles quantitatively (Azam *et al.*, 1983). For example, frequent bacterial cross-contamination may be occurred during diversity analysis, and tendency to obtain insufficient amounts of bacterial cell may hinder signification of the whole bacterial diversity in its local area (Azam *et al.*, 1983; Klindworth *et al.*, 2012).

Historically, the enumeration method was applied to quantify bacteria cells. However, in later years, this method opposes several disadvantages. For instance, plate counts, serial dilution, or phase-contrast microscopy mainly gave ten percent of actual number estimation (Klindworth *et al.*, 2012). The actual numbers of estimated bacterial biomass are usually discarded (Klindworth *et al.*, 2012). Therefore, a culture-independent survey has been developed to address Bacteria and Archaea significant fraction in order to improve microbial diversity analysis (Klindworth *et al.*, 2012).

Formerly, cloning and sequencing of the 16S ribosomal DNA gene (16S rDNA) or 16S ribosomal RNA gene (16S rRNA) by using conserved broad-range PCR primers were commonly used (Bastien *et al.*, 2008). With the advent of massively parallel sequencing technologies, PCR amplicons direct sequencing was applied to the

systems such as Roche 454 GS20 pyrosequencing and Illumina™ Miseq (Fadrosh *et al.*, 2014)

In this chapter, the methodology is discussed briefly based on the justification of research design, sample selection, sampling activity, biodiversity analysis and physical-geochemical analysis.

3.2 Research Design

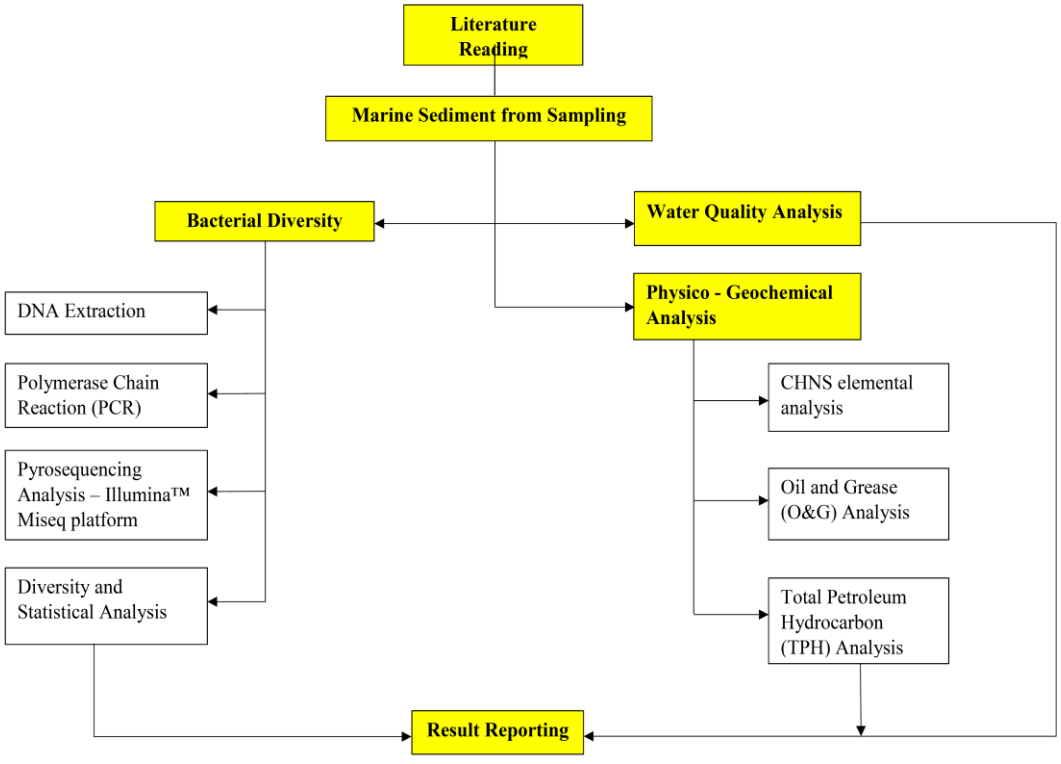


Figure 3.1 Diagram of overall of research design flow

As shown in Figure 3.1, this study is divided into six sections where every element involved is made to follow conceptual framework (section 2.7). Basically, this experiment was commenced by performing sediment sampling, where all sediment collected is divided into two polyethylene bags for two analyses: - 1) Bacteria identification, 2) Water quality analysis and 3) Physical-Geochemical analysis. This

experiment is not designed to include *in-vitro* bacteria cultivation (e.g. cell incubation on specified marine agar) as it may reduce chances to obtain sufficient bacterial cells and, there are higher chances of obtaining massive contaminant genus.

The Physical-geochemical analysis was designed to only follows bacteria phylogenetic findings to minimize undesirable cost and time. Based on the physical-geochemical findings, the overall bacteria dispersion via environmental condition could be estimated. Subsequently, all data that is obtained in every analysis are then compared with several references before it is ready for research reporting.

This research is considered a pre-elementary study and may encounter several shortcomings in the result and discussion. We believed that this research will help us to determine the right DNA primer configuration for local marine bacteria identification. To overcome any data inadequacies, the NGS are designed to portray bacteria diversity based on statistical method. At least 5000 of DNA sequences must be identified per sample, in order to obtain a satisfactory α-diversity value. Every finding that is achieved in this study will be used to improve the future research planned.

3.2.1 Sample Selection: Attached Marine Bacteria

There are two bacteria characteristics that need attention upon sampling activity. First, the bacteria that is being isolated naturally, must have the energy to utilize any food sources in order to survive in the warm environment. Secondly, the bacteria must naturally have an ample cellular activity, or has a great cells concentration, that ensures a sufficient extractable DNA amount for replication and transcription procedures. Based on discussion in subsection 2.4.1, the attached marine bacteria were chosen for phylogenetic analysis because it has an active metabolic in warmer condition (Mohit *et al.*, 2014; Irriberi *et al.*, 1987), had a deeper phylum-level diversity (Mohit *et al.*, 2014), larger in size (Acinas *et al.*, 1999), and more locally concentrated (Fernández-Gómez *et al.*, 2013) compares to free-living state bacterium.

Based on sampling strategy, the easiest way to obtain attached bacteria is through the fresh marine sediment. Therefore, sampling protocol was established to extract attached marine bacteria directly from sediment that has been stored in a 4°C chiller by using the DNA extraction kit. This procedure is being explained in details in the section 3.4

3.2.2 Selection of 16S rDNA Hypervariable Region (V)

This research is being designed to compare partial sequence regions with an approximate length of 400bp, delineated for a common domain-specific bacteria primer. The outcomes of PCR analysis are then compared virtually with the available 16S rRNA gene sequences report in the RDP database. In general, RDP report displays a comprehensive bacteria culture taxonomy. The bacterial sequence comparisons were mainly conducted to observe its OTU richness, parametric and non-parametric OTU richness, OTU clustering accuracy, and phylum community structure. Furthermore, the RDP report will identify proximity of 16S rDNA gene partial region(s) and its distance cut-off value for a clearer marine bacterial community report in the purpose area.

Therefore, in this study, the V3-V4 regions are being designated as targeted V regions, because is more divergent and offer a richer phylogenetic resolution than other V regions. A richer phylogenetic resolution is important for microbiomes analysis of specialized habitats such as - an intestinal tract of animal and humans, the Lumens, anaerobic digesters, and biological wastewater treatment reactors - where great diversity exists at low tax (Kim *et al.*, 2011).

The RDP and other databases, mostly deposited the V1 to V4 partial sequences from 16S rDNA region than the downstream regions. Thus, it has more sequences database to compare and generate a reliable phylogenetic analysis. The conclusions that derive from this study were drawn based on a comparison of short partial 16S rDNA sequences that is being recovered from one, or few habitats. As such, these studies do not apply to broad environments (Kim *et al.*, 2011).

3.2.3 Selection of Pyrosequencing Analysis

In 2006, Roche's 454-pyrosequencing became the first high-throughput sequencing technology that successfully applied for large-scale biodiversity analysis and, becomes the key to uncovering 'rare biosphere'. 16S rDNA/RNA analysis by 454-pyrosequencing technology (Roche) requires the V1-V3 regional target when using the FLX Titanium system. Meanwhile, the V1-V4 region should be targeted when using newest 454 FLX model (Kim *et al.,* 2011). Continuous development of the technology offers reading a DNA sequence length up to 1000bp, improvement in throughput and resolution of 16S rDNA sequencing. Since then, additional high-throughput sequencing has become commercially available (Klindworth *et al.*, 2012).

The Illumina™ sequencer was introduced later in 2006 offers a cheaper per base cost and has comparatively high sequencing depth despite having short read lengths (Liu *et al.,* 2012) which become a popular choice to conduct biodiversity studies, especially in Malaysia. Based on a comparison of the mechanism of four different sequences described in Table 3.1, Illumina™ (e.g: Hiseq2000) has the most flexible sequencing engines that provides biggest output and lower reagent cost (Liu *et al.*, 2012).

This research is conducted to identify overall bacteria diversity in a local environment, where identification of species is not the uppermost priority. As mentioned in 3.1.1, several V regions (e.g: V1-V3 or the V1-V4) that are short in DNA base pairs (bp) are more divergent. Thus, it can provide more phylogenetic resolution that other V region. Moreover, identification of species requires years of continuous investigation as it involves a lengthier DNA base pair and exorbitant operation cost in just to achieve at least 99.99 % sequences accuracy (e.g. Sanger 3730xl sequencer).

In conclusion, this research only requires a short but divergent V region and it offers cheaper cost operations. Therefore, Illumina™ was chosen for this study as it meets these research requirements.

Table 3.1: Advantages and mechanism of sequencers (adapted from Liu *et al.*, 2012)

Sequencer	454 GS FLX	HiSeq2000	SOLiDv4	Sanger 3730xl
Sequencing mechanism	Pyrosequencing	Sequencing by synthesis	Ligation and two-base coding	Dideoxy chain termination
Read length	700bp	50SE, 50PE. 101PE	50 + 35bp or 50 + 50bp	400~900bp
Accuracy	99.9%*	98%, (100PE)	99.94% *	99.999%
Output data / run	1M	3G	1200~1400M	-
Time / run	0.7 Gb	600 Gb	120 Gb	1.9~84Kb
Advantage	Read length, fast	High throughput	Accuracy	High quality, long read length
Disadvantage	Error rate with polybase more than 6, high cost, low throughput	Short read assembly	Short read assembly	High-cost low throughput

*Raw data

(1) All the data is taken from daily average performance runs in BGI. The average daily sequence data output is about 8Tb in BGI when about 80% sequencers (mainly HiSeq2000) are running
(2) The reagent cost of 454 GS FLX Titanium is calculated based on the sequencing of 400 bp; the reagent cost of HiSeq 2000 is calculated based on the sequencing of 200 bp; the reagent cost of SOLiDv4 is calculated based on the sequencing of 85 bp.
(3) HiSeq 2000 is more flexible in sequencing types like 50SE, 50PE, or 101PE.
(4) SOLiD has high accuracy especially when coverage is more than 30x, so it is widely used in detecting variations in resequencing, targeted resequencing, and transcriptome sequencing. Lanes can be independently run to reduce cost.

3.3 Sampling collection

In this study, three fresh sea sediments from a shallow sedimentary layer in off-Terengganu (TSD) are collected, in order to obtain attached state bacterium. Based on information depicted in Table 3.2, the first sampling station (1) signified as the shallowest coastline in the off-Terengganu and was positioned approximately 4.01km from Pulau Duyong's piers. The second sampling station (2) that signified a shallower benthic coastline are positioned in southeast bearing; approximately 6.27km from the initial points.

It is assumed that, less sedimentation flux occurs in this area; as it is positioned far from the breakwater lees. The third station (3) is positioned approximately 8.27km from the initial points in a northeast direction. It is expected that no visible turbidity and undesirable nutrient flux occur at this sampling point.

Table 3.2: Location information for sampling activity

Sampling Sites	Longitude (E)	Latitude (N)	Time of sampling (hours)	Depth (m)	Approximate distance from initial points [+](km)
1	103°09.954E	5°20.413N	0945	± 15	4.01
2	103°09.309E	5°20.354N	1125	± 21	6.27
3	103°09.342E	5°20.603N	1350	± 55	8.27

+ The initial points are located in the Pulau Duyong Harbour, Kuala Terengganu. Approximately 4.01 kilometer from the first sampling point.

By using a Smith McIntyre grab sampler ($0.1m^3$ of jaw grab size) depicted in Figure 3.3, sampling activity was conducted on 30th of November 2014 based on Holme and McIntyre (1984) methodology. All samples that are being collected are carefully handled and kept in a double-layered polyethylene bag before it was stored in a -25°C freezer until further analysis.

No specific permits required for the described sampling because it does not involve endangered species and does not occur within a designated marine protected the area and private reserved parking (Marziah *et al.*, 2016).

Additional Notes

This research only managed to obtain three samples due to financial constraint and bad weather that has occurred during sampling activity. A future re-sampling in Off-Terengganu will be conducted in 2018, where the sampling point will be increased; from a three-point stations to a ten-point stations.

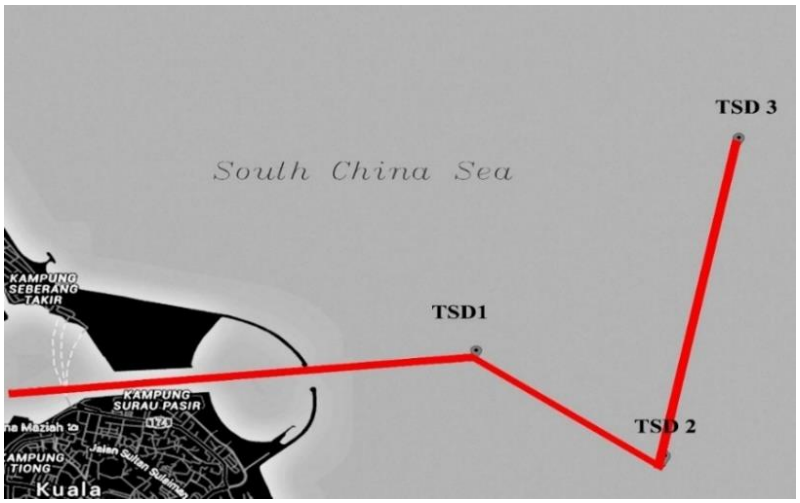


Figure 3.2 Illustration of sampling point in off-Terengganu coastline[+] (5°30N)

+Two northeastern sampling points (TSD1 and TSD3) and one southeast sampling point (2) (Image was adapted from Marziah 2015a).

