ความหลากหลายของแบคทีเรียในทะเลอันดามันประเทศไทยโดยวิธีเพาะเลี้ยงและไม่เพาะเลี้ยง

นางสาวคุณลักษณ์ คิดเหมาะ

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

BACTERIAL DIVERSITY IN ANDAMAN SEA, THAILAND BY CULTURE-DEPENDENT AND

CULTURE-INDEPENDENT METHODS

Miss Khunnalack Khitmoh

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Industrial Microbiology

Department of Microbiology

Faculty of Science

Chulalongkorn University

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Field of Study	Industrial Microbiology
Thesis Advisor	Assistant Professor Naraporn Somboonna, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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--External Examiner

(Sissades Tongsima, Ph.D.)

คุณลักษณ์ คิดเหมาะ : ความหลากหลายของแบคทีเรียในทะเลอันดามันประเทศไทย โดยวิธีเพาะเลี้ยงและไม่เพาะเลี้ยง. (BACTERIAL DIVERSITY IN ANDAMAN SEA, THAILAND BY CULTURE-DEPENDENT AND CULTURE-INDEPENDENT METHODS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นราพร สมบูรณ์นะ, 111 หน้า.

พื้นที่ส่วนใหญ่ของโลกประกอบด้วยบริเวณที่เป็นทะเลและมหาสมุทรเชื่อมต่อกัน ประมาณ 3 ใน 4 (71%) ของพื้นผิวโลก ประเทศไทยมีอาณาเขตติดกับทะเล 2 ส่วน คือ ทะเลฝั่ง อ่าวไทย และฝั่งทะเลอันดามัน เนื่องจากตั้งอยู่บริเวณแนวเส้นศูนย์สูตรทำให้ประเทศไทยมี ภูมิอากาศร้อนชื้นจึงส่งผลให้ทะเลไทยมีความอุดมสมบูรณ์ทางชีวภาพของจุลินทรีย์มากมาย แต่ วิธีการเพาะเลี้ยงเชื้อแบบดั้งเดิมนั้นสามารถค้นพบจุลินทรีย์ได้เพียง 1% ของจุลินทรีย์ทั้งหมด เนื่องจากข้อจำกัดการเจริญเติบโตของจุลินทรีย์ภายใต้สภาวะในห้องปฏิบัติการ ดังนั้นจุลินทรีย์อีก กว่า 99% อาจถูกค้นพบได้โดยวิธีเมตาจีโนมิคส์ (metagenomics) ซึ่งไม่ต้องอาศัยวิธีการ เพาะเลี้ยงเชื้อ งานวิจัยนี้ได้นำวิธีเมตาจีโนมิคส์ มาใช้ร่วมกับการไพโรซีเควนยีนไรโบโซมอลอาร์ เอ็นเอชนิด 16S เพื่อให้ได้ความหลากหลายของจุลินทรีย์ที่แท้จริงและเข้าใจระบบนิเวศของ จุลินทรีย์ในทะเลอันดามัน ประเทศไทย ในงานวิจัยนี้ได้ศึกษาน้ำทะเลในระยะห่างจากชายฝั่งที่ แตกต่างกันคือที่ 08°56.004'N 98°05.857'E (PT) ระดับความลึก 30 เมตร และ 08°51.096'N 97°31.207'E (TC) ที่ผิวน้ำและระดับความลึก 30, 100 และ 150 เมตร ตำแหน่ง PT และ TC มี ระดับความลึกประมาณ 35 เมตรและ 155 เมตรตามลำดับ ผลที่ได้จากวิธีไม่เพาะเลี้ยงพบว่าได้ จำนวนสายพันธุ์มากมายที่ไม่พบด้วยวิธีเพาะเลี้ยง วิทยานิพนธ์ฉบับนี้ช่วยให้เข้าใจโครงสร้าง ประชากรแบคทีเรียและอาร์เคียและศักยภาพหน้าที่ในระบบนิเวศของจุลินทรีย์เหล่านี้ในทะเลที่ ตำแหน่ง PT และ TC

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต ....
สาขาวิชา	จุลชีววิทยาทางอุตสาหกรรม	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก ....
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KHUNNALACK KHITMOH: BACTERIAL DIVERSITY IN ANDAMAN SEA, THAILAND BY CULTURE-DEPENDENT AND CULTURE-INDEPENDENT METHODS. ADVISOR: ASST. PROF. NARAPORN SOMBOONNA, Ph.D., 111 pp.

Thailand is surrounded by Andaman Sea and Gulf of Thailand. As Thailand is located around an equator with hot and rainy climate, Thai marine comprise abundant microbial biodiversity. Yet, conventional or standard method cultural procedures could reveal < 1% of all microbiota due to limited capacity of the microorganismal growth under laboratory condition. Presently, the other > 99% of the microbiota could be revealed by culture-independent approach known metagenomics. This research utilized culture-dependent and culture-independent methods (metagenomics combined with pyrotagged 16S rDNA sequencing) to attain a complete picture of the true prokaryotic diversity of this environment, and better understand the ecosystem of bacteria and archaea in the southeast Andaman Sea off the west coast of Thailand. The seawater samples of two different distances from the Andaman coast: 08°56.004'N 98°05.857'E (PT) at 30-meter depth and 08°51.096'N 97°31.207'E (TC) on the surface and at 30, 100, 150-meter depth were investigated. The PT and TC sites had the seafloor depths of approximately 35 meters and 155 meters, respectively. The latter represents the farther seashore distance sample. The culture-dependent and culture-independent data demonstrated that the culture-dependent method provided far fewer number of isolates than by the culture-independent method, which could detect species that were missed by plating and also highlighted several uncultured bacteria. Subsequently, this thesis helped better understand the aquatic bacterial and archaeal population structures, and their metabolic potentials at PT and TC.

Department:	Microbiology	Student's Signature	....
Field of Study:	Industrial Microbiology	Advisor's Signature	....
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ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

μl	Microliter
μm	Micrometer
bp	Base pair
CFU	Colony forming unit
EDTA	Ethylenediaminetetraacetic acid
g	Gram
hr	Hour
kb	Kilobase pair
Km	Kilometer
l	Liter
min	Minute
mL	Milliliter
NGS	Next-generation sequencing
$^{\circ}$C	Degree Celsius
PCR	Polymerase chain reaction
rpm	Revolution per minute
rDNA	Ribosomal deoxyribonucleic acid
UV	Ultraviolet

CHAPER I

INTRODUCION

1.1 Background

Biological diversity means the variability among living organisms from all resources, including diversity within species and between species, and their relative frequencies. Diversity infers similarities and differences in species compositions and species richness, of which they are part of the complex ecology (Kratochwil, 1999). When assessing biological systems, all microbial and organismal community structures must be considered.

Microorganisms are tiny living things unseen by naked eyes, but are ubiquitous and comprise a majority of species on earth. They have a collective mass greater than all on the planet. They play major roles in diverse functional systems in various ecosystems. Even though we cannot see them, they have a huge impact on us and our environments: fresh water, soil, lakes, open oceans, air and even inside and on human body (Button et al., 1993; Watve et al., 2000; Connon et al., 2002; Tringe et al., 2005; Steen et al., 2010). In fact, we couldn't live without them. Microbes shaped the Earth and continuously changed the environmental conditions by complex interaction with other species they live with. They can also adapt to global environmental changes, and were suggested to help other organisms adapt to the changes better (Pavlov and Ehrenberg, 2013; Damkiaer et al., 2013). Microorganisms thereby represent another key to understand the complex, entire ecosystems.

Open oceans comprise the largest ecosystem on Earth. Due to their large volume and vast area coverage, they serve the primary food source on Earth, especially bottom-living organisms. They represent a main of the ocean food web. It is clear that the marine environment is so enormous that microbial biodiversity remains largely unstudied. Marine microbes are also sources for many biotechnologically products. Yet, they play a crucial role in decomposition of organic matter and cycling of nutrients.

The diversity of aquatic bacteria was found varying, according to biotic and abiotic factors: seasons, sea level, climate, hydrography, sunlight, salinity, oxygen

concentration, temperature, and availability of limiting inorganic substances. Studies showed that microbial structural diversity and activity vary in soils and aquatic systems, and upon environmental changes. For instances, the enrichment of ammonia-oxidizing bacteria response to nitrogen deposition were associated with the higher potential for nitrification. This demonstrates free-living soil bacteria community structure were closely related with nitrogen and carbon dioxide levels (Zak et al., 2000; Horz et al., 2004; Bowatte et al., 2008; Liu et al., 2009). Moreover, the population growth rates and relative abundance of bacteria were found associated with nature fruitfulness (Van der Gucht et al., 2007). All these recent findings were discovered by culture-independent experiments.

Unlike traditional cultivation methods that could assess only 1% of all microorganisms (Amann et al., 1990; Ferrari, 2005; Giovannoni and Stingl, 2005), recent studies utilized culture-independent approach and found more than 99% of microorganisms in nature (Amann et al., 1990; Fuhrman and Campbell, 1998; Schloss and Handelsman, 2008). The reasons are because culture-independent methods do not depend on bacterial growth and culture media. Therefore, the method is believed to provide and unbiased way to disclose actual microbiota in the sample. Recently, metagenomics and metatranscriptomics have been popular used and in combination with next generation sequencing (NGS). These methods allow scientists to directly analyze natural microbial communities, including their genes, metabolites and their interactions in the ecosystem. Yet, for microbial diversity analysis, metagenomic derived 16S rDNA is often preferred, since the greater species sequencing coverage is gained when only an amplicon is sequencing interested instead of an entire genome of each species in the environment (Acinas et al, 2004). Beside the 16s rDNA is considered a 'gold standard' for prokaryotic species identification. The use of NGS has increases the power of sequencing, allowing 16S rDNA sequence databases sufficient to represent hopefully the true microbiota at speed (Tringe and Hugenholtz, 2008; Baker 2010). To date, the microbiota databases for several global ocean sites have been revealed. Studied areas include Indian Ocean, North Sea, Baltic Sea, Sargasso Sea, Pacific and Indian Oceans. These include freshwater and marine aquatic sites. Still the aquatic microbial database for Thai

maritime zone remains very limited. No microbial database of the Andaman Sea, Thailand, is ever available. Hence, this study investigated prokaryotic diversity in the Andaman Sea, Thailand, by culture-dependent and culture-independent methods. This is for the first time to attain the database of the microbial structural diversity and associated metabolic potentials based on metagenomic derived 16S rDNA sequencing, representing the seawater samples at 30-meter depth of 15 kilometers from the Andaman coast (PT site: 98o05.857'E) and at <5, 30, 100, and 150-meter depth of 52 kilometers from the Andaman coast (TC site: 97o31.207'E). PT and TC sites occupy the seafloor depths of approximately 35-meter and 155-meter, respectively. The latter represents the farther seashore distance sample. Together, this sample collection also allowed comparison of prokaryotic structures associated with seashore distances (PT 30-meter vs. TC 30-meter), and sea depth (TC 0-meter vs. TC 30-meter vs. TC 100-meter vs. TC 150-meter). Different offshore distances are differentially polluted by human activities, and differentially affected by water runoff of the mangrove forests of Thai shores. Different sea levels involve different amount of sunlight and stratified water. Moreover, the study compared the PT and TC database against those belonged 75 other global ocean sites to help better understand the global ocean, microbial ecosystem and the potential metabolic network along the regions.

1.2 Objectives

1. To study bacterial diversity by culture-dependent and culture-independent (metagenomics) methods at different offshore distances and sea depths in the Andaman Sea, Thailand

2. To obtain bacteria database and better understand the ecosystem of bacteria in the Andaman Sea, Thailand

1.3 Hypothesis

A combination of traditional culture and metagenomic-derived 16S rDNA sequencing methods serve a powerful tool to attain bacterial databases of PT and TC sites. And because different offshore distances and sea levels could contribute to different microbial profiles, those culture-dependent and culture-independent results representing these microbial profiles should be different.

CHAPER II

LITERATURE REVIEW

2.1 Andaman Sea

Andaman Sea located in the Indian Ocean is biologically rich in species diversity and species richness. Indian Ocean is known one of the major aquatic biological abundance on Earth. It is also one of the 25 hot spots of highly endangered eco-regions of the world (NIO 2008). The hydrography in the southeast Andaman Sea is dynamic, comprising stratified inflow freshwater from Irrawaddy River, India, and salt nutrient-rich water runoff from the mangrove forests of Thai shores (Satapoomin et al., 2004).

Thailand is located in a biodiversity hotspot region called "Indo-Burma", which includes Vietnam, Thailand, Cambodia, Laos, Myanmar, and portions of eastern India and southern China. The hotspot includes the coast extending thousands of miles along the South China Sea, Gulf of Thailand, Andaman Sea, and the Bay of Bengal (www.enviroliteracy.org/article.php/498.html). The west coast of Thailand faces the southeast of Andaman Sea is a mangrove forest. This enhances the biodiversity of the southeast Andaman Sea, Thailand.