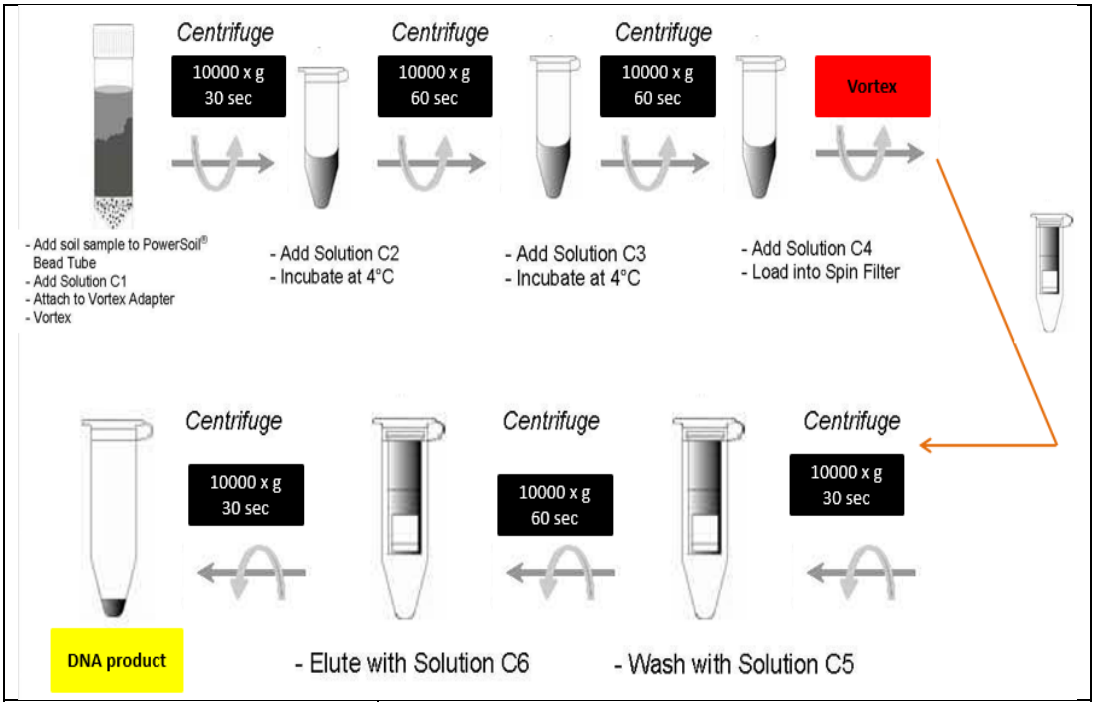
Figure 3.3 Example of Smith-MycIntyre Grab

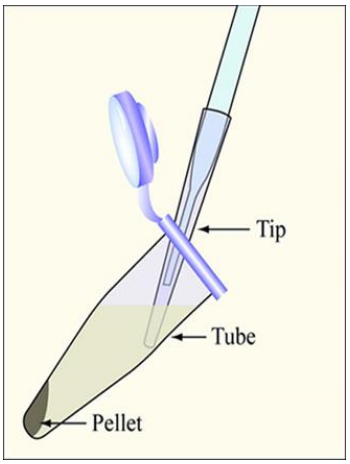
(Image Courtesy of Biota Korea)

3.4 Isolation and Bacteria Characterization

Genomic DNA from one gram of sediment was extracted by using the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's protocols depicted in Table 3.3 below:

Table 3.3: DNA Extraction Protocol



Protocol	Description
Sample (Add to Power Bead Tubes)	Seabed Sediment - 0.25g
Homogenize	1. Gently vortex to mix 2. Add 60µl of Solution C1 3. Vortex horizontally with maximum speed for 10 minutes 4. Centrifuge tubes at 10,000xg for 30 seconds at room temp. (RT) 5. Transfer the supernatant to a clean 2 ml Collection Tube
Extraction 	1. Add 250µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes 2. Centrifuge the tubes at RT 3. Avoid the pallet, transfer 600µl of supernatant to a clean 2 ml Collection 4. Add 200µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes 5. Centrifuge the tube for 1 min. at 10000 x g 6. Transfer 750µl supernatant into a clean 2ml Collection Tube 7. Add 1200ul Solution C4 to the supernatant and vortex for 5 seconds 8. Load 675µl onto a Spin Filter and Centrifuge at 10000 x g for 1 min. at RT. 9. Discard the flow through and add 675µl of supernatant to the Spin filter and centrifuge at 10000 x g for 1 min. at RT

	10. Load the remaining supernatant onto the spin Filter and centrifuge at 10000 x g for 1 min at RT 11. Add 500ul of Solution C5 and centrifuge for 1 min at 10000 x g , RT 12. Discard the flow through 13. Centrifuge again at RT for 1 min at 10000 x g
DNA Elution and Storage 	1. Carefully place spin filter in a clean 2ml Collection Tube 2. Add 100µl of Solution C6 to the center of the white membrane. 3. Centrifuge at room temperature for 30 seconds at 10000 x g 4. Discard the Spin filter. DNA (±90µl) in the tube will be stored in (-20°C to -80°C)

There are two methods that are used to identify a fragment of extracting bacteria DNA: -

First, 3µm DNA elution that is extracted and purified from sea sediment sample are mixed with 3µm blue dye DNA indicator (molecular grade). Subsequently, 6µm of this mixture are then carefully pipetted into 0.8 percent agarose gel block that is being immersed in TAE buffer solution in an electrophoresis tank (refer to Figure 3.4). Electrophoresis process was then conducted for 15 minutes before the agarose gel is viewed under UV light to identify DNA fragment. The result of DNA fragment is shown in Figure 3.5.

Second, the PCR analysis was conducted per standard protocol. The components that are used for PCR reaction are listed in Table 3.4. A primer pair that is used for this analysis is the longest universal primer pair designated: - **8F** (AGAGTTTGATCCTGGCTCAG) and **1492R** (GGTTACCTTGTTACGACTT).

Next, the PCR reaction mixture is then undergone PCR analysis, where four segments of thermal cycling protocols are designated according to Table 3.5. In the DNA replication section (segment 2), we have increased the cycle loop into 50 cycles

from a standard 25 cycles (normal cycle) to provide ample time for DNA fragments to anneal effectively.

Table 3.4: Components for PCR Reaction

Components	**Volume (µl)**	**Final Concentration**
10X Reaction Buffer	2.5	1x
10mM dNTPs mix	0.5	0.2 um
Forward primer (10uM)	1	0.4 um
Reverse primer (10uM)	1	0.4 um
DNA polymerase (2.5 /µl)	0.5	1.25 U
Double distilled water	14.5	n/a
Genomic DNA	5	n/a
	Total: 25µl	

Table 3.5: Thermal Cycling Protocol

Segment	**No. of Cycles**	**Temperature**	**Duration**
1	1	94°C	5 min
2	~~25~~ **50**	94°C (Denaturizing)	30 sec
		55.5°C (Annealing)	45 sec
		72°C (Extension)	30 sec
3	1	72°C	5 min
4	-	4°C	infinity

The NGS analysis was conducted in Sangon Biotech Co., Ltd., that is based in Shanghai, China. Upon arrival, the DNA substrate was carefully quantified with Qubit® 2.0 DNA Kit (Invitrogen by Thermo-Scientific Inc., Waltham, MA, USA) to ensure sufficient DNA products obtained for Polymerase Chain Reaction (PCR) amplification. The primer that is used for amplifies the DNA product for 16SrDNA V3-V4 region analysis are set as 341F (5'CCTACGGGNGGCWGCAG3') and 805R (5'GACTACHVGGGTATCTAATCC 3').

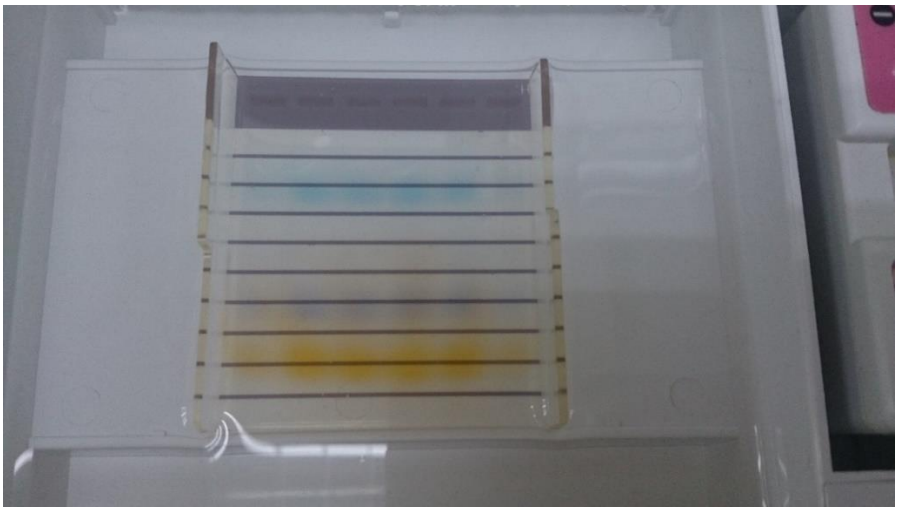


Figure 3.4 Results from Dye separation in the Electrophoresis procedure.

Dye separation indicates that Electrophoresis procedure was conducted properly. Turquoise dye indicates that DNA products are probably presence in the mixtures.

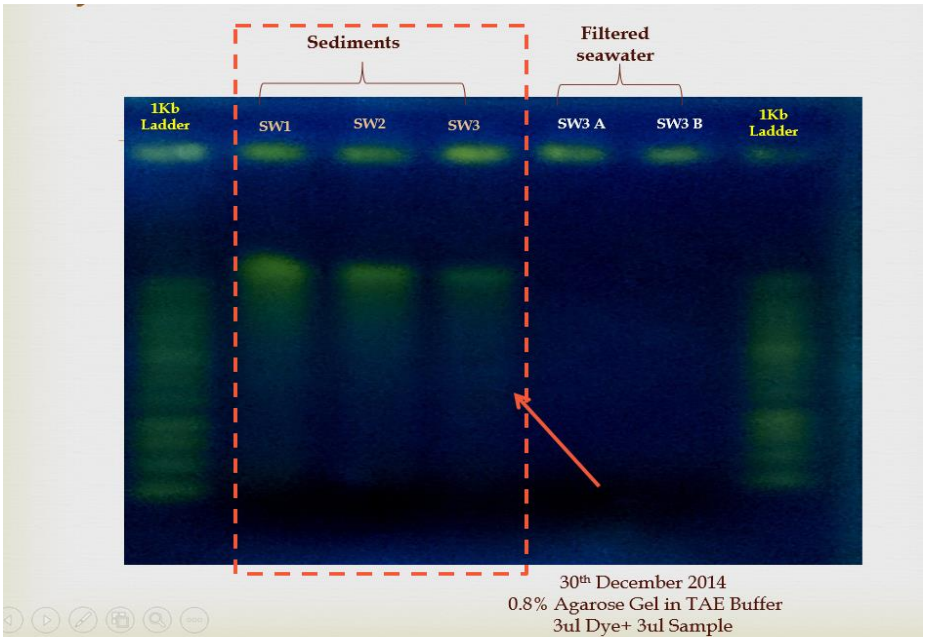


Figure 3.5 Results from PCR screening for TSD1 (SW1), TSD2 (SW2) and TSD3 (SW3).

Results indicate bacteria DNA fragment are adequately presence. Therefore, it is suitable to undergo NGS analysis. Sample SW3A and SW3B indicates no DNA fragment detected

The amplified product integrity was tested and recovered with the agarose gel electrophoresis and Sangon agarose recovery kit (Sangon Biotech Co., Ltd., Shanghai, China). Subsequently, DNA recovery products are then quantified again before it

mixed into a concentration of 1:1 ratio by using Qubit® 2.0 Fluorometer (Invitrogen by Thermo-Scientific Inc. Waltham, MA, USA). Upon obtaining desirable DNA concentration ratio, the DNA substrate administered into the Illumina® Miseq platform (Illumina Inc., San Diego, CA) by Sangon Biotech Co., Ltd. (Shanghai, China) for pyrosequencing analysis.

Subsequently, results that contain thousands of whole genome sequence data was then deposited in the NCBIs - Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) with temporary submission ID (SUB1112034). (Marziah *et al.*, 2016)

3.5 DNA Sequence Analysis

A total of 37,363 sequences that span 16S rDNA V3-V4 hypervariable regions were identified and filtered using the Illumina Miseq™ platform (Illumina Inc., San Diego, CA). Random sequences, ambiguous residues, and sequence lengths of than 150 bp were eliminated. Quality control (QC) for the raw sequences was performed with PRINSEQ-lite 0.19.5 to truncate the low-quality data and improve the merge ratio for subsequent sequences. By using Flash v1.2.7, the raw sequence fragment was merged in a dual-terminal to form a single primer.

Subsequently, short, low-complexity and low-quality primer fragments were eliminated by PRINSEQ-lite 0.19.5 software. Correction of sequencing errors was performed with pre-cluster software and was integrated with the Mothur software. Subsequently, chimeras and extraterritorial sequences of the target area were removed by the Uchime software by using SILVA data as the template. By the time the QC ended, primer length was successfully aligned between 400-500bp, with an average of 450bp (Marziah *et al.*, 2016). All V3 and V4 optimized sequence reads were determined by RDP classifier 16S (Wang *et al.*, 2007) and Silva 16S (Quast *et al.*, 2013).

3.5.1 Diversity and Statistical Analysis

The sequence parameter that is customized for similarity and Operational Taxonomic Unit (OTU) was set to 97% coverage of genus probability. For the first step, OTU clustering was performed by using UCLUST v.1.1.579 to select the longest reads from the clean sequence as seed sequences (Edgar, 2010). In the second step, - a sequence with similarity to the seed sequence within the threshold range - was then selected. Finally, all the sequences obtained from the first and second steps were classified into one OTU category. All three steps of the above process were repeated until all the sequences were successfully classified (Marziah *et al.*, 2016).

The taxonomic unit was classified with the RDP classifier based on Bergey's taxonomy by using a Bayesian assignment calculation to calculate the probability of each sequence being assigned to the rank on the genus level. One representative sequence with the highest OTU abundance was automatically distinguished by the RDP classifier to categorize the species, with the default value of taxonomy threshold being 0.8/0.5. A cluster of multiple sequences based on the distance between sequences, OTU classifications, and the similarity of the sequence threshold value was determined by the Mothur™ software.

Subsequently, all sequence clusters were calculated based on the α-diversity index analysis (based on Richness index, Shannon index, ACE index, Chao1 index). The rarefaction curve value and graph were generated based on 97% of the sequence similarity threshold of every species, genus, and family level analyzed. β-diversity index analysis was excluded from the diversity study due to data deficiency (which requires at least three samples to generate a satisfactory β-diversity index) (Marziah *et al.*, 2016).

All the effective genus identification was then calculated with α-diversity index parameter (based on Richness index, Shannon index, ACE index and Chao1 index). Subsequently, all bacteria taxonomy that has been identified, was then phylogenetically illustrated with the RDP classifier 16S (Wang *et al.*, 2007) and Silva 16S (Quast *et al.*, 2013) and Microsoft® Excel 2013.