2.2 Biodiversity

Biodiversity is a fundamental factor in healthy ecosystems of nature and human life. Biodiversity is complex covering many organismal levels, from microbes that cannot be seen by naked eyes, to organism and plants. Species composition and species richness are considered in biodiversity. Every species, neither how small the size nor how few the member is, all share certain roles in ecosystem (Shah, 2011). To keep any ecosystem in healthy balance, appropriate compositions and relative frequencies of species are essential.
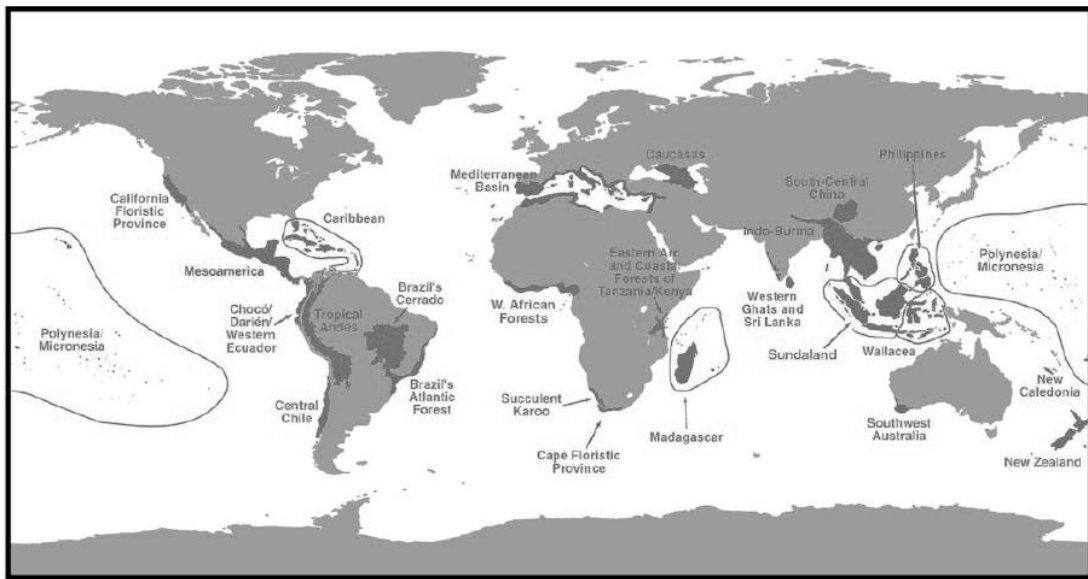
Figure 2.1 Edited from: World hotspot areas as circled (Myers et al., 2000)

Bacteria are ubiquitous, and their biodiversity also affect the abundance of other species, as well as the food web and organic/inorganic cycling of the system. Bacteria are so widespread, they may be found on the tops of mountains, the bottom of the deepest oceans, in the guts of animals, and even in the frozen rocks and ice of Antarctica. Bacteria play important roles in the global ecosystem. The ecosystem, both on land and in the water, depends heavily upon the activity of bacteria. The cycling of nutrients such as carbon, nitrogen, and sulfur. They act as decomposers at the end of food chains and food webs. If there were no bacteria, the environment would have been polluted and full of harmful microorganisms (Duarte et al., 2009; Van, 2011). Interestingly, our bodies frequently live in symbiosis with these bacteria: for example, bacteria such as *E.coli* in our gut synthesize Vitamin K, a vitamin important for blood clotting. Individuals taking certain antibiotics may experience a nearly 3/4 reduction in Vitamin K production (Hao and Lee, 2004).

Factors affecting bacterial diversity include biotic and abiotic factors. Biotic factors are all living things in the environment that interact among one another, either directly or indirectly, and affect the ecosystem and the uniqueness of the habitat area. Abiotic factors, the non-living components, include the physical and chemical components in the environment. Some of the significant abiotic factors

include dissolved oxygen, toxic metal contamination, nitrogen content, temperature, sunlight, pressures, salinity, nutrient availability, and alkalinity. Every factor may cause the change of bacterial diversity in nature or in the experiment (Torsvik et al., 1998; Theron and Cloete, 2000).

2.3 Bacterial diversity in aquatic environments

Water covers 75% of the earth's surface, the largest habitat space for living organisms both by volume and area coverage (Bernan, 1997; Das et al., 2006; Yadav, 2013). The world ocean has a total coastline of 312,000 km (193,000 miles) and a volume of 137 x 10^6 km^3. Albeit diverse aquatic environments throughout the world, microorganisms were present. Aquatic bacteria thrive not only in the surface waters, but also in the lower and abyssal depths, not only coastal but also the offshore and open ocean, and not only blue water but also coral reefs and hot thermal vents floor (Qasim, 1999). Aquatic bacteria also thrive for marine vs. freshwater habitats, and for different water conditions: salinity, pH, climate, tidal movement, sunlight, season, dissolved oxygen, and organic/inorganic matters. Natural disaster and manmade activities also affect aquatic bacterial diversity. Benefits of aquatic bacteria were such as being potential resources for important biotechnological compounds and their bioremediation capabilities. Bacterial play crucial roles in decomposition of organic matter and cycling of nutrients, the major food source for bottom-living organisms (Das et al., 2006).

For marine bacteria, the majority was found gram-negative bacteria, because gram-negative cell wall could better adapt to survive in alkaline-different marine environments (Zobell, 1946; Gonzalez and Moran, 1997; Taylor et al., 2007; Menezes et al., 2009; Wilson et al., 2010). Bacteria have rapid developmental life cycles, allowing them to adapt more quickly to changing environments and potentially to become widespread (Poole et al., 2003; Aller et al., 2008) found that archaea tend to have the lower diversity than the bacteria in the same environment, since archaea could use resources of the environment in a more restrictive way than the bacteria. With advances in culture-independent studies, the bacterial communities of deep

water were found even more complex than previously reported (Mitchell et al., 2006). Further, the marine bacterial diversity was found fluctuate among seasons in a repeatedly year by year, and the bacterial diversity was different across the globe along different latitudinal gradients, unlike that seen for macro-organisms (Joshua et al., 2013).

For freshwater bacteria, compared to marine environments, freshwater has received less attention, despite the fact that freshwater is a limited resource and has greater effects on humans (Zinger et al. 2011). The community belong the freshwater sediment was more evenly distributed, while the marine sediment was more biased, with a dominance of *Gammaproteobacteria* and *Deltaproteobacteria* (Wang et al. 2012). It has been reported that the microbial community structure is driven mainly by salinity at the global scale (Lozupone and Knight, 2007). Nevertheless, recent meta-analyses showed that there was also a high bacterial and archaeal diversity in inland freshwater environments (Auguet et al. 2010; Barber and Casamayor, 2010).