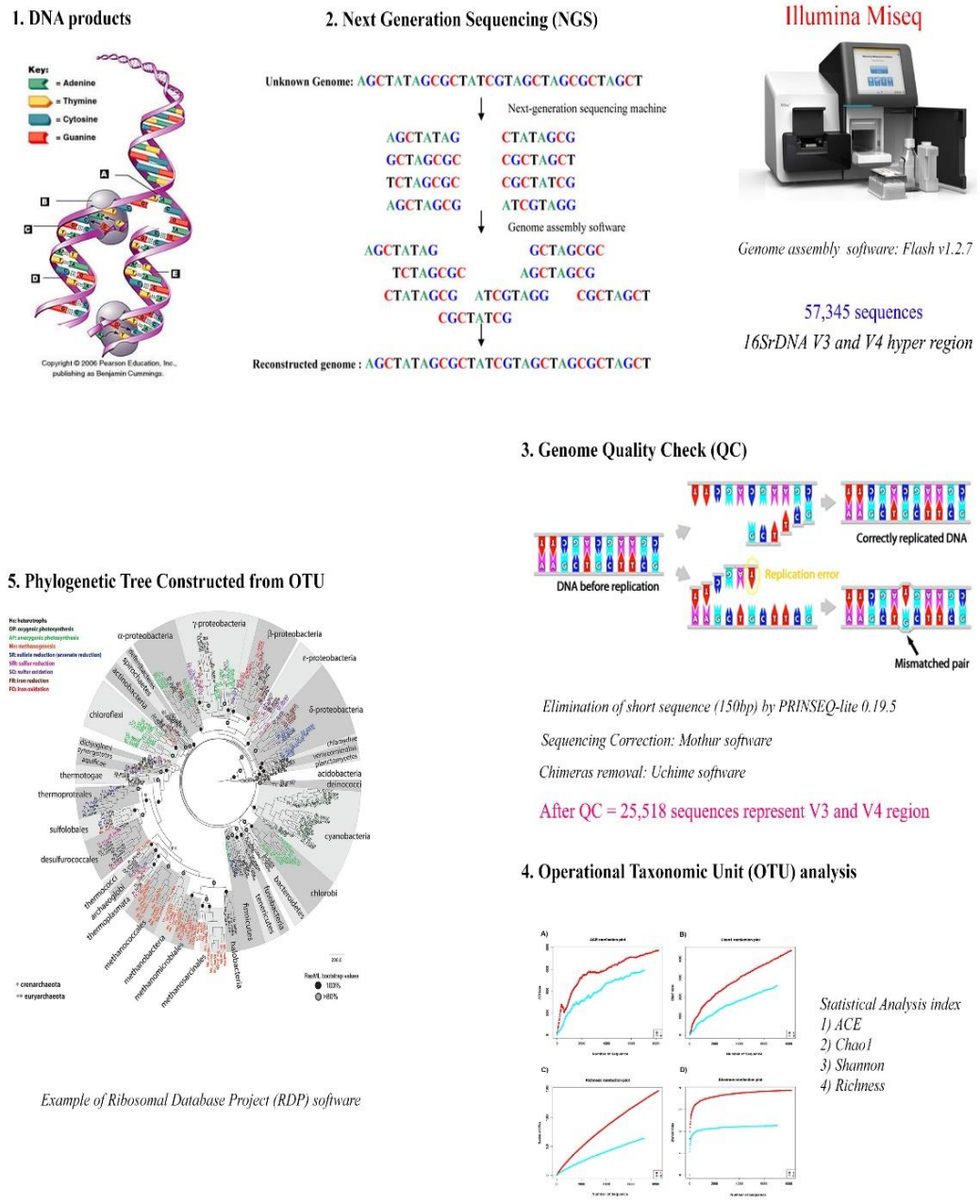


Figure 3.6 Diagram of overall progress in microbial pyrosequencing analysis via Next Generation Sequence (NGS) method

3.6 Physical-Chemical Analysis

For the identification of local physical-geochemical concentrations, an understanding of ambient background and baseline concentrations of metals in sediments is extremely important. The sediment geochemical baseline values can be used to assess the quality of dredged materials, remedial rehabilitation of contaminated sites and ecological risk assessment (Atgin *et al.*, 2000). The physical-geochemical signature of sediments is useful as the indicator to signify the local environmental state. For instance, physical-geochemical findings in the coastline water may demonstrate an intriguing chemical pollutant (e.g. where it might have influenced by a nearby river basin) that has been contaminated anthropogenically; in contrast to a pristine seawater such as in remote islands.

In this study, the physical-geochemical analysis was designed based on bacterial community findings in off-Terengganu sampling station. Specifically, physical-geochemical parameters for this study are chosen based on local nutrient preferences of the dominant bacterial community (e.g. Sulphur-degrading bacteria).

3.6.1 Water Quality Analysis

The Hydrolab Sonde DS5X Multiparameter was used to evaluate in-situ water quality, with seven parameters analysed: - Temperature, pH, Specific Conductivity, Salinity, Total Dissolve Solid (TSD), Turbidity, and Dissolved Oxygen (DO). The multi-parameter probe was cleaned and calibrated prior to each sampling session. Eleven to 12 readings for each parameter were obtained in a single point where every output was directly linked (by GPS) and recorded into the Aqualab Hydras 3 LT Software for Microsoft® Windows 7. Statistical analysis was performed with SPSS 16.0 for Microsoft® Windows 7. The results were interpreted based on Pearson correlation with $P \leq 0.05$ and $P \leq 0.01$ being considered as significant (Marziah *et al.*, 2016).

3.6.2 CHNS Elemental Analysis

Carbon, hydrogen, nitrogen, and sulfur are the common natural element that is essential for life survival and its ratio are mainly reflected from the geographical distinctive in the selected areas. The reason for selecting CHNS elemental analysis is mainly to investigate any possible bacteria affinity towards certain CHNS element. For example, there are possibility that sulfur-degrading bacteria is expected to thrive in the hydrothermal vent (Wright *et al.*, 2013; Dahle *et al.*, 2015; Inagaki *et al.*, 2004) and volcanic region (Wang *et al.*, 2015b) due to the abundance of sulfide or sulphate mineral generated from the Earth's magma chamber (Inagaki *et al.*, 2004).

Rapid identification of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) in the sediment sample was performed by using the Vario MACRO ™ cube CHNS (Elementary, Deutschland). The sediment samples were air-dried in a 50°C oven and then ground, sieved (<2mm), and homogenized according to the ISO 2004 protocol. The sulphur determination was conducted according to the ISO 2005 protocol (Marziah *et al.*, 2016).

3.6.3 Oil and Grease (O&G) Analysis

Based on several claims, anthropogenic activities solely pollute the global sea coastline, where it is frequently associated with the hydrocarbon spill. The oil and grease (O&G) analysis was the first method introduced to trace any hydrocarbon compound the in surveyed areas before further experiment will be conducted. Oil and grease determination was conducted by using a partition-gravimetric method. Specifically, the Hexane Extractable Method - USEPA 1664 (EPA 1999) was used. The oil and grease in the sediment were extracted from water and then attached to n-Hexane solvent. The solvent was allowed to evaporate slightly before transferring it to a pre-weighed culture tube. The solvent was further evaporated completely until dry. The culture tubes were then weighed again (EPA 1999, Bucci *et al.*, 2015; Marziah *et al.*, 2016).

3.6.4 Total Petroleum Hydrocarbon (TPH) Analysis

Total Petroleum Hydrocarbon (TPH) analysis was introduced to identify hydrocarbon variations in the surveyed area, in order to understand its relations to anthropogenic-based pollution. TPH analysis was implemented if the surveyed area was verified to have O&G traces. TPH was measured based on the USEPA 8015B test method (EPA, 2000). Ten grams of chilled fresh sediment were transferred into vials with a solid cap and a Teflon septum. Twenty ml of n-Pentane solution was added to the same vial and mixed homogeneously by centrifugation for 15 minutes. The mixture was allowed to settle for one hour at room temperature and then considered ready for Gas Chromatography with Flame Ionization Detector (GC-FID) analysis. Each sample mixture was passed through the Agilent J&W Capillary (DB-5 30m x 0.25mm x 0.25µm) into the Agilent 7890A GC-FID with a carrier gas (Helium) – constant flow rate of 40cm/sec was recorded. Considering performed internal Quality Control ±5% as the acceptance criterion (Marziah *et al.*, 2016).

3.6.5 TOC Analysis

TOC in the sediment is an indicator of the organic pollution and biological productivity in selected areas. It plays an important role in nutrient release and accumulation in the water. Rich organic carbon indicates active biological productivity in selected areas. However, excessive organic carbon will eventually produce an anoxic condition in the water column and sediments which in turn affect the productivity in the selected area (Rosnan, 1990).

For wet oxidation phase, the revised Walkley-Black titration was applied in accordance with clause 3 of BS 1377: Part 3 (BSI, 1990). About 2000 g of sediment that is been previously dried in 70°C oven is weighed and treated with 10.00 cm^3 of 1.000 N potassium dichromate solution followed by the rapid addition of 20 cm^3 of concentrated sulphuric acid containing 0.5g of silver sulphate, to precipitate chloride ions. Samples were allowed to cool uniformly at room temperature for 30 minutes

(inside 20°C fume hood). The mixture was then diluted with 200 cm^3 of double-distilled water and 10 cm3 of orthophosphoric was added subsequently (BSI, 1990).

Finally, the excess potassium dichromate solution (after adding a further 1.00 cm3) was back-titrated with 0.5 n iron (II) sulphate solution by using barium diphenylamine sulphonate as an indicator. Standardization of the iron (II) sulphate solution was performed at the beginning of each analysis using 20 cm3 of double-distilled water instead of the sediment sample (BSI, 1990). Organic carbon in sediment was determined as:

$$\text{Organic carbon (\%)} = \frac{11\left(1-\frac{y}{x}\right)0.39}{m}$$

Where *x* is the volume (cm^3) of iron (II) sulphate used in the standardization, *y* is the volume (cm^3) of iron (II) sulphate used in the titration, m is the mass (g) of sediment used in the sample determination. The results were quantified to 0.01%.

To obtain a rapid determination, Sediment drying step are considered optional. The titration analysis was also performed using fresh and undried sediment samples. The results were corrected to match the water content in the samples, and determined with other portions (BSI, 1990).

3.7 Supplementary Data – Sediment Quality Study

Data sources for enlisted sediment quality study in subpoints below are taken directly from the Environmental Impact Assessment (EIA) report that is displayed in the Malaysia Department of Environment (DOE) library in Putrajaya. Every EIA

report chosen are referred based on appropriate locations such as oil and gas field, pristine seawater, and river estuary.

In this study, TOC, Oil & Grease (HEM), TPH, and CHNS elemental reports are compared with supplementary data enlisted. In addition, Redox Potential, In-situ water quality, Metals, and Polynuclear Aromatic Hydrocarbon (PAH) information are studied to support research findings.

3.7.1 EIA - Redox Potential

The redox potential study is conducted to measure the availability of oxygen in the interstitial water in the sediment. The lower redox value indicates a high utilization of oxygen. A negative reading of redox indicates that all freely available oxygen has been removed. Also, oxygen bound to inorganic compounds such as sulphides; where it is commonly being degraded by a marine bacterium. Oxygen reaches the sub-surface layers of sediment in the pore water via connections with the overlying water column. Water does not percolate through fine and/or compacted sediment efficiently. Therefore, oxygen supply in that sediment is limited. Organic materials in the sediment will also create an oxygen demand because of anaerobic decomposition. Currently. There is no recommended limit for Redox Potential in the United State National Oceanic and Atmospheric Administration Screening Quick Reference Tables (US NOAA SQuiRTs).

3.7.2 EIA - Total Organic Carbon

TOC method that is depicted in the EIA reports is different from the TOC method conducted in this study. Samples were analysed using MS 678: Part 1 to 4: 1980 methods where approximately 0.1g of dried and pulverized sample was digested on a hot plate (low heat setting) with dilute nitric acid till dryness. The sample was then combusted at 1350°C. Evolved carbon was then determined using an Infrared detector cell. The results were quantified to 0.01%

3.7.3 EIA - Oil and Grease

Oil and grease were determined using the APHA 5520E method where samples were dried and subjected with n-Hexane in a Soxhlet apparatus. The residue remaining after evaporation was weighed to determine its oil and grease content.

CHAPTER 4

RESULTS

4.1 Background

In this chapter, the results of 16SrDNA based Next Generation Sequences (NGS) analysis that includes RDP illustrations of bacteria diversity and phylogenetic tree analysis will be discussed. Subsequently, it follows with the physical-geochemical report for all sampling areas. Discussions that relate to research findings will be further deliberate in the Chapter Five: Discussion.

4.2 Biodiversity Report

A total of 57,345 raw sequences was successfully obtained from the Illumina™ Miseq genome assembling analysis. Subsequently, about 25,518 of cleaned effective sequences based on 16S rDNA V3-V4 were successfully obtained and grouped into 3301 unique OTUs (Operational Taxonomic Unit) where one OTU denotes a sequence with an identical value equal to or higher than 97% (Zhu *et al.*, 2013 and, Wang *et al.*, 2015). Based on OTU classification depicted in Table 4.1, TSD1 demonstrate a higher marine bacteria species richness and evenness comparable to TSD2 and TSD3.

Table 4.1: The list of α-diversity index cumulative results. (For TSD1, TSD2, and TSD3)

Sample ID	Seq. Num	OTU Num	Shannon index	ACE Index	Chao1 Index	Coverage Index
TSD1	8210	1496	3.8097	8407.9424	4711.624	0.865652
TSD2	10250	1145	2.8476	7421.287	3566.971	0.91961
TSD3	7058	660	2.0567	5949.7505	2538.676	0.92675

4.3 Phylogenetic Identification

Illustration of bacterial phylogenetic profiles is described in Figure 4.1. Results indicated that Proteobacteria tops the overall phylum abundance in all surveyed areas with 85.25% (TSD1), 88.38% (TSD2) and, 94.3% (TSD3). These results also show that unclassified phylum is the second-most-abundant phylum detected with 5% (TSD1), 1.46% (TSD2) and 2018% (TSD3) respectively, accompanied by the phylum Bacteroides with 2% (TSD1), 5.63% (TSD2) and, 0.72% (TSD3) respectively. This result also shows a high phylum diversity reported in TSD1 compares to TSD2 and TSD3.

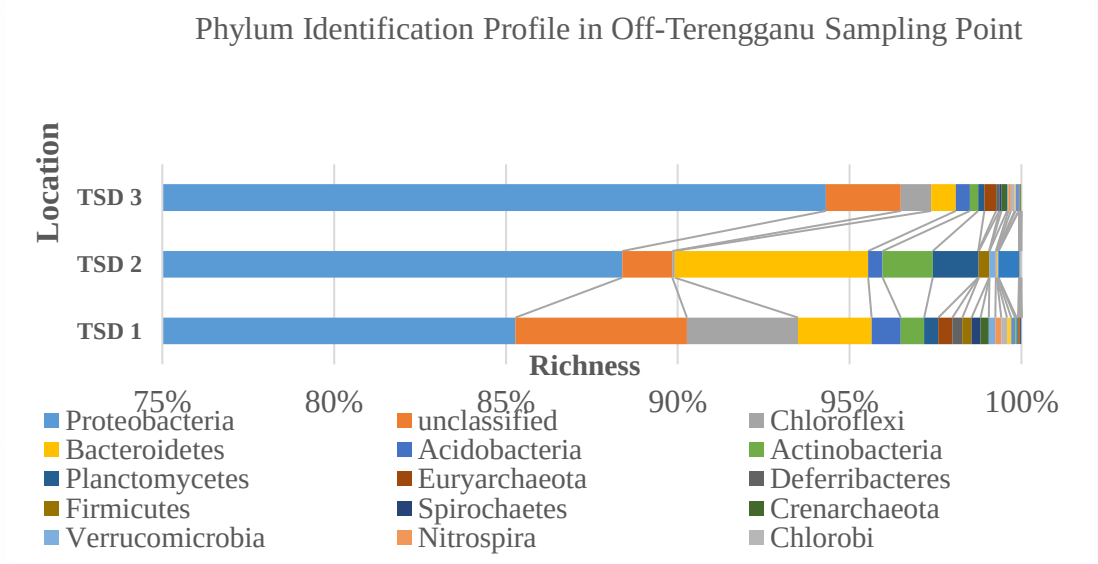


Figure 4.1 Illustration of Metagenomic Profile indicates Proteobacteria dominations in all sampling stations[+].