2.4 Culture-dependent methods

Previously, bacterial biodiversity was studied by our well-known and long adopted culture-dependent approach that highly depends on the medium and incubation condition used for propagation and isolation (Janssen, 2006). This method was commonly used for the study of the structure of the bacterial communities in various environments. Before the advent of DNA sequencing, bacteria were classified based on their shapes and biochemical properties. Bacteria were enumerated for their natural abundance based on plate counts, serial dilutions, and phase-contrast microscopy. While the latter method is not depend on the culture medium and incubation condition, the others remain related to the selectivity of the nutrient media and culture conditions which lead to favoring only a fraction of the inhabiting bacterial community (Al-Mailem et al., 2014). This thereby yielded estimates of 0.1% of the actual numbers of the true bacterial biomass, and drove advances in microbiology (Amann et al., 1990; Jannasch and Jones, 1959).

To increase the chances of previously uncultured bacteria, media with different compositions and nutrient concentrations should be used (Sipkema et al., 2011). Such cultivation-based approaches have previously been used to isolate aquatic bacteria with antimicrobial activities such as *Crambe crambe* (marine sponge) (Muscholl-Silberhorn et al., 2008; Kennedy et al., 2010; O'Halloran et al., 2011), DNA-DNA hybridization technique for estuarine bacterioplankton (Kisand and Wikner, 2003), study diversity within the obligate marine actinomycete by inoculate onto medium plus natural seawater and antifungal agent cycloheximide was adapted to reduce the number of unwanted bacteria. (Mincer et al., 2005). The cultivation-dependent strategy may therefore underestimate the richness in samples, which is overcome by culture-independent methods.

2.5 Culture-independent methods

Recently, metagenomics arises as the technique independent of culture, and are accepted by scientist the more powerful way for microbial database obtainment. Metagenomics, a term first dubbed by Handelsman in 1998 as analyze the genomes of microbial communities recovered directly from the environment. Most naturally occurring bacteria cannot be cultured and therefore cannot be analyzed by traditional methods. Subsequently, aquatic sample can be metagenomic analyzed by first extraction of total metagenomes, followed by shotgun sequencing, directed NGS, or targeted NGS. The latter method comprises library construction of specific amplicons to target NGS to give huge coverage of targeted amplicons to ensure sufficient microbiota coverage upon NGS. For analyzing prokaryotic (bacteria and archaea) diversity, targeted 16S rDNA is preferred as the gene is a gold standard for prokaryotic species identification. The 16S rDNA is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions (V) that are frequently used in phylogenetic clustering and species classifications are V3 and V4 (Claesson and O'Toole, 2010; Vasileiadis et al., 2012). For many years, 'metagenomics' was often connected with the analysis of environmental samples. Now metagenomics become broadly connected with human

health studies and many applications (Qin et al., 2010; Miller et al., 2013). Only ~0.1% of microorganisms are cultivable, therefore metagenomic researches dramatically broaden our knowledge of environments.

2.6 Next-generation sequencing (NGS)

First generation DNA Sequencing, or Sanger sequencing, approach relies on DNA replication to identify which of the four A, T, C, or G nucleotide bases that make up the genetic code were at each position in a fragment of DNA. Researchers could read almost a thousand nucleotides of DNA sequence in a reaction. This sequencing requires a specific primer to start the read at a specific location along the DNA template, and record the different labels for each nucleotide within the sequence. So, this technology still required too much DNA, reagents, effort, and times to meet the needs of genomic researchers. Besides, since completion of the first human genome sequence by shotgun sequencing in 2003 (Singh, 2013), the demand for the cheaper and faster sequencing method had driven the development of high-throughput sequencing (next-generation sequencing) technologies that parallelize the sequencing process, producing thousands or millions of sequences concurrently. High-throughput sequencing technologies are intended to lower the cost of DNA sequencing beyond what is possible with standard dye-terminator methods.

As a result, new technique that enabled sequencing of multiple DNA strands from less material, hence cheaper cost, arose in the early 2000s. These platforms perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced simultaneously. Massively parallel sequencing technology facilitates high-throughput sequencing, which allows an entire genome to be sequenced in a day. This technique is known second-generation sequencing method, or next-generation sequencing (NGS). Since the introduction of NGS technology, a major transformation in the way scientists extract genetic information from biological system has changed, and caused considerable advancements in a wide range of scientific fields: a more complete picture of biological things and biological systems from the genetic view.

NGS platforms include massively parallel signature sequencing (MPSS), this method incorporated a parallelized, adapter/ligation-mediated, bead-based sequencing technology. Polony sequencing combined an in vitro paired-tag library with emulsion PCR, an automated microscope, and ligation-based sequencing chemistry to sequence. Illumina (Solexa) sequencing method based on reversible dye-terminators technology, and engineered polymerases. SOLiD sequencing, this sequencing by ligation of DNA ligase for matching sequences results in a signal of the nucleotide at that position. Ion torrent semiconductor sequencing, this method of sequencing is based on the detection of hydrogen ions that are released during the polymerization of DNA, DNA nanoball sequencing, use rolling circle replication to amplify small fragments of genomic DNA into DNA nanoballs then unchained sequencing to determine the nucleotide sequence. Heliscope single molecule sequencing, use DNA fragments with added poly-A tail adapters which are attached to the flow cell surface. Single molecule real time (SMRT) sequencing, performed with use of unmodified polymerase and fluorescently labeled nucleotides which is detached from the nucleotide at its incorporation into the DNA strand. And 454 pyrosequencing uses luciferase to generate light for detection of the individual nucleotides added to the nascent DNA, and the combined data are used to generate sequence read-outs. (Berglund et al., 2011; Gogol-Doring et al., 2012).

2.7 Pyrosequencing

Pål Nyrén tried to improve traditional sequencing method, while he was a post-doctoral. He found photosynthetic bacterium (*Rhodospirillum rubrum*) that has bioluminescent enzymes that can utilize pyrophosphate to drive luminescence. Therefore, pyrosequencing was developed (Nyrén, 2006) (Figure 2.2).

Pyrosequencing is a DNA sequencing technique that detects the release of pyrophosphate (PPi) during DNA synthesis. Four different nucleotides (A, T, G, C) are added stepwise to the immobilized primed DNA template via a polymerase enzyme. As a result, a pyrophosphate (PPi) molecule(s) is released during nucleotide

incorporation by polymerase, and subsequently converted to ATP by ATP sulfurylase which provides the energy to luciferase to oxidize luciferin and generate light. Visible light is generated that is proportional to the number of incorporated nucleotides during which a luciferin molecule is oxidized. After each nucleotide addition, the nucleotides are continuously degraded by nucleotide degrading enzyme allowing addition of subsequent nucleotide. Because the added nucleotide is known, the sequence of the template can be determined (Figure 2.3). The nucleic acid molecule can be either RNA or DNA (Ronaghi et al., 1996, 1998; Ronaghi, 2001).

Pyrotagged pyrosequencing has been used in many metagenomic studies. For examples, Seong et al. (2010) found barcoded pyrosequencing approach can be a powerful tool for characterizing the microbiota in fermented food ecosystems compared with classical molecular ecological approaches, such as PCR-DGGE. Henk et al. (2011) applied massive parallel 16S rRNA gene tag sequencing to identify the diversity and biochemical complexity of coastal microbial mats at the northwestern part of the North Sea coast of the Dutch barrier island Schiermonnikoog.

$$(NA)_n + \text{Nucleotide} \xrightarrow{\text{Polymerase}} (NA)_{n+1} + PPi$$

$$PPi + APS \xrightarrow{\text{ATP sulfurylase}} ATP + SO_4^{-2}$$

$$ATP + \text{Luciferin} + O_2 \xrightarrow{\text{Polymerase}} AMP + PPi + \text{Oxyluciferin} + CO_2 + \text{Light}$$
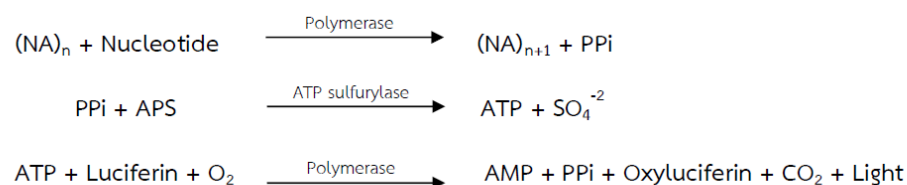
Figure 2.2 Principle of pyrosequencing reaction
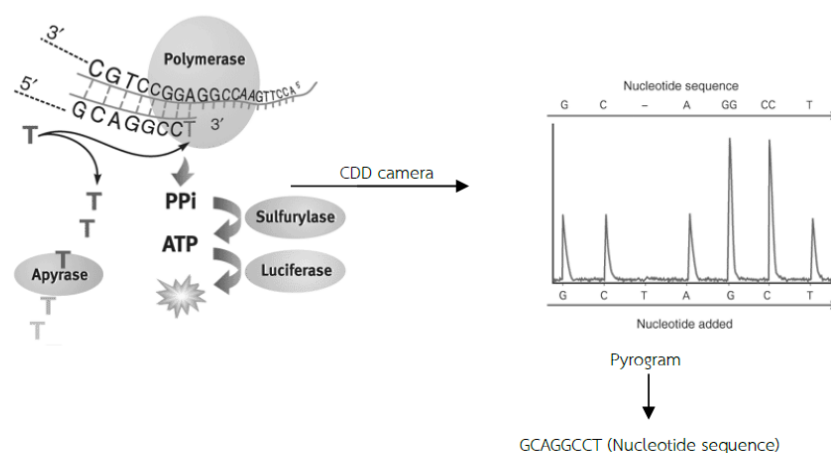
Figure 2.3 Example of pyrosequencing output. Double peak heights indicate incorporations of two nucleotides in a row

CHAPTER III

METERIALS AND METHODS

3.1 Instruments

Autoclave: Kokusan, Shizuoka, Japan

Balances: L2200P and A200S, Sartorius, New York, USA

Bench-top centrifuge: Mikro20, Hettich, Germany

Biosafety PCR Cabinet

Conductivity Meter: Mettler-Toledo, Bangkok, Thailand

Freezer 4°C: MISUBISHI, Tokyo, Japan

Deep freezer -20°C: SANYO, Osaka, Japan

Deep freezer -80°C: SANYO, Osaka, Japan

DNA Thermo Cycle: G-STORM, Scientific Promotion Co., Ltd., Bangkok, Thailand

Agarose Gel Electrophoresis System: GE-100, Hangzhou Bioer Technology CO., LTD.
       Hangzhou, China

Gel Documentation: Gel DOC 2000^{TM}, Bio-Rad Laboratories, California, USA

Genome Sequencer-FLX: Roche, Burgess Hill, UK

Laminar flow: BossTech, Hampshire, UK

Makrolon (polycarbonate): Polypropylene filter support, Goettingen, German

Microcentrifuge: Hettich, Massachusetts, USA; and WiseSpin CF-10, DAIHAN Scientific,
       Seoul, Korea

Microscope: Olympus, USA

Nanodrop spectrophotometer: nanodrop2000, Thermo Scientific, New York, UK

Refrigerated centrifuge: JR-21, Beckman Instrument Inc., USA

Rotary vacuum evaporation: EYELA, Japan

Salinity Refractometer: RHS-35ATC Index Instruments Limited, Cambridgeshire, UK

Supercomputer: Pro 3330 MT, HP, California, USA

Thermo-block: TDB-120, BIOSAN, Riga, Latvia

UV transilluminator: HANGZHOU BIOER TECHNOLOGY CO., LTD., Hangzhou, China

Vortex mixer: VM-10, DAIHAN Scientific, Seoul, Korea

pH meter

3.2 Chemicals

Double distilled water

0.5xTBE buffer

1.0xTBE buffer

70% ethanol

0.85% NaCl

Agarose powder: AMRESCO®, Ohio, USA

Ethidium Bromide: AMRESCO®, Ohio, USA

GeneRuler™100 bp Plus DNA Ladder: Invitrogen, New York, USA)