+ With overall phylum abundant by 85% in 1, 88.38% in 2 and 94.3%

As mentioned in section 2.4.2, Proteobacteria phylum consist of six classes where each of the genera has it is own characteristics. For this survey, comparison of Proteobacteria phylum genera is depicted in Figure 4.2 where it shows at Epsilonproteobacteria predominates Proteobacteria phylum community in TSD1 (60%) and, TSD3 (88%). However, Epsilonproteobacteria is barely discernible in TSD2 (0.04%). Instead, the Gammaproteobacteria class predominates the bacterial community in TSD2 with 78.67% of effective sequences identified. A Gammaproteobacteria community in TSD1 and TSD3 were slightly abundant with 15% and 5% of effective sequences identified respectively.

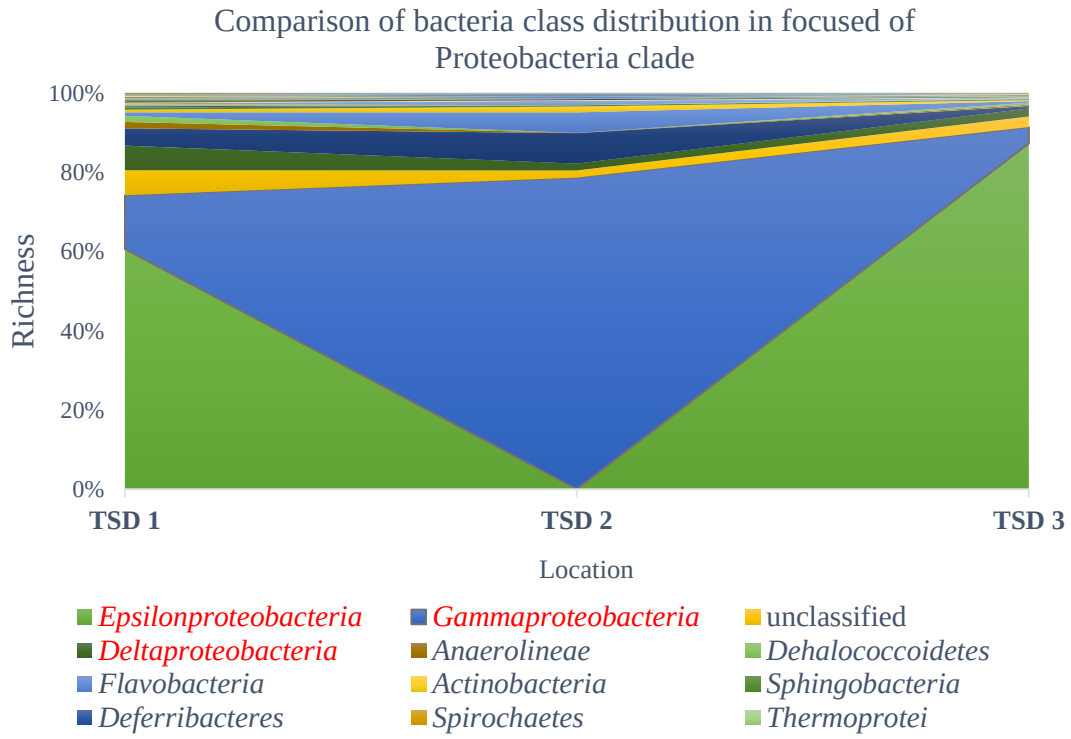


Figure 4.2 Comparison of genera distribution among Proteobacteria phylum (red font) in all sampling locations

Other Proteobacteria group variant was also identified in the study. Deltaproteobacteria community is scantily identified at all sampling points with 6.24%, 1.73%, and 2.08%, respectively, and Alphaproteobacteria, being the least bacteria community identified in all locations with 4.4%, 7.71%, and 0.58% respectively. These findings also suggest that the unclassified phylum is the second

most abundant phylum identified by 4.99%, 1.46%, and 2.18%, respectively, followed by Chloroflexi phylum with 3.23%, 0.07 and 0.88% respectively.

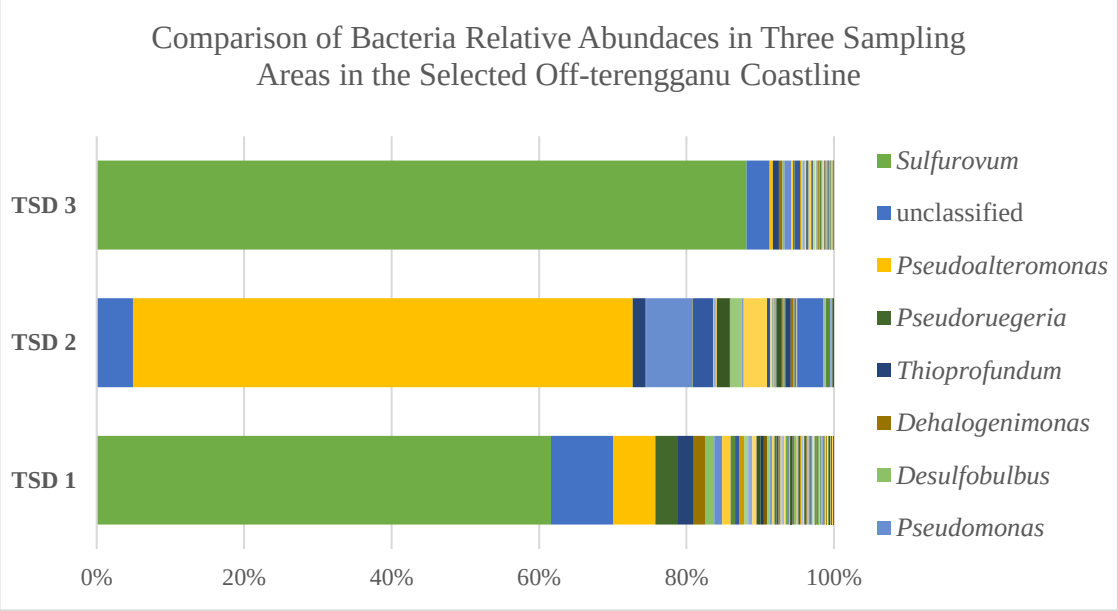


Figure 4.3 Comparison of marine bacteria abundances in three sampling areas off-Terengganu Coastline.

The 100% stacked bar was generated from a 100-selected genus from a total of 335 genera identified, where nine of genus depicted in the figure represent the dominant species found in the respective locations (Image was adapted from Marziah 2015b).

In a genus identification perspective, Figure 4.3 indicates that *Sulfurovum* sp. was the only genus that covers the entire Epsilonproteobacteria class in both surveyed areas (60% in TSD1 and 88% in TSD2). This genus was characterized by its egg-like coccoidal shape and capable of oxidizing sulphur for food and survival (Inagaki *et al.*, 2004 and, Takai *et al.*, 2004).

Pseudoalteromonas sp. had dominated gammaproteobacteria class identified in the TSD 2 with 62.02%. Meanwhile, TSD1 contains several Gammaproteobacteria class bacteria, such as *Thioprofundum*, *Desulfobulbus*, *Desulfovirga*, *Desulfobacterium*, *Desulfosalsimonas*, *Sulfurimonas*, *Sulfuricurvum*, and *Thermodesulfovibrio*, which accounts 13.59% of the effects sequence identified.

4.4 Water Quality Analysis

Water quality analysis indicates that the temperature, pH, Sp. Conductivity, Salinity, TDS and LDO values in all surveyed areas are significant and does not demonstrate an abnormal value except for Turbidity. Table 4.2 shows a moderate nephelometric turbidity unit (NTUs) value in the TSD1 (4.1818 NTUs) and lesser value (1.32 NTUs) were reported in TSD 2. However, TSD3 significantly has minimum turbidity value (0.32 NTUs). It is believed that TSD1 are prone to accumulate high sedimentation, which penetrates the water bodies from a storm water runoff or high bank erosion rates from a concrete surface such as breakwater lees, roads, and bridges (EPA 2005). Minimum NTUs value in TSD3 is predictable since the sediment layer is stable as it was remotely located from the breakwater (Qi and Gao 2015).

Table 4.2: Results of seawater quality analysis

No	*Parameters*	*TSD1*	*TSD2*	*TSD3*
Seawater In-Situ Water Quality Analysis				
1	Temp (°C)	30.55 ± 0.03	30.20 ± 0.07	30.12 ± 0.11
2	pH	7.84 ± 0.02	7.90 ± 0.01	7.93 ± 0.0
4	Turbidity (NTUs)	4.18 ± 0.85	1.32 ± 0.83	0.32 ± 0.02
5	Salinity (ppt)	40.74 ± 0.08	40.56 ± 0.41	40.73 ± 0.08
6	TDS (g/L)	38.86 ± 0.07	38.66 ± 0.35	38.80 ± 0.09
7	DO (mg/L)	6.64 ± 0.06	6.79 ± 0.02	6.77 ± 0.07

Historically, a high turbidity value will reduce the amount of light reaching the seabed, which inhibits submerged aquatic plant growth. Consequently, it affects several aquatic species, which are dependent on them, such as fish and shellfish. High turbidity levels can also affect the ability of fish gills to absorb dissolved oxygen. Higher dissolved oxygen concentrations are expected around coral reefs due to photosynthesis and aeration from eddies and breaking waves.

These DO levels can fluctuate from 4-15 mg/L, though they usually remain around 5-8 mg/L, between day photosynthesis production and night plant respiration cycles (Kemker, 2013). In terms of air saturation, this means that dissolved oxygen

near coral reefs can easily range from 40-200% (Kemker, 2013). Based on Table 4.2, dissolve oxygen in all three sampling was merely an oxygenated water and it is sufficient to support life aquatic photosynthesis and respirations. Therefore, it is assumed that turbidity does not cause a severe aquatic live depletion in TSD1.

4.5 Physical-Geochemical Analysis

Contaminants that derive from urban development, industrial, agricultural activities, atmospheric deposition, and natural geological sources, usually accumulate in the sediments; up to several times of the background concentrations. The sediment also serves as the potential storage to more than 90 percent of the heavy metal loads (Calmano *et al.*, 1993); for both the inorganic and organic contaminants (Sumith *et al.*, 2009; Reczynski *et al.*, 2010). This toxic metal accumulation is hazardous and affect the sustainability of the natural resources such as water, plants, and aquatic animals. Particle-reactive heavy metals that enters the water bodies, may be quickly adsorbed onto suspended matter; eventually, move to the bottom sediment layers.

The tropical region in the east coast of Peninsular Malaysia is undergoing rapid development in the industry sector and urbanization, especially in the coastal areas of the South China Sea. Industrial effluent, municipal discharge, agricultural runoff, and past mining waste materials may result in contamination of the food chain when entering the river system. It is, therefore, important to document the prevailing concentrations, distribution, and geochemistry of the elements to monitor any changes caused by anthropogenic activities in the future.

In Malaysia, there are currently no comprehensive sediment reference values available to establish levels of potentially toxic elements. Hence, this work is significant in understanding the geochemical baselines of the major and trace elements by presenting detailed documentation of the current state of the tropical river, estuary, and lake sediments of the northeast coastal region of Peninsular Malaysia.

The average concentrations of the measured elements were also compared with the environmental guideline and geochemical baseline values established for sediments around the world (Sultan & Shazili, 2010).

4.5.1 Total Organic Carbon (TOC)

Based on Table 4.3, Total Organic Carbon (TOC) sedimentary value in TSD sediments are increase with depth. In correspondences to zero water turbidity value in TSD3, it is assumed that sunlight effectively penetrates the clear water column (Marziah *et al.*, 2016). This supports photosynthesis, thus creating a better marine food-chain environment, and generating high organic matter from the cell remains (Marziah *et al.*, 2016; Bell *et al.*, 2015; Saraswathy *et al.*, 2015) where it demonstrates high TOC value (Bendtsen *et al.*, 2015).

Table 4.3: Results of TOC analysis in the Off-Terengganu

	Locations		
Units	**TSD1**	**TSD2**	**TSD3**
wt%	0.46	0.50	0.52

Table 4.4: Comparison of TOC value in Off-Terengganu with other locations

	Locations					
Units	TSD Off-Terengganu	Paka River, Terengganu	Sarawak Gas Field	Pristine island, Terengganu	EEZ oil rig, Terengganu	Sarawak oil rig
wt%	0.52%♣	0.20% Ψ	3.40% Ψ	<0.1% Ψ	2.20% Ψ	0.50% Ψ

♣ Results that are produced in this study

Ψ Results that are excerpted from several EIA reports courtesy of DOE Malaysia

notes: all data provided are calculated based on wet basis

However, there is no concrete evidence that links *Sulfurovum sp.* abundance with the high TOC in 1 and 3 sedimentary layer. Nevertheless, there is no evidence to link TOC and *Sulfurovum sp.* in the 2 since this genus is not detected in the NGS analysis (Marziah *et al.*, 2016).

Table 4.4 describes the comparison of TOC value with four locations in Malaysian's water. This table shows that TOC value in all locations is considered normal. It is expected that the TOC value is high in oil and gas platform due to hydrocarbon effluent that is being released into the water column.

A scant TOC value in the pristine island indicates that this area only accumulated a low organic pollutant. For instance, only one small pier is established in this area where it serves occasional vessel transportation per month.

4.5.2 CHNS Elemental Analysis

The CHNS analysis was mainly performed to observe the overall elemental composition in the surveyed area. Based on Table 4.4, all four of the main elemental ratios, including sulfur, were scarcely identified. Further analysis is, therefore, necessary to investigate sulfur concentration to demonstrate a convincing association of the *Sulfurovum* sp. with the sulfur content in the surveyed area.

Table 4.5: Elemental results in all sampling points in Off-Terengganu

No	*Parameters*	*TSD1*	*TSD2*	*TSD3*
Sediment Elemental Analysis (CHNS)				
1	Carbon (%)	1.86	2.75	1.25
2	Hydrogen (%)	1.017	1.353	0.035
3	Nitrogen (%)	0.99	0.58	0.58
4	Sulphur (%)	0.916	1.046	0.212

Table 4.6: Comparison of elemental results in Off-Terengganu with five reference data provided by Vario MACRO™

		Locations					
Fractions	units	Off-Terengganu	Waste Ψ	NCS Coal Ψ	Soil Ψ	Biomass Ψ	Heavy oil Ψ
C	mg/kg	18600	555500	783500	13410	559500	8447000
H	mg/kg	10170	74150	45370	-	-	107100
N	mg/kg	9900	8430	13460	1270	35600	3390
S	mg/kg	9160	1320	13770	220	21200	-

Ψ Result are provided by Vario MARCO™ manufacturer

Although the sulphur ratio is to some extent higher from other elements, it is assumed that the surveyed area is not a hydrothermal region as the value is infinitesimal. There are probabilities that hydrocarbon impurities contribute to sulfide fractions in the region of interest. Therefore, Hexane Extractable Method (HEM) analysis is conducted to find the sulfur correlation with oil and grease (O&G) compound in all TSD sediments.

4.5.3 Hexane Extracted Method (HEM) and Total Petroleum Analysis (TPH)

Based on the Hexane Extracted Method (HEM) analysis, the sediment's oil and grease fragments are fairly identified by 0.47% (1), 0.16% (TSD2) and 0.08% (3). Since HEM assessment shows a promising value, it was necessary to thoroughly quantify hydrocarbon compounds by using TPH (total petroleum hydrocarbon) analysis (Bucci *et al.*, 2015). The outcome of TPH analysis confirmed the existence of gasoline (C_4– C_9), diesel (C_{10}-C_{19}), and organic oil (C_{20}–C_{36}) fractions in 1 at 0.05 ppm, 0.10 ppm, and 0.22 ppm respectively and 0.01 ppm, 0.12 ppm, and, 0.21 ppm respectively in TSD2. Conversely, 3 sediment only traced diesel fraction (C10-C19), and organic oil fraction (C20 – C36) at 0.11 ppm and 0.29 ppm respectively. No asphalt/bitumen fraction (C37 – C44) was detected in all three samples.