1 kb Plus DNA ladder: Invitrogen, USA

Glycerol

Isopropanol: MERCK, Darmstadt, Germany

3.3 Supplies

Microcentrifuge tubes: Bioline, Massachusetts, USA

Micropipette: Labnet International, Inc., New Jersey, USA

Petri Dish: Thermo Fisher Scientific, Inc., New York, USA

Blade

Cheesecloth

Forceps

Glassware

Laboratory bottles

0.22 micron membrane filters: Whatman International, UK

Scissors

Stainless spoons and spatula

3.4 Kits

Metagenomic DNA Isolation Kit for Water, EPICENTRE, Wisconsin, USA

GF-1 Bacterial DNA Extraction Kit: Vivantis, California, USA

EmeraldAmp® GT PCR Master Mix: TAKARA BIO INC., Shiga, Japan

PureLink® Quick Gel Extraction Kit: Invitrogen, New York, USA

3.5 Sample collection

Water samples were collected independently three times each at least 20 L from the Andaman Sea into a separated sterile glass container on 10 January 2012 (PT site) 7 May 2012 and (TC site), around 02:00 - 05:00 pm (Figure 3.1). On-site measurement of water properties included temperature, salinity, conductivity, dissolved oxygen and pH. Samples collection and on-site measurement of water properties were done by Dr. Doonyapong Wongsawaeng (Faculty of Engineering, Chulalongkorn University). The PT and TC sites share similar latitude but longitudes by about 37 kilometers from the Andaman coast, hence representing one good comparative pair of study for marine prokaryotic ecology due to seashore distances. Additionally, the water samples at TC were collected at surface, 30-m, 100-m and 150-m levels; hence representing good comparative sample for prokaryotic diversity based on sea levels. All collected samples were stored in 4°C and were processed for the next step within 14 days. Note each 20 L seawater sample was processed separately for DNA extraction and amplicon library construction, and pooled upon NGS. Each 0.5 L seawater sample was processed for culture experiments.
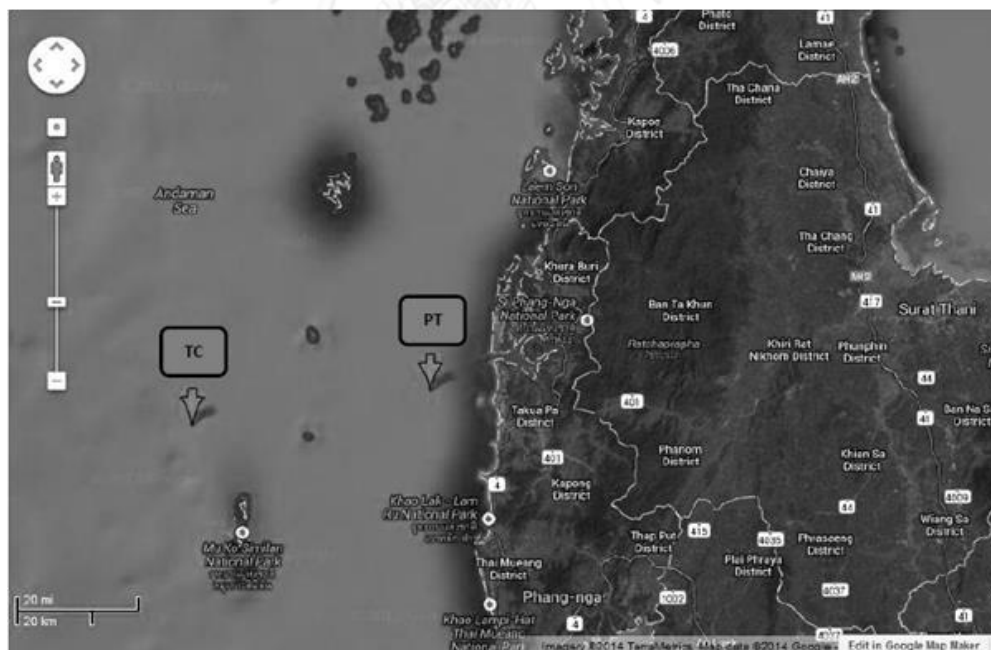
Figure 3.1 Oceanographic positions of PT and TC sites relative to Andaman coast, Thailand. The map was from Google Satellite Map, retrieved on May 2014.

3.6 Metagenomic DNA extraction and DNA quality examination

Pour each water sample (4 L) through four-layered sterile cheesecloth to remove large-size organisms of ≥ 30 μm and debris. Then, pour through a sterile 0.22 μm filter membrane (Merck Millipore, Massachusetts, USA) to capture only microorganisms and debris of ≥ 0.22 μm in sizes (Figure 3.2). Total nucleic acids were isolated by Metagenomic DNA Isolation Kit for Water (Epicentre, Wisconsin, USA), following the manufacturer's instructions. Firstly, wash off the microbes trapped on the membrane using Filter Wash Buffer and transfer the cell suspension to a clean microcentrifuge tube, centrifuge, and discard the supernatant. Resuspend the cell pellet in TE Buffer, and then add Ready-Lyse Lysozyme Solution and RNase A to the cell suspension. After incubate at 37℃ for 30 minutes add Meta-Lysis Solution (2x) and Proteinase K. Incubate at 65℃ for 15 minutes. Cool down on ice for 3-5 minutes. Add MPC Protein Precipitation Reagent Next, transfer the supernatant to a clean microcentrifuge tube and discard the pellet. Clean the pellet by 70% ethanol, air-dry the pellet at room temperature. Finally, resuspend the DNA pellet in 50 μl of TE Buffer (Baker et al., 2011)(Appendix F). The isolated fragments should be randomly sheared and appeared approximately 40 kb. Analysis of DNA concentration and quality by agarose gel electrophoresis and Nanodrop spectrophotometer at 260 and 280 nm.

Figure 3.2 Filtration systems consisted of four-layered sterile cheesecloth (left) and 0.22 μm filter membrane (right)

3.7 Cultivation of bacteria

Bacteria were cultured by direct plate count and concentrate plate count. For direct plate count, seawater were 1/10 serially diluted with 0.85% NaCl, and 0.1 mL of each diluent (10^{0}-10^{-4}) as inoculated in duplicate onto solid salt nutrient agar (SN: 2.7% NaCl, 0.3% beef extract, 0.5% peptone, 1.5% agar, pH ~6.8) (Satpute et al. 2008; Kakizaki et al. 2008). For concentrate plate count, 500 mL of seawater were poured through four-layered sterile cheesecloth and filtered through 0.22 µm filter membrane. The microorganisms were rinsed from the filtered membrane by 0.85% NaCl, 1/10 serial dilution was performed (10^{0}-10^{-4}), and 0.1 mL of each diluent were inoculated in replicates onto SN agar. All plates were incubated at 28-30°C for 1-5 days. Plates with 30-300 colonies were counted for colony forming unit (CFU). Colonies with unique morphologies were streaked for isolate colonies, and were determined species by morphology, gram strain, and colony 16S rRNA gene PCR sequencing.

3.8 Colony PCR of 16S ribosomal RNA gene and sequencing

We amplified and sequenced the 16S rRNA gene from each isolate colony. The single colony was re-suspended in 0.85% NaCl, boiled at 95°C for 10 minutes to allow membrane Lysis, let cool on ice 3-5 minutes and brief-centrifuge for 5 seconds, and then gently mix by pipette (Appendix G). Then transfer 8 µl into PCR mixture. 16S rRNA gene PCR was performed using universal prokaryotic, 16S rRNA gene primers 338F and 786R (Table 3.1). For some clone that no PCR result was shown, we used GF-1 Bacterial DNA Extraction Kit to extract bacterial DNA from each isolated colony. Then use the extracted DNA from the kit to do PCR with the 16S rRNA gene primers 338F and 786R. Thermocycling parameters were as follows: initial activation of the DNA-Polymerase for 4 min at 95°C, followed by 30 cycles of 45 sec denaturation at 94°C, annealing for 50 sec at 50°C and extension for 1 min 30 sec at 72°C (Somboonna et al., 2012). After amplification, the 459 bp size product was confirmed by agarose gel electrophoresis using 1.5% agarose gels. Gel was run at 100 V constant for 30 minutes. The single band of colony PCR was commercially Sanger-sequenced

using ABI3730XL (Macrogen Inc., Korea). Species were identified by BLASTN with 10^{-4} E-value cut off, unless stated.