Table 4.7: Result of Physical-Geochemical analyses

No	Parameters	TSD1	TSD2	TSD3
Sediment Oil and Grease Analysis				
1	Hexane Extractable Method (HEM) (%)	0.47	0.16	0.08
Sediment Total Petroleum Hydrocarbon (TPH) Analysis				
2	$C_6 – C_9$ (ppm)	0.05	0.01	*ND
3	$C_{10} – C_{19}$ (ppm)	0.10	0.12	0.11
4	$C_{20} – C_{36}$ (ppm)	0.22	0.21	0.29
5	$C_{37} – C_{44}$ (ppm)	*ND	*ND	*ND

*ND= Not detected

HEM analysis results are then compared with other HEM values taken from five EIA studies conducted in Malaysians water. Baku River is located about 160 kilometres south from Kuala Terengganu, is a river estuary that is connected to a coastline city named Paka in Dungun district. Paka River is subject to environmental impact assessment study before jetty expansion and tourism based constructions are instigated.

The Perhentian Island is signified as a pristine water environment – where the data that represents the sediment, is nearly free from any anthropogenic impact. The Sarawak gas field represents a natural gas production platform. The Exclusive Economic Zone (EEZ) oil rig and Sarawak oil rig represent an oil drill based platform.

Table 4.8: Comparisons on HEM analysis in Off-Terengganu with other selected locations

	Locations					
Units	Off-Terengganu	Paka River, Terengganu	Sarawak Gas Field	Perhentian Is., Terengganu	EEZ oil rig, Terengganu	Sarawak oil rig
mg/kg	4.7	< 1.0	<10.0	<0.1	<1000	3230

Based on HEM comparison depicted in table 4.7, the Sarawak oil rig station has the highest hydrocarbon traces reported. Followed by EEZ oil rig and the TSD respectively. It is assumed that a moderate HEM value in TSD is due to hydrocarbon accretion in its surroundings. This value probably influenced by numerous of vessel's activity inside the jetty. The location of Sarawak oil rig and EEZ oil rig are remoted from coastline with an approximate distance of 150 kilometres. It is reported that HEM value in both oil rigs sediment is mainly influenced from crude oil smear prior to drilling activity.

Although Paka River has a plenty number of jetties, maritime activities in its surroundings are minimal, most clean water resources is located on its upstream river. Nevertheless, no distinguished breakwater structure that protects the coastline are linked to the Paka River estuary. Therefore, it is assumed that the high HEM value in

Off-Terengganu is influenced by a hefty drainage system, Kuala Terengganu river estuaries, jetties, and several active constructions.

Comparisons of Total Petroleum Hydrocarbon (TPH) analysis depicted in Table 4.8 indicate that all hydrocarbon fractions, that is measured in TSD are considered minimally comparable to oil rigs platform and gas platform. Based on the EIA reported that is released in the Sarawak gas field. There is no visible hydrocarbon fraction reported in selected areas because the gas field does not produce/distilled crude oil. The presences of hydrocarbon compound are assumedly from mineral oil and diesel No precise TPH parameter values are provided for Paka River. Therefore, it is assumed that both Off-Terengganu and Paka River have a minimal hydrocarbon fraction in its surface sediment.

Table 4.9: Comparison of TPH analysis in Off-Terengganu with selected locations

		Locations					
Fractions	Units	Off-Terengganu	Paka River, Terengganu	Sarawak Gas Field	Pristine island, Terengganu	EEZ oil rig, Terengganu	Sarawak oil rig
C_6-C_9	mg/kg	0.05	<1.0	<5.0	ND	<5.0	<100.0
C_{10} - C_{14}	mg/kg	0.1	<1.0	<50.0	ND	<50.0	<100.0
C_{15} - C_{28}	mg/kg	0.22	<1.0	<100.0	ND	<100.0	<100.0
C_{29}-C_{36}	mg/kg	ND	<1.0	<100.0	ND	<100.0	<100.0
C_{37} – C_{44}	mg/kg	ND	ND	<100.00	ND	<100.0	<100.0

ND – Not Detected / No Data

4.6 Results from other Physical-Geochemical Supplementary Data.

Table 4.10: Total Hg, methyl Hg and Hg (II) (ng g-1 dry wt) in marine sediment from Off-Terengganu (Courtesy of Kannan & Falandysz, 1996)

Sample no.	Total Hg.	Methyl Hg.	Hg (II)	%MeHg[a]	%Hg (II)[b]
1	20	0.0053	0.32	0.27	1.6
2	127	0.037	13.2	0.03	10.4
3	40	0.052	2.52	0.13	6.3
4	55	0.01	3.2	0.02	5.8
mean	61 ± 47	0.038±0.02	4.81±5.73	0.11±0.12	6.0±3.6

[a] percentage of methyl Hg in total Hg
[b] percentage of Hg(II) in total Hg

To understand the relationship of sulfur-degrading bacteria (sub-section 4.3) and hydrocarbon pollutants (subsection 4.5.3) in the surveyed area, additional physical-geochemical properties that might theorize this phenomenon are investigated. Initially, identification of mercury is vital since it is used as a pollutant sign in sea coastline (Kannan & Falandysz, 1996).

Mercury is locally found in volcanoes, forest fire, cinnabar (ore) and fossil fuels such as coal and petroleum. Based on Table 4.9, mercury concentration in every sample is minimal with less than 10% of the total Hg reported back in 1996. Secondly, identification of redox potential (Eh) value determines free oxygen condition in the sediment. Redox potential - also known as reduction potential, is a measure of the tendency of a chemical species to acquire electrons and thereby be reduced. Each chemical species has its own intrinsic reduction potential; the more positive the potential, the greater the species' affinity for electrons and tendency to be reduced. Reduction potential is measured in volts (V), or millivolts (mV).

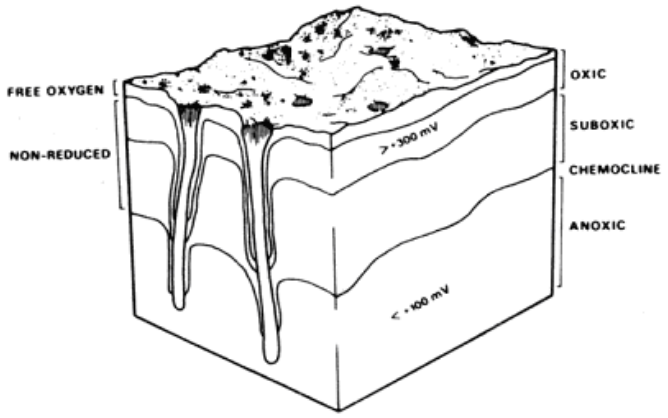


Figure 4.4 Illustration of oxygen availability in the sediment based on Redox Potential value

Table 4.11: Comparison of Redox Potential (Eh) value in the surrounding of Off-Terengganu with other selected locations

	Locations						
units	Off-Terengganu	Paka River, Terengganu	Sarawak Gas Field	Pristine island, Terengganu	EEZ oil rig, Terengganu	Sarawak oil rig	Dungun estuary
mV	-51.00 ♣	ND	357.09 Ψ	370.32 Ψ	194 Ψ	-254 Ψ	285.7 Ψ

♣ Results are excerpted from (Sultan *et al.*, 2011)
Ψ Results are excerpted from several EIA reports courtesy of DOE, Malaysia
ND No data

Redox potential (Eh) in the Off-Terengganu surroundings probably signify sub-oxic condition in the survey points. Based on phylogenetic identification results (sub-section 4.3), *Sulfurovum* sp. that is found abundant in TSD 1 and TSD 2, mainly lives in the anoxic environment, specifically in the deep-sea region or anoxic reactor (Liu *et al.*, 2016). *Pseudoalteromonas* sp. on the other hand, able to grow in both oxic (Zhang *et al.*, 2016), and anoxic (Wu *et al.*, 2016) conditions.

Based on Table 4.10 descriptions, Sarawak oil rig has the lowest Eh value because, in principal, active carbon (hydrocarbon) adsorbs free oxygen on the marine sediment (Xue *et al.*, 2016). Therefore, the area is considered anoxic. It is also indicated in the presences of sulphate-reducing bacteria because any sulphur or hydrocarbon compounds naturally emits hydrogen sulphate (Stark *et al.*, 2016; Groysman, 2017). Other areas depicted in Table 4.10 have a positive Eh value which is an indication of no contamination were reported. Based on CHNS elemental reports (subsection 4.5.2) and Hydrocarbon analysis, it is assumed all three surfaces sediment has both sulphur and hydrocarbon trace - which is perpetual for *Sulfurovum* and *Pseudoalteromonas* to degrade sulphate as its energy sources.

4.7 Potential of Disease Outbreak Towards Human

Although there is no conclusive finding to describe *Sulfurovum* and virulence factor in human, high interest in finding pathogenic niches in Epsilonproteobacteria groups are increased in the past few years. It has recently been demonstrated that *Sulfurovum* was carrying a similar gene with *Helicobacter pylori* - a notable pathogenic bacterium that causing gastroenteritis in human (Gupta, 2006; Nakagawa, 2007). However, both *Sulfurovum* and *Helicobacter* have different niches and regimes in nature (Nakagawa, 2007; Nothaft & Szymanski, 2010).

Based on MOLE-BLAST query shown in Figure 4.5, it is projected that two *Sulfurovum* genera. namely *Sulfurovum lithotrophicum and Sulfurovum aggregans Mochim33* are virtually identical with the dominated *Sulfurovum* genus in Off-Terengganu, by 98 percent and 96 percent identical scores respectively with zero

expectant (E) value score. However, deep sequence analysis by using Sanger 3730xl or SOLiDv4 will correctly determine *Sulfurovum* species discovered in Off-Terengganu because both requires a longer 16S rDNA sequence to achieve at least 99.99 percent accuracy.

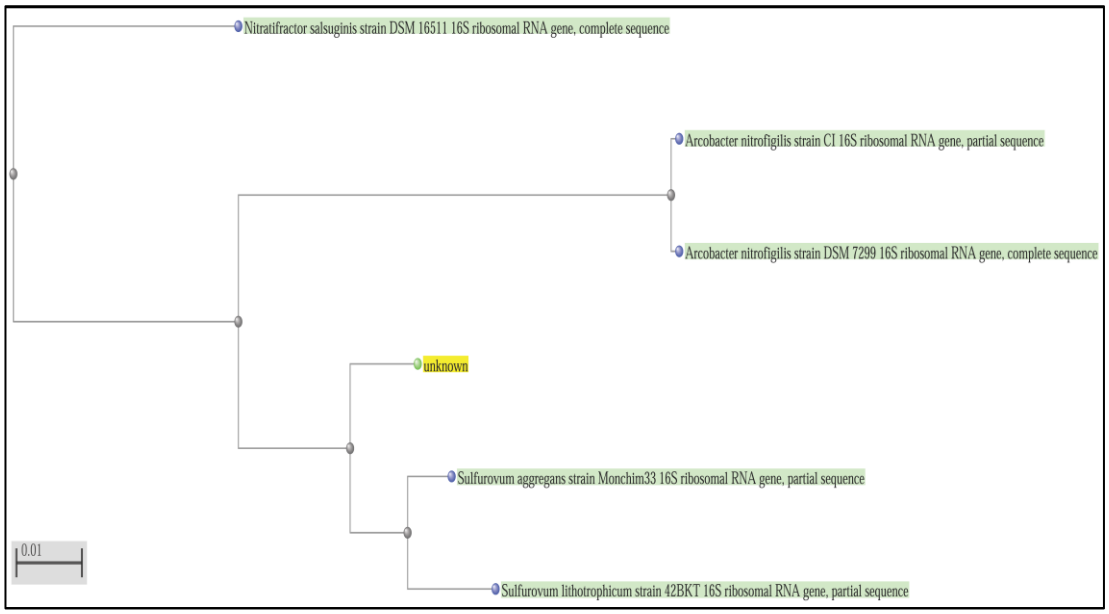


Figure 4.5 Illustration of *Sulfurovum* sp. sequences query based on phylogenetic tree under 0.75 maximum sequence difference+.

+Diagram shows that Sulfurovum aggregans Monchim33 and Sulfurovum lithotrophicum are virtually identtical as Sulfurovum sp. genus that has been extracted from Off-Terengganu surface sediment

In the previous study, Nakagawa *et al.* (2007) have claimed that Epsilonproteobacteria holds *Sulfurovum lithotrophicum– a helibacteraceae* family has a similar genus sequence with other Espilonproteobacteria that also represent deep-sea hydrothermal vent species, namely *Caminibacter medialanticus* - a *Nautiliaceae* family (Mitchell *et al.*, 2014). Both genuses chemosynthetic mechanisms have been studied by Pérez-Rodríguez *et al.* (2015) to distinguish its capability to cause disease towards human

In the deep-sea hydrothermal, chemosynthetic substrate in Epsilonproteobacteria colony is exposed to steep thermal and redox gradients. In many bacteria, substrate attachment, biofilm formation, expression of virulence genes and

host colonization are partly controlled via a cell density-dependent mechanism involving signal molecules, known as quorum sensing. In general, Epsilonproteobacteria quorum sensing has been investigated only in human pathogens that use the luxS/autoinducer-2 (AI-2) mechanism to control the expression of some of these functions (Pérez-Rodríguez *et al.*, 2015).

The result that is released by Pérez-Rodríguez *et al.*, (2015) suggested that luxS is conserved, in the Epsilonproteobacteria group. Pathogenic and mesophilic members of this group, inherited luxS from a thermophilic ancestor. This study also show that, the luxS gene in *Sulfurovum lithotrophicum* and *Caminibacter mediatlanticus* are expressed, and a quorum-sensing signal is produced. Finally, luxS transcripts are detected in Epsilonproteobacteria-dominated biofilm communities, collected from deep-sea hydrothermal vents.

Taken together, this finding indicates that the LuxS enzyme from epsilonproteobacterium lineage is originated in high-temperature geothermal environments and in vent Epsilonproteobacteria. The luxS expression is linked to the production of AI-2 signals, which are likely produced *in-situ* at deep-sea vents. Pérez-Rodríguez *et al.*, (2015) concluded that the luxS gene is part of the ancestral epsilon proteobacteria genome and represents an evolutionary link that connects thermophiles to human pathogens.

In the subsections 2.6.4, it is mentioned that several *Pseudoalteromonas* species caused shell disease syndrome in crabs (Ramos and Rowley 2004; Sweet and Bateman 2016). Its pathogenicity towards human remains unknown. Several studies reveal that *Pseudoalteromonas* sp. is being investigated extensively for antibiotic medication for a human such as *Pseudoalteromonas phenolics* sp. nov. O-BC30 (Isnansetyo and Kamei 2003), *Pseudoalteromonas tunicata* KCTC 12086 T (=O-BC30T) (Sivasubramaniam and Vijayapriya 2011; Choe *et al.*, 2016).

Disease events in the marine environment not only impact directly on the host population. However, it can also result in ecosystem-wide impacts due to, for example, the mass mortality of keystone species (Burge *et al.*, 2013). These events are predicted

to increase with global climate change and elevating anthropogenic pressures (Gattuso *et al.*, 2015). Hence, there is an urgent need to generate data that speak to both the causes and the environmental factors mitigating disease in marine systems (Egan and Gardiner 2016).