Table 3.1 Oligonucleotide primers

Primer	Forward (5'→3')	Reverse (3'→5')
16S rRNA	ACTCCTACGGGAGGCAGCAG	CTACCAGGGTATCTAATC
PT-30M	*ACATCGAG*ACTCCTACGGGAGGCAGCAG	*ACATCGAG*CTACCAGGGTATCTAATC
TC-surface	*TCTCTGTG*ACTCCTACGGGAGGCAGCAG	*TCTCTGTG*CTACCAGGGTATCTAATC
TC-30M	*TCTACTCG*ACTCCTACGGGAGGCAGCAG	*TCTACTCG*CTACCAGGGT ATCTAATC
TC-100M	*TAGTAGCGA*ACTCCTACGGGAGGCAGCAG	*TAGTAGCG*CTACCAGGGTATCTAATC
TC-150M	*AGACGACG*ACTCCTACGGGAGGCAGCAG	*AGACGACG*CTACCAGGGTATCTAATC

* *Italic sequence* represents pyrotagged sequence.

3.9 Pyrotagged 16S rRNA gene amplification

Primers for pyrotagged 16S rDNA were listed in Table 3.1. Universal prokaryotic 338F and 786R target the 16S rDNA at variable V3 and V4 regions. 8 nucleotides pyrotagged sequence was for labeling for pyrosequencing. A 50 µl PCR reaction included 1 EmeraldAmp® GT PCR Master Mix (TAKARA, Shiga, Japan), each primer 0.3 µM, and 100 ng metagenomic DNA. The thermal cycling profiles were 4 min at 95℃ followed by 30-35 cycles of 94℃ 45 s, 50℃ 5 s, 72 ℃ 1:30 min, and a final extension at 72℃ 10 min. The amplicon should had 466 nucleotides in length according to *Escherichia coli* strain MYL-4 (GenBank Accession No. HQ738475). The PCR products can be stored short-term at -20℃.

3.10 Gel purification and pyrosequencing of PT and TC pyrotagged 16S rDNA fragments

The 466 bp amplicon was examined using 1.75% agarose gel electrophoresis. Gel was run at 100 V for 20 min and 50 V for 30 min, and visualized under UV transilluminator. The 466 bp band was cut by clean blades, and purified using

PureLink® Quick Gel Extraction Kit (Invitrogen, New York, USA). Firstly, cut the gel piece containing the DNA fragment of interest into clean microcentrifuge tube. Incubate the gel slice and Gel Solubilization Buffer at 60°C. Pipet the dissolved gel piece into a Quick Gel Extraction Column. Centrifuge, discard the flow-through and replace into the Wash Tube. Add Wash Buffer containing ethanol to clean the gel. Then, centrifuge to remove any residual Wash Buffer. Place into a Recovery Tube. Add Elution Buffer to elude DNA, incubate the column at room temperature. The Recovery Tube contains the purified DNA. Store the purified DNA (Appendix H). Pooled 116 ng each of TC-pyrotagged 16S rDNA fragments and 60 ng each of PT-pyrotagged 16S rDNA fragments were pooled and pyrosequenced on an eight-lane Picotiter plate. Pyrosequencing was performed using the 454 GS FLX system (Roche, Branford, CT) by in-house NGS facilities at National Center for Genetic Engineering and Biotechnology, according to manufacturer's protocols.

3.11 16S rRNA gene sequence analysis

The sequences of less than 50 nucleotides in length were removed. 16S rRNA gene sequences were categorized PT and TC, and for TC what sample depth, based on the appended pyrotag sequences. An annotated read infers the read with $\leq 10^{-4}$ E-values (except TC-30M used $\leq 10^{-2}$ E-values) by BLASTN (Altschul et al., 1997) against NCBI non-redundant (Sayers et al., 2012), RDP (Maidak et al., 2001) and Greengenes (DeSantis *et al.*, 2006; McDonald et al., 2012) database. Domain and phylum composition were visualized by mg-RAST (Overbeek et al., 2005; Meyer et al., 2008). Genus and species compositions was computed in excel and Heatmap. Sequence alignments were performed by PHYLIP 3.69 and neighbor-joining tree was constructed (Felsenstein 1993; Xiao et al., 2013). For functional subsystem determination, sequences were generated by mg-RAST (Jeffries et al., 2011). For Global Ocean Sampling (GOS) sites comparison, the GOS profiles were downloaded from https://portal.camera.calit2.net/gridsphere/gridsphere (Vener et al., 2004; Rusch et al., 2007; Yooseph et al., 2010). Tha Wang and Tham Phang profiles were from our lab database. Thetayc and Bray-Curtis indices were computed to prioritize population structure similarity among pairs of samples.

CHAPER IV

RESULTS

4.1 Water characteristics of PT and TC samples

At different seashore distances, PT-30M had lower dissolved oxygen (DO) and acidity (pH) than TC-30M. At TC but with different sea levels, temperature, DO and pH showed a decreasing trend as the sea level was deeper (Table 4.1). These differences in niche environmental conditions could affect the microbial biodiversity.

Table 4.1 Characteristics of water samples from PT and TC sites

Station name	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Latitude (N)	$08^{\circ}56.004'$	$08^{\circ}51.096'$	$08^{\circ}51.096'$	$08^{\circ}51.096'$	$08^{\circ}51.096'$
Longitude (E)	$098^{\circ}05.857'$	$097^{\circ}31.207'$	$097^{\circ}31.207'$	$097^{\circ}31.207'$	$097^{\circ}31.207'$
Sampling Depth (m)	30	5	30	100	150
Temperature ($^{\circ}$C)	27.80	N/A*	28.40	22.44	19.99
Conductivity (µS/cm)	52675	N/A	53357	48993	49510
Salinity (parts per thousand: ppt)	32.66	N/A	32.73	34.32	34.43
Dissolved oxygen (mg/L)	5.02	N/A	6.87	6.50	4.30
pH	7.79	N/A	8.04	7.80	7.80

*N/A represents data not available.

4.2 Cultured isolates of bacteria from TC site

Direct and concentrate plate count methods could cultivate, isolate and propagate bacteria from TC site. Both methods showed roughly equivalent colony number. Numbers of total colonies (CFUs/mL seawater) were displayed in table 4.2. The average CFUs/mL of TC-surface 4.04 x 10^3 CFUs/mL, TC-30M 6.46 x 10^3 CFUs/mL, TC-100M 1.79 x 10^4 CFUs/mL, and TC-150M 5.09 x 10^3 CFUs/mL.