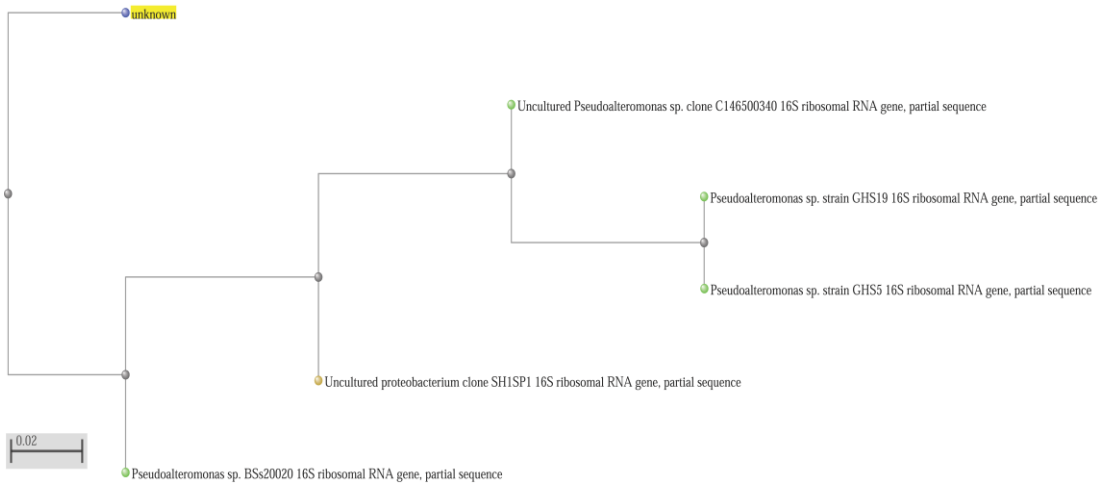


Figure 4.6 Illustration of *Pseudoalteromonas* sp. sequences query based on phylogenetic tree under 0.75 maximum sequence difference+.

+Diagram shows that Pseudoalteromonas sp. strain GHS19 and Pseudoalteromonas sp. strain GHS5 are virtually identtical as Pseudoalteromonas sp. genus that has been extracted from Off-Terengganu surface sediment

Knowledge in marine diseases complexity requires deep investigation on the human microbiome field. In the past decade, research into human disease has suggested that many chronic diseases (including skin, bowel, and lung disorders) are driven by a disturbance (or shift) in the natural microbial (i.e., Dysbiosis = A microbial community shift that has a negative impact on the host.) rather than a singular etiological agent (Althani *et al.*, 2015)

4.8 Data Repository

The sequence data from this research have been deposited in the NCBI's Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra) with the temporary submission ID of (SUB1112034).

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Background

Microbiological activity in the marine sediments is partially responsible for marine primary production and overall geochemical process (Danovaro *et al.*, 2015). In general, most of the bacterial communities that dwell in the coastal sediments are derived with a specific purpose. For instance, 50% of the deposited mineral in the coastline setting are mineralized via sulfate reduction (Païssé *et al.*, 2010; Jorgensen, 1982). In such environment, sulfur-degrading bacteria are mainly responsible for utilized sulfate compound (Suárez-Suárez *et al.*, 2011).

In concurrence of literature reviews and research findings, Proteobacteria phylum dominates the overall marine bacterial community in off-Terengganu sampling location by an astounding 85.25 to 94.3% of effective sequences identified. Subsequently, the unclassified phylum scantily followed the abundance list with a range of 1.46 to 4.99%. This chapter will focus on deliberating the research findings. Any data correlations are addressed based on the research objective.

5.2 Objective One: Bacteria Abundance in The Off-Terengganu Sedimentary Layer

Bacterial identification is considered as a fundamental microbiology assessment to obtain an unambiguous bacterial characteristic. Identifying the dominant bacteria genus helps to strengthen its overall dispersion trend in the region of interest.

The Neritic zone that is equivalent to a sea coastline feature mainly fills with a sea grass and it has a unique preference. Other than providing a beneficiary dissolved oxygen concentration, the seagrass meadow primarily accumulates the organic matter and provides a shelter for various elusive marine lives. Nevertheless, any nutrients influenced by a nearby estuary (e.g. Kuala Terengganu River) will accumulate in the seagrass meadow. Therefore, it makes a perfect source of food for marine life (Baden *et al.*, 2010; Jankowska *et al.*, 2015). As the sediment goes far from the coastline, the seagrass vegetation in its local environment is lessening due to organic nutrient limitation. Such condition will create a food privation – perhaps, affect the overall bacteria's abundant (Garcia-Martínez *et al.*, 2008, Jankowska *et al.*, 2015).

In the Chapter 3, section 3.2, it is stated that three sampling points were randomly selected to signify a different coastline sediment depth and distance from initial points. Based on OTU count, TSD1 that signify the shallowest coastline displays the highest marine bacterial diversity of 1496 species. Subsequently, it follows by TSD2 and TSD3 with 1145 and 660 species respectively. The relegation of bacterial community in off-Terengganu versus depth shows that organic nutrient in its local environment may determine bacterial community within the sediment.

The bacterial community in the TSD1 decreased ostensibly with distance from initial points. In principal, nutrient concentration is a decline with depth prior to the sea current interference and littoral depth. Thus, the nutrient availability in a deeper seabed is extremely scarce (Garcia-Martínez *et al.*, 2008; Jankowska *et al.*, 2015). Identification of marine bacteria is crucial to determine species inclinations towards nutrient availability in the seawater. In this study, the NGS analysis demonstrates huge phylum class dissimilarities at every sampling point. The Epsilonproteobacteria class is being identified dominantly in TSD1 and TSD3 with 60.63% and 87.33% respectively. The Gammaproteobacteria class bacteria has been dominated the TSD2 bacteria community with 78.67%. Although both classes belong to the same phylum, each of the class demonstrates a different characteristic and ecological preference. In this study, two of marine bacterial has demonstrated its domination where *Sulfurovum* sp. is high abundance in TSD1 and TSD3. *Pseudoalteromonas* sp. on the other hand, thrives only in TSD2.

5.3 Objective Two: Identification of dominant bacterial species in a selected coastline sedimentary layer

A natural toxic rendering process mainly conducted by marine bacteria to reduce water toxicity (Herbert, 1999). Generally, most of the marine bacteria community in the seawater is designed to regulate the organic compound as its own food source in which eventually conserved the sea grass productivities. For example, the marine bacteria regulate the nitrogen and phosphorus compound that derives from wastewater or agricultural waste in the estuaries (Zehr & Ward, 2002).

Phylogenies findings in section 4.3 describe a vast bacterial community that expresses sulfur utilization of its energy was dominant in all three sampling stations. Figure 4.3 has indicated that *Sulfurovum* sp. was the only genus that have been dominated the entire Epsilonproteobacteria class in both surveyed areas (60% in TSD1 and 88% in TSD2). This genus was characterized by its egg-like coccoidal shape and capable of oxidizing sulphur for survival (Inagaki *et al.*, 2004; Takai *et al.*, 2004). Meanwhile, Pseudoalteromonas sp. has been dominated TSD 2 sediment surface by 62.02% effective sequence identified. The result also shows a variety sulphur-degrading bacteria are present in TSD2 which is: - *Thioprofundum*, *Desulfobulbus, Desulfovirga, Desulfobacterium, Desulfosalsimonas, Sulfurimonas, Sulfuricurvum,* and *Thermodesulfovibrio*.

For the past two decades, several speculations are being addressed to identify Epsilonproteobacteria interaction with organic pollutant, after several genera in its class is being identified in a polluted coastline and open water (Nakagawa *et al.*, 2005). In a recent study, Epsilonproteobacteria class demonstrates a visible interaction with the organic compound in the polluted region (Lin *et al.*, 2014; Bolhuis *et al.*, 2014).

The outcome of this study describes that *Sulfurovum* sp. is the only genus that covers the entire Epsilonproteobacteria phylogeny profile in both surveyed areas. *Sulfurovum* sp. is a gram-negative, non-motile genus that is categorized under sulphur-oxidizing chemoautotrophic genera and was first isolated from a deep-sea hydrothermal vent in Okinawa, Japan (Inagaki *et al.*, 2004). It prefers a moderate temperature of between 20°C to 45°C and a medium salinity (Willey *et al.*, 2008).

Although the metabolic properties for most of Sulfurovum sp. remain indecisive, one of the strains, *Sulfurovum* 42BKTT grew chemolithoautotrophically with elemental sulphur or thiosulfate as the sole electron donor and oxygen (optimum 5 % in the gas phase) or nitrate as the electron acceptor. The G + C content of its genomic DNA was 48.0 mol% (Inagaki *et al.*, 2004). In a recent finding, the *Sulfurovum* sp. inhibit its growth in freshwater rivers (Hubert *et al.*, 2011), high turbidity waters, and acidic conditions (Bolhuis *et al.*, 2014). Therefore, it is probable that the high turbidity value at TSD1 may likely be the main cause of *Sulfurovum* sp. abundance inhibition compares to TSD2

Generally, sulphur-oxidizing bacteria abundant in the oil reservoirs are affected by temperature, mineralization, permeability and, water displacement (Lin *et al.*, 2014). Certain heavy metals effluent such as barium, iron, and manganese (which mainly discharged from hydrocarbon energy plants) stimulates these bacterial groups (Yeung *et al.*, 2011). In a natural environment, the *Sulfurovum* sp. Was discovered in hydrocarbon-polluted coastal seawater, such as at a coal oil point in California, USA (Håvelsrud *et al.*, 2011), Berre lagoon in France (Paissé *et al.*, 2008), and Busan Northport in South Korea (Subha *et al.*, 2014). Moreover, *Sulfurovum* sp. Is also a predominant species that are being identified in deep hydrothermal vents (Wright *et al.*, 2013; Dahle *et al.*, 2015; Inagaki *et al.*, 2004), shallow hydrothermal vents (Giovannelli *et al.*, 2013), volcanic regions (Wang *et al.*, 2015b), caves, sinkholes, and sulphide compounds (Nakagawa *et al.*, 2005; Jones *et al.*, 2010; Handley *et al.*, 2012).

Sulfurovum sp. metabolic versatility was recently recognized where several studies indicate its role in degrading aromatic hydrocarbons (Lin *et al.*, 2014; Håvelsrud *et al.*, 2011; Paissé *et al.*, 2008; Paissé *et al.*, 2010), benzene, phenols, and toluene (Kleinsteuber *et al.*, 2008). Furthermore, *Sulfurovum* sp., together with other sulphur-oxidizing bacteria has the capability to produce active surfactants (Xiu *et al.*, 2010; Grabowski *et al.*, 2005).

However, none of the above studies exhibit high *Sulfurovum* sp. abundance in a hydrocarbon pollutant compared to its abundance in this report at the Off-Terengganu coastline (Marziah *et al.*, 2016). For the past two decades, several

speculations escalate the possibility of Epsilonproteobacteria interaction with organic pollutant after several genera in its class is being acknowledged in a polluted coastline and open water (Nakagawa *et al.*, 2005). In a recent study, Epsilonproteobacteria class demonstrates a noticeable interaction with the organic compound in the polluted region (Lin *et al.*, 2014; Bolhuis *et al.*, 2014)

5.3.1 Local Physical-Geochemical Reports

To strengthen sulphur-degrading bacteria relationship with sulphide richness in its surface sediment, several physical-geochemical analyses was performed. CHNS, HEM, and TPH analyses indicate the proportion of targeted organic compound are diminishing with depth - correspondingly reflected with bacteria community's declination (Garcia-Martínez *et al.*, 2008; Jankowska *et al.*, 2015). However, this such analysis itself needs further investigation.

In this study, the CHNS analysis demonstrates a deficient sulphur and carbon ratio. Therefore, there is no concrete theory to support the existence of hydrothermal vents and volcanic composite in TSD sampling point. It is impossible to predict *Sulfurovum* interaction based on both criteria. Perhaps, *Sulfurovum* genus that was identified TSD has a dissimilar DNA assembly compared to its ancestor's genes. It is difficult to obtain a *Sulfurovum* culture for laboratory analysis as it can easily perish upon sampling due to environment jolt. Previous research indicates that *Sulfurovum* able to survive in both aerobic (Wang *et al.*, 2015) and anaerobic (Dahle *et al.*, 2015) condition and prefers a moderate temperature between 20 to 45°C and medium salinity environment (Willey *et al.*, 2008).

However, *Sulfurovum* is not expected to thrive in a natural freshwater (Hubert *et al.*, 2011), high turbidity and acidic estuaries (Bolhuis *et al.*, 2014). Since the TSD1 has a moderate turbidity rate, it is probably the main reason for *Sulfurovum* shortages (-26.67%) in contrast to TSD3. Perhaps, the available nutrient sources are limited due to competition with aggressive sulphur-oxidizing bacteria such as *Pseudoalteromonas*.

No conscientious reasons could be deliberated prior to these research findings. Fundamentally, it is unexpected to perceive *Surfuvorum* sp. abundances in shallow and, non-hydrothermal region, which is TSD1 and TSD3. Upon its first identification in the deep-sea region, no conceivable data to explain its cell mechanism has been released. Contrariwise, *Sulfurovum* inhibitions in TSD2 are very dubious. Based on the bacterial nature itself, there is a probability that *Sulfurovum* unable to compete with other sulphur-oxidizing bacterial species (Inagaki. *et al.*, 2004). Perhaps, the marine bacteria community in TSD2 comprises of many bacteria predatory types that overwhelmed *Sulfurovum* growth. Therefore, this research requires further investigation to distinguish *Sulfurovum* molecular characteristic.

5.3.2 Mercury pollutions in Off-Terengganu

Historically, mercury (Hg) compound was detected in the Off-Terengganu surroundings two decades ago. Although the concentration value is considered manageable (20 - 127 ng $^{g-1}$ dry wt), mercury existence in Off-Terengganu may suggest anthropogenic pollution has occurred for several years and caused the anoxic condition in its neritic sediment. Kannan & Falandyz (1996) describes that a lower proportion of total Hg suggests that most Hg in anoxic marine sediments form strong complexes with sulphide and precipitate as mercury sulphide (Hgs).

The biogeochemistry of methyl mercury production is complicated. Its unique biogeochemical cycle and involvement of several factors in the local environment such as - oxygen, temperature, pH, organic matters, and sulphate are crucial. Future studies should account for all these parameters, in order to understand the mercury biogeochemical cycling in the marine environment.

The presence of high sulphate concentrations in sea water (millimolar amounts) and consequently in marine sediments influences various microbial processes. In sulphate-rich anaerobic (anoxic) marine sediments, mercuric ions are bound to hydrogen sulfide and become less available for microbial methylation (Capone & Kiene, 1988). Furthermore, sulphate may interfere with methylation of Hg through its

effect on the redox potential. Based on the previous study by Sultan *et al.* (2011), redox potential (Eh) value in several areas that are near to Off-Terengganu coastline is in between -51mV to 100mV; where it is mainly influenced by runoff from the Terengganu river basin. It is expected that the exact Eh value in all three sampling stations may account to -100 mg or more, since the reduction of sulphate mainly occurs at ~ – 200 mV (Kannan & Falandyz, 1996).

Another possible factor that contributes to negative Eu value is the occurrence of oxygen adsorption from the sediment surface layers; Which is mainly caused by an active hydrocarbon compound (identification is based on TPH results is section 4.5.3) (Xue *et al.*, 2016). Therefore, oxygen depletion in surface sediment will suppress anaerobic bacteria community growth and gives an advantage for anaerobic bacteria specifically *Sulfurovum* sp. and *Pseudoalteromonas* sp. to dominate in such condition.

5.4 Objective Three: To Identify, Among Those Dominant Species, A Potential Waterborne Bacterium That Causes Disease Towards the Human

For this objective, the metabolic capability of two dominant bacterial genera discussed to identify its capability to induce infection in humans and, animals. Before any discussion is made to achieve bacteria pathogenesis capability, below are descriptions of both bacterium molecular characteristics: -

5.4.1 *Sulfurovum* sp.

In the deep-sea hydrothermal, chemosynthetic substrate in Epsilonproteobacteria colony is exposed to steep thermal and redox gradients. In many bacteria, substrate attachment, biofilm formation, expression of virulence genes and host colonization are partly controlled via a cell density-dependent mechanism involving signal molecules, known as quorum sensing. In general, Epsilonproteobacteria quorum sensing has been investigated only in human pathogens

that use the luxS/autoinducer-2 (AI-2) mechanism to control the expression of some of these functions (Pérez-Rodríguez *et al.*, 2015).

The result that is released by Pérez-Rodríguez *et al.*, (2015) suggested that luxS is conserved in Epsilonproteobacteria class. Pathogenic and mesophilic members of this class inherited luxS from a thermophilic ancestor. In addition, this study shows that the luxS gene in *Sulfurovum lithotrophicum* and *Caminibacter mediatlanticus* are expressed — and a quorum-sensing signal is produced. Finally, luxS transcripts are detected in Epsilonproteobacteria-dominated biofilm community; that is collected from a deep-sea hydrothermal vents.

Taken together, these findings indicate that the epsilonproteobacterium lineage of the LuxS enzyme is originated in high-temperature geothermal environments and that, in vent Epsilonproteobacteria, the luxS expression is linked to the production of AI-2 signals, which are likely produced in situ at deep-sea vents. Therefore, Pérez-Rodríguez *et al.*, (2015) conclude that the luxS gene is part of the ancestral epsilon proteobacteria genome and represents an evolutionary link that connects thermophiles to human pathogens.

5.4.2 *Pseudoalteromoas* sp.

Conversely, *Pseudoalteromas* dominates the overall bacterial community in TSD2 and somehow extremely limited in TSD1 and TSD3. This genus was considered as a normal free-living bacterium in the seawater where certain of its species can cause Shell Disease Syndrome especially in crabs (Ramos & Rowley, 2004). Their existence in the seawater is beneficial for antimicrobial properties to counter Methicillin-resistant Staphylococcus aureus (MRSA) (Isnansetyo & Kamei 2003) and a coral pathogen; *Vibrio shiloi* (Nissimov *et al.*, 2008). Based on the sampling route depicted in Figure 3.2, TSD2 have located near to a coral-featured Pulau Kapas; which probably portrays *Pseudoalteromonas* association in a delicate coral prone zone.

Currently, there are no equitable data to demonstrate *Pseudoalteromonas* abundance in a sulphur composite area (e.g. volcanic region). Contrariwise, a recent study indicates that certain *Pseudoalteromonas* phenotype exhibit an aggressive degradation behaviour when it was introduced to the hydrocarbon rich medium (Chatterje, 2015; King *et al.*, 2015). There is the probability that *Pseudoalteromonas* abundance in TSD2 is influenced by the monsoonal direction. However, it requires further investigation to recognise the sea current patterns in the surveyed area.

Generally, it is expected that marine bacteria phylogenetic in all TSD sampling areas indicate community likeness to thrive from a hydrocarbon pollutant source. Further NGS analysis is necessary to expand the 16SrDNA variant such as V6-V9 in order to merge the exact species phenotypic. In overall, the NGS and physical-geochemical findings correspond to the third research hypothesis for this study: - Bacteria abundance in each littoral zone is reflected by nutrient availability, which is hydrocarbon pollutant and sulphur element.

5.5 Anthropogenic Pollution Concerns in The Off-Terengganu Coastline

It is widely reported that hydrocarbon based spillage (e.g. Petroleum) is the main cause of anthropogenic pollution in the marine coastline (Mistch, 2010; Suárez-Suárez *et al.*, 2011). Its occurrences are rapid, frequent and, unpredictable (Suárez-Suárez *et al.*, 2011). Subsequently, it leads to a dreadful ecological perturbation (Berthe-Conti & Nachtkamp, 2010). For instance, petroleum is a complex mixture of an organic compound with over 17,000 distinct components (Head *et al.*, 2006) and it was classified into aromatic and aliphatic hydrocarbons.

For more than 30 years, the aromatic hydrocarbon is broadly studied because it is stable, toxic and has a high affinity for sediment (McElroy *et al.*, 1989). It is reported that only a small fraction of aromatic hydrocarbon is naturally dissipated in the seawater, where the rest are formed into droplets, suspended organic and inorganic particles (Berthe-Conti & Nachtkamp, 2010). The consequences of hydrocarbon pollution have enforced the United State Environmental Protection Agency (USEPA)

to legalize several experiments that are applicable to monitor environmental impact assessment (EIA); particularly, the HEM method (USEPA 1664) and the TPH method (USEPA 8105B).

In this study, the HEM analysis indicates that both surveyed areas a slightly polluted with oil and grease compound. Subsequently, the TPH analysis confirms the existences of gasoline, diesel, and mineral oil concentration; are caused by oil spills from fishing vessels and high-speed boats (Marziah *et al.*, 2016). Based on available information, about 2216 units of the fishing vessels were registered specifically in the surveyed area (Kuala Terengganu district) in the year of 2001, whereas 316 units are the outboard powered vessel and the rest were numerous Inboard-Powered vessel type (Information on Fisheries Management in Malaysia, 2001). The inboard powered motor was fuelled mainly by gasoline or diesel while the outboard motor are fuelled with gasoline, with 0 - 10% of ethanol alcohol blended fuel.

Other criteria that probably contributes to augmentations of anthropogenic pollution in the surveyed area is the Pulau Duyung breakwater (refer to Figure 2.6). Generally, the breakwater was built to reduce the wave intensity in the inshore water as part of the coastal defence or an anchorage protection from the weather and longshore drift effects (Marziah *et al.*, 2016; Jonsson *et al.*, 2006). However, the breakwater structure has its unintended consequences towards the sediment (Jonsson, *et al.*, 2006) because the dissipation of energy and relatively calm water created in the lee of the breakwaters often encourages accretion of sediment and salient to build up (Van Rijn, 2010). Furthermore, if excessive rainfall occurs inside the breakwater, the storm will cause a runoff and eventually trapped in the breakwater (Butt, 2013). For example, a 13.4 km breakwater structure on the Long Beach, CA coastline was built circa the 1970s to protect the U.S Naval ships in World War II. Although the harbour is already closed since 1996, the pollution that accumulates in the breakwater still remains, and it is harmful to locals and tourist to swim around it (Butt, 2013). Therefore, further investigations are needed to predict if runoff water that was trapped in the Pulau Duyong breakwater is the main cause of hydrocarbon accumulation in both water column and sediment.

The impact of oil spill in the marine ecosystem depends on the history of the local environmental pollution itself. For instance, bacteria that were adapted from a previous oil spill will occur faster than in a pristine environment due to metabolically readiness to utilize a hydrocarbon compound (Païssé *et al.*, 2008). A few hydrocarbon and sulphur degraded bacteria was tested for its natural bioremediation potential by attempting to distribute bacteria colony to degrade residual oil in the coastal environment naturally (Païssé *et al.*, 2010).

There is a strong probability that TSD1 is positioned in an active wave dissipation area because of water turbidity. Therefore, the sedimentary layer of TSD1 may have richer particulate matter retention of anthropogenic pollutants in the breakwater opening (Marziah *et al.*, 2016).

5.6 Research Conclusions

This research marks the first benthic bacterial community insight in the off-Terengganu where it shows that two sulphur-oxidized bacteria dominates all three sampling points namely *Sulfurovum* sp. and *Pseudoalteromonas* sp. The findings also describe that marine bacteria community in the Off-Terengganu is prominently abundance in the shallowest sampling point and it gradually dwindles as the subsequent sampling point are far-off to the open water and deeper from the initial sampling point.

Subsequently, plenty of rare bacteria biospheres that are categorized as a sulphur-oxidized genus was identified in the surveyed area; that generally relies on sulphide as nutrient sources. In general, sulphur and sulphide resources in the seawater come from a hydrothermal vent, volcanic region, and hydrocarbon oil. This research exhibits a small trace of the sulphur element in all sampling stations - With no conceivable resource that derives from hydrothermal vent and volcanic compounds. The HEM and TPH analysis exhibit hydrocarbon compound in the surveyed areas. Therefore, it is possible that sulphate emission is derived from petroleum contaminant

and has developed anoxic sedimentary layer – which in overall, supports anaerobic bacteria growth.

Historically, sulphate sources in Off-Terengganu come from total mercury (total Hg) pollution. Based on Hannan & Falandyz (1996) claims, other that developing anoxic sedimentary layer in all sampling stations, low total Hg proportion suggests that most Hg in anoxic marine sediments form strong complexes with sulphide and precipitate as mercury sulphide (Hgs). Therefore, an anoxic, or anaerobic condition in all marine sediment supports *Sulfurovum* sp. and *Pseudoalteromonas* sp. growth and has suppressed aerobic bacteria community growth.

The *Sulfurovum* predomination novelty in the Off-Terengganu coastline is evident because this genus was historically detected in deep-sea hydrothermal vents and volcanic regions (Marziah *et al.*, 2016). This study is depicted as one of the highest *Sulfurovum* sp. distributions ever reported in a natural environment, showing the broadening versatility of its genus in adapting to a different environmental condition. *Sulfurovum* existence in a shallow sedimentary layer, is astonishing in terms of attaining anaerobic condition to promote its proliferation.

In overall, *Sulfurovum* sp. domination in Off-Terengganu possibly has been influenced by sulfite emission that derives from petroleum contaminant and HgS. A native marine bacterium such as *Sulfurovum* may have altered its molecular expression to subsist in previous marine pollution. If pollution is reverted in the same local environment, the bacteria has its metabolic readiness to utilize available organic compound (Marziah *et al.*, 2016), permits proliferation or simply, preserve its energy (Païssé *et al.*, 2008). In overall, a full-length *Sulfurovum* sp. sequence will improve a species identification.

There are no conclusive findings to elucidate *Pseudoalteromonas* abundances in the TSD2, compares to TSD1 and TSD3. However, it is confirmed that this species was expected to occur in the marine sediments since it is a native littoral marine bacterium. There is a possibility that *Pseudoalteromonas* appears to be dominant due

to its aggressive hydrocarbon oxidizer behaviour where it might easily eradicate other hydrocarbon oxidizer genus in its local community.

There is no adequate information to deliberate the probability of both dominant bacteria to cause illness in human. However, a recent research suggested that *Sulfurovum* sp. and other Epsilonproteobacterium class quorum sensing has been compared with other human pathogens that use the luxS/autoinducer-2 (AI-2) mechanism to control the expression of some of these functions (Pérez-Rodríguez *et al.*, 2015). Currently, *Sulfurovum* sp. carries similar genes as *Helicobacter pylori,* which are a prominent species that caused gastroenteric infection in a human, no insignificant differences in their niches and regimes to *Helicobacter* species (Gupta 2006; Nothaft and Szymanski 2010).

Pseudoalteromonas sp., on the other hand, shows no correlation to elicit illness in human. In fact, *Pseudoalteromonas* is being widely investigated to produce an antibiotic compound to combat clinical bacterial infection in human.

To date, only *Sulfurovum* sp. chemosynthetic mechanisms are being studied extensively to distinguish its capability to trigger a disease in human. In conclusion, knowledge of its true pathogenic influence in human remains unknown.

5.7 Recommendations

Culturing a live *Sulfurovum* sp. in the artificial environment is extremely difficult. Therefore, all available DNA products obtained from the off-Terengganu is exploited for additional molecular investigation - to broaden its species coverage by implementing a different 16SrDNA hypervariable region such as V6 - V9 and proteomics. Furthermore, the additional molecular analysis may increase the chances to recognize a substantial amount of unknown phylum that was reported in the surveyed area (Marziah *et al.*, 2016).

Theoretically, anthropogenic pollution in a marine ecosystem is dependent on the history of environmental pollution itself. However, there are no conclusive findings to identify precise anthropogenic ranges along the Off-Terengganu coastline (Marziah *et al.*, 2016). Physical-geochemical findings in this thesis are irrefutably supported excerpted data (E.g. Eh value, mercury concentration, heavy metal analysis, and PAH value), gathered from the DOE library, and scientific journals.

This research findings proposed that all TSD-related surface bathymetry is anoxic as it supports the sustainability of anaerobic and sulphur-oxidizing bacteria community. Therefore, the accurate Eh value in sampling location is necessary to seek further significant facts that reflect existing physical-geochemical findings.

Finally, this study also requires extensive abiotic analysis, such as Polycyclic Aromatic Hydrocarbon (PAH) that is beneficial to investigate *Sulfurovum* sp. interaction with carcinogenic and toxic compounds for clearer geochemical evidence, dispersion scale, and species variations in its local environment.

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APPENDIX A

THEORY OF BIOLOGY

Table A 1 The domain and fundamental principles of the theory of biology

Domain
The diversity and complexity of living systems, including causes and consequences
Principles
1. Life consists of open, non-equilibrial systems that are persistent
2. The cell is the fundamental unit of life
3. Life requires a system to store, use, and transmit information
4. Living systems vary in their composition and structure at all levels
5. Living systems consist of complex sets of interacting parts
6. The complexity of living systems leads to emergent properties.
7. The complexity of living systems creates a role for contingency
8. The persistence of living systems requires that they are capable of change over time
9. Living systems come from other living systems
10. Life originated from non-life

APPENDIX B

THEORY OF CELLS

Table B1 The domain and fundamental principles of the theory of cells

Domain	
Cells and the causes of their structure, function, and variation	
Principles	
1.	Cells are highly ordered, bounded systems
2.	Cells are composed of heterogeneous parts consisting of subsystems that act to localize resources and processes
3.	Cells are regulated by a network of biochemical and supermolecular interactions
4.	Cells interact with their external environment, including with other cells
5.	Cells exchange matter through boundaries consisting of semipermeable membranes.
6.	Cells require an external source, either chemical or electromagnetic.
7.	Cells use energy to create concentration gradients of ions and molecules.
8.	New cells are formed from other existing cells.
9.	Cells contain all of the information necessary for their own construction, operation, and replication.
10.	The properties of cells are the result of evolution.

APPENDIX C

THEORY OF ORGANISMS

Table C1 The domain and fundamental principles of the theory of organisms

Domain	
Individual and the causes of their structure, function, and variation	
Principles	
1.	An individual organism actively maintaines its structural and functional integrity
2.	All organism are composed of cells at some point in their life cycle.
3.	Organismal maintenance at one level requires change at other levels.
4.	Organismal functions trade-off against each other.
5.	Organismal maintenance is a functions of interactions with the abiotic and biotic environment
6.	Organisms require external sources of materials and energy for maintenance, growth, and reproductions.
7.	Because organism are changeable, external influences can force change
8.	Heterogeneity of resources in space and time leads to variation in ontogeny and life history patterns
9.	Organismal reproduction is both a cause and consequences of evolutionary processes
10.	The properties of organisms are the result of evolution

APPENDIX D

THEORY OF GENETICS

Table D1 The domain and fundamental principles of the theory of genetics

Domain	
The patterns and processes of the use, storage, and transmittal of information in organisms	
Principles	
1.	Offspring resemble their parents
2.	The fidelity of information transmittal requires an error correction system.
3.	Because life is the product of natural selection, the information system must capable to produce new information.
4.	The imperfections of error correction create new information.
5.	The exchange and recombination of information among individuals create new information.
6.	Random processes play an importance role in information transmittal, error correction, and the exchange of information among individuals.
7.	The systems of information usage must be robust to errors.
8.	Information usage is context dependent.
9.	The properties of information systems are the result of evolution

APPENDIX E

THEORY OF ECOLOGY

Table D2 The domain and fundamental principles of the theory of ecology

Domain	
The spatial and temporal patterns of the distribution and abudance or organism, including causes and consequences	
Principles	
1.	Organism are distributed unevenly in space and time
2.	Organism interact with their abiotic and biotic environments
3.	Variation in the characteristic of organism results in heterogeneity of ecological patterns and processes.
4.	The distribution of organism and their interactions depend on contingencies
5.	Environmental conditions are heterogenous in space and time.
6.	Resource are finit and heterogenous in space and time
7.	Birth rates and death rates are a consequence of interactions with the abiotc and biotic environment.
8.	The ecological properties of species are the results of evolution.

APPENDIX F

LIST OF PUBLICATIONS

2016

Marziah, Z., Mahadzir, A., Musa, M.N., Azhim, A. and, Hara, H. (2016) Abundance of sulfur degrading bacteria in a benthic bacterial community of shallow sea sediment in the Off-Terengganu Coast of the South China Sea. *MicrobiologyOpen*. 5(X):xxx-xxx doi:10.1002/mbo3.380 (2.21)

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Marziah, Z., Mahadzir, A. and, Musa, M.N. (2015, August). Ciguatera Poisoning and its Potential Incidence Risks of OTEC Operation in Tropical Reef Coastal Waters. *Proceedings of 3rd International Ocean Thermal Energy Conversion (OTEC) Symposium 2015*. 8th October 2015. ISBN: 978-983-44732-5-9

Akbariah Mahadzir and **Marziah Zahar**. (2015, August). OTEC Spin-Off Industries and Socio-Economic Transformation. Future Energy: Is OTEC the Solution, points, myForesight ® - Malaysia Industry-Government Group for High Technology (MIGHT), 3: 22-23. DOI: 10.13140/RG.2.1.4679.8166

Marziah Zahar and Noor Fazreen Dzulkafli. (2015, August). Marine Microbe: Secrets from The Ocean. Future Energy: Is OTEC the Solution, myForesight ® - Malaysia Industry-Government Group for High Technology (MIGHT), 3: 36-37. DOI: 10.13140/RG.2.1.3868.8086

Z. Marziah, A. Azhim, A. Mahadzir, M.N. Musa, A. Bakar Jaafar. 2015. Potential of Deep Seawater Aquaculture for Economic Transformation in Sabah, Malaysia. 10th Asian Control Conference. *IEEE Control Systems Society*. 31st May – 03rd June 2015. Pg: 132. DOI: 10.1109/ASCC.2015.7244687

2014

Z. Marziah and A. Azhim. 2014. Marine Biological Assessment in Offshore Water. *1st Biologically Inspired System and Technology Symposium*. August 6-7th 2014.

APPENDIX G

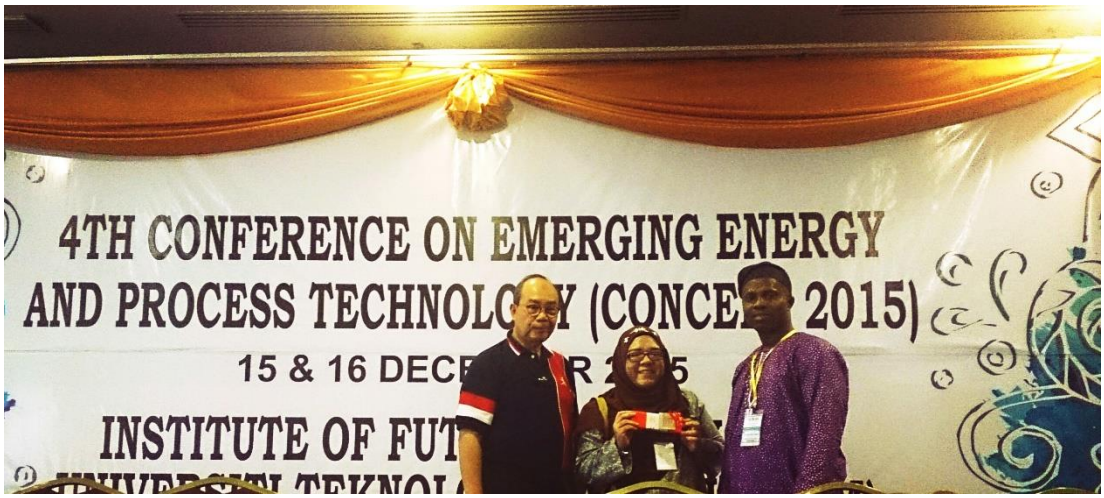
LIST OF SEVERAL BACTERIA DIVERSITY IN THE SOUTH CHINA SEA

Region	Country	Species	Host / Sample	Sampling Location
North SCS	**China**	1. *Aeromonas* sp. 2. *Pseudomonas* sp. 3. *Photobacterium* sp. 4. *Vibrio* sp. 5. *Enterobacter* sp. 6. *Bacillus* sp. 7. *Acinetobacter* sp. 8. *Cytophaga* sp. 9. *Lutibacteriu* sp. 10. *Moraxella* sp. 11. *Flavobacterium* sp. 12. *Xanthomonas* sp. 13. *Chromobacterium* sp. 14. *Alcaligenes* sp.	Seawater	Dapeng Bay (DP) (Jiang *et al.*, 2010)
	Philippines	1. *Vibrio* sp.• 2. *Vibrio parahaemolyticus*• 3. *Vibrio harveyi*• 4. *Pseudomonas* sp.• 5. *Pseudomonas aeruginosa*• 6. *Pseudoalteromonas* sp.• 7. *Pseudoalteromonas viridis*• 8. *Ruegeria lacuscaerulensis*• 9. *Roseobacter gallaeciensis*• 10. *Pelagibacter* sp.• 11. *Ponticoccus* sp.• 12. *Alphaproteobacterium*• 13. *Halobacillus* sp.• 14. *Bacillus pumilus*• 15. *Microbacterium esteraromaticum*• 16. *Algoriphagus* sp.• 17. *Coccinimonas marina* •	Seawater•	Bolinao, Pangasinan Northern Phillipines• (Manset *et al.*, 2013)
	Taiwan	1. *Oceanicola marinus* 2. *Pseudidiomarina taiwanensis* ˌ 3. *Vibrio vulnificus* ˇ 4. *Vibrio ruber* ʷ 5. *Vibrio fischeri*^ 6. *Vibrio logel*^ 7. *Vibrio harveyi*^ 8. *Vibrio vulnificus*^ 9. *Vibrio splendidius*^ 10. *Vibrio orientalis*^ 11. *Vibrio cholera*^ 12. *Shewanella hanedai*^ 13. *Shewanella woodyi*^ 14. *Photobacterium leiognathi*^ 15. *Photobacterium phosphoreum*^	1. Seawater 2. Seawaterˌ 3. Seawaterˇ 4. Seawaterʷ 5. Seawater^	1. Eluanbi coast, Pingtung County, (Lin *et al.*, 2007) 2. ˌAn-Ping Harbour (Jean *et al.*, 2006) 3. ˇunspecified location (Goo and Wan 1995) 4. ʷKeelung (Shieh *et al.*, 2003) 5. ^unspecified location (Chiu *et al.*, 2007)

	Vietnam	1. *Bacillus* sp.+ 2. *Vibrio* sp.+ 3. *Pseudomonas* sp.+ 4. *Pseudoalteromonas* sp.+ 5. *Marinococcus* sp.+ 6. *Halobacillus* sp.+ 7. *Shewanella* sp.+ 8. *Sulfitobacter* sp.+	+Cultivated Mollusk in Seawater 1. *Crassostrea lugubris* 2. *Perna viridis*	1. + Gulf of Nha Trang Lagoon (Beleneva *et al.*, 2007)
South SCS	**West(Peninsular) Malaysia**	1. *Vibrio parahaemolyticus*♦ 2. *Bacillus megaterium* ♦ 3. *Shewanella sp.* ♦ 4. *Escherichia coli* ♦ 5. *Salinimonas chungwhensis*♦ 6. *Alteromonas sp.* ♦ 7. *Alteromonasalvinellae*♦ 8. *Pseudomonas sp.*□ 9. *Enterobacter agglomerans*□ 10. *Klebsiella pneumonia*□ 11. *Acinetobacter sp.*□ 12. *Flavobacterium* sp. □ 13. *Escherichia coli*♠ 14. *Vibrio parahaemolyticus*♠ 15. *Salmonella Typhi*♠	1. Seawater♦ 2. *Acropora cervicornis* (Coral)□ 3. Seawater♠	1. ♦(You *et al.*, 2012) 2. □East Coast of Peninsular (Kalimutho *et al.*, 2007) 3. ♠Kuantan, Pahang (Lee *et al.*, 2011)
	East Malaysia (Sabah) **- West coast**	1. *Vibrio harveyito*× 2. *Vibrio parahaemolyticus** 3. *Vibrio alginolyticus**	1. × Marine net cage, seawater - Asia seabass (*Latescalcarifer*) - Brown marble grouper (*Epinephelusfuscoguttatus*) - Red snapper (*Lutjanus sp.*) - Hybrid grouper (*E.fusguttatus x E. lanceolatus*) 2. * Marine net cage, seawater	1. ×Aquaculture facility, Sulaman Bay, West Coast Sabah (Albert and Ransangan 2013) 2. *West Coast Sabah (Ransangan *et al.*, 2013)
	East Malaysia (Sarawak) **-West coast**	1. *Faecal coliforms* 2. *Escherichia coli* 3. *Faecal coli* 4. *Faecal streptococci* 5. *Thalassospira profundimaris* (Carbazole degrader) [s] 6. *Kordiimonas gwangyanggensis* (closely related) *Alphaproteobacteria* OC6ST[s]	1. Seawater 2. Seawater [s] 3. Seawater [s]	1. Tanjung Batu beach, Bintulu Sarawak (Appan 1991) 2. Miri, Sarawak (Rani 2011) 3. [s] Zhulkarnain (2014)

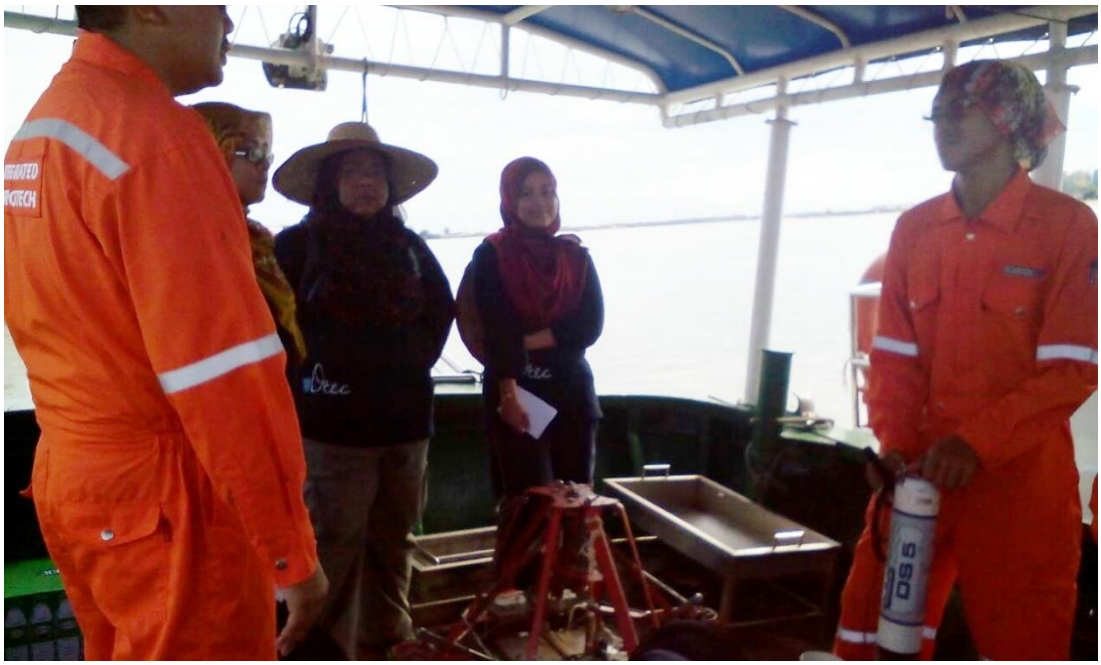
APPENDIX H

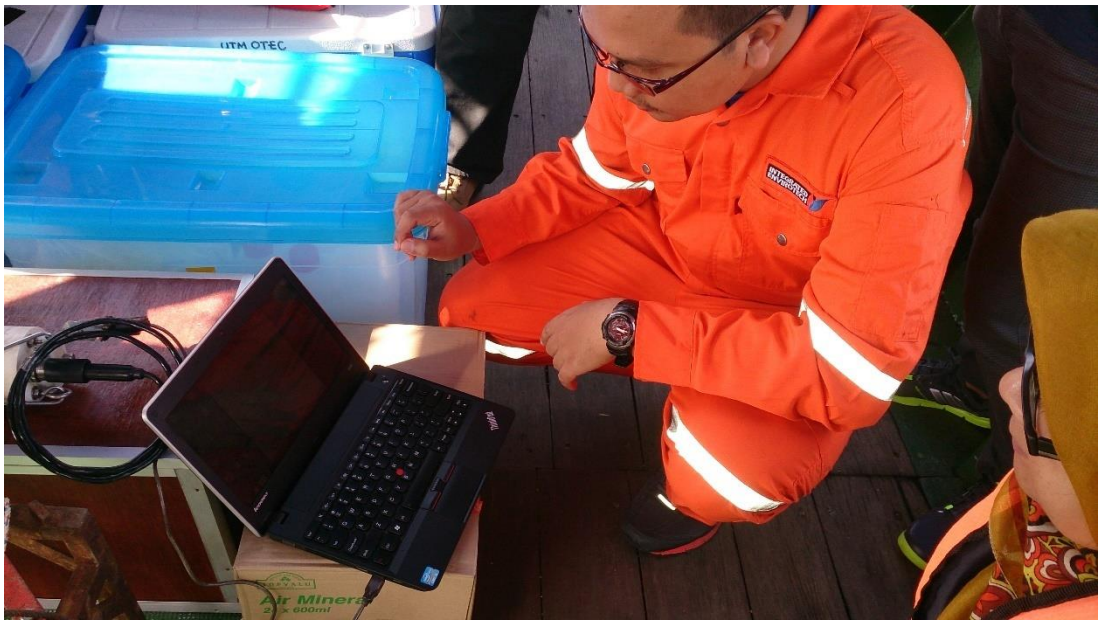
GALLERY: BEST ORAL PRESENTER (CATALYST II) - CONCEPT 2015



APPENDIX I

GALLERY: SAMPLING ACTIVITY IN OFF-TERENGGANU




UTM OTEC