Table 4.2 Bacteria diversity by culture-dependent methods

Sample	Cultivation method	Bacterial diversity			
		Number of total colonies (CFUs/mL seawater)		Number of distinct colony type	
		aerobe	anaerobe	aerobe	anaerobe
TC-surface	Direct plate count	4.9 x 10^3	1 x 10^3	6	4
	Concentrate plate count	6.2 x 10^3	0	1	0
TC-30M	Direct plate count	1.1 x 10^5	4.05 x 10^3	4	4
	Concentrate plate count	1.08 x 10^5	0	5	0
TC-100M	Direct plate count	3.65 x 10^3	3.05 x 10^4	2	2
	Concentrate plate count	3.45 x 10^4	2.85 x 10^3	2	3
TC-150M	Direct plate count	8.7 x 10^3	3.8 x 10^4	2	2
	Concentrate plate count	6.5 x 10^3	4.75 x 10^3	3	2

Each distinct morphological colony was recorded, gram stained, and colony PCRed and sequenced of the V3-V4 16S rRNA gene. Figure 4.1 showed 1.55% agarose gel electrophoresis confirmed a single product at 456 bp. Note when the amplicon showed more than a single band (i.e. 'a' and 'g' in Figure 4.1b), the band at 456 bp was excised, purified DNA from the gel, and sequenced. Sequencing results were in Table 4.3.

a. TC-surface
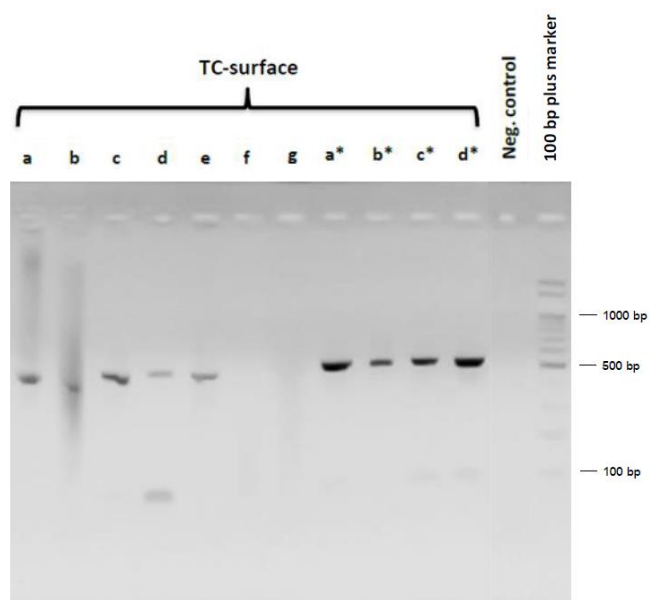
b. TC-30M
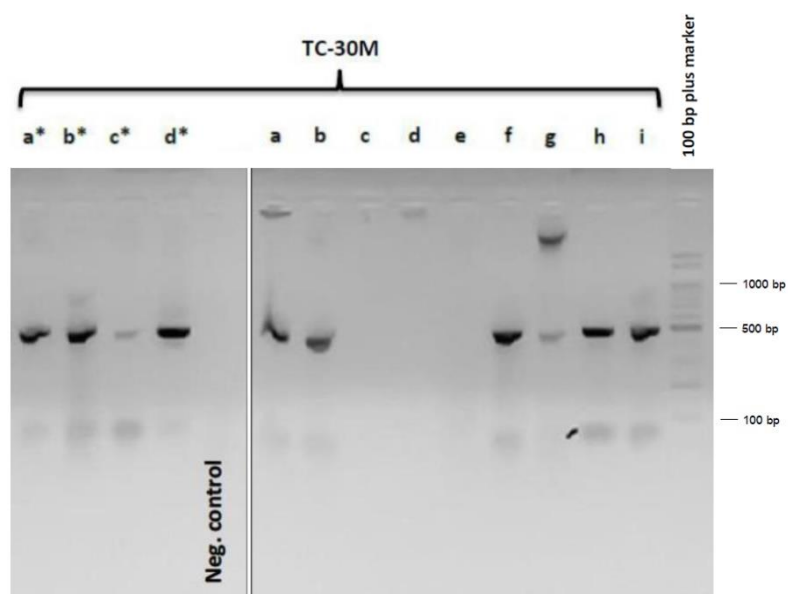
c. TC-100M

d. TC-150M
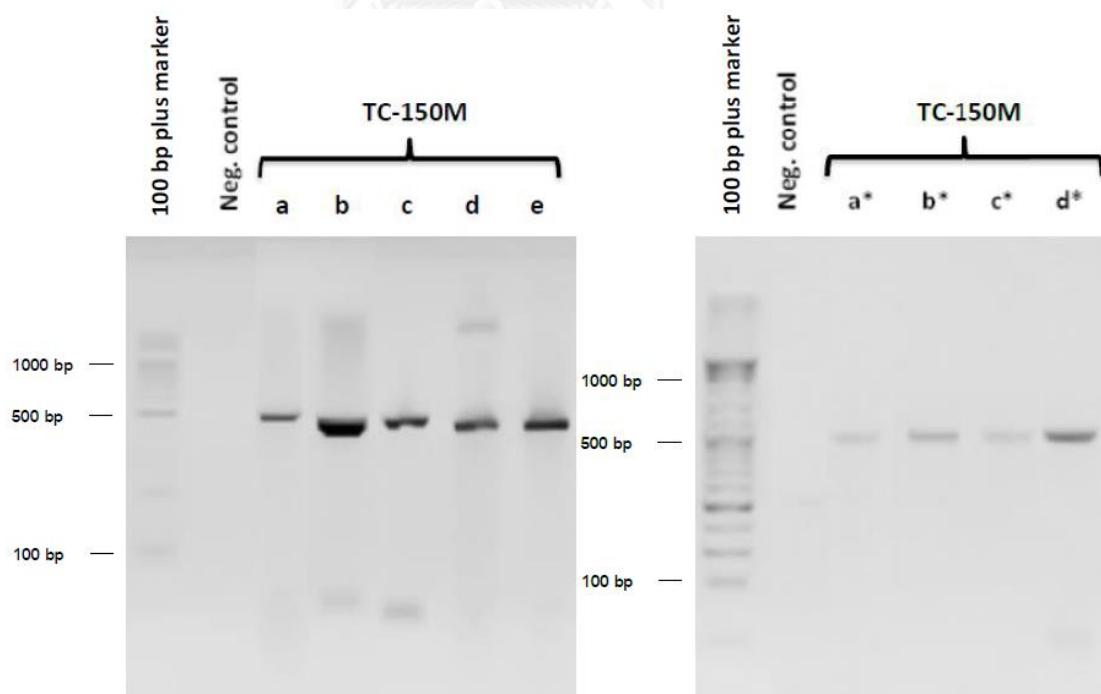
Figure 4.1 Colony PCR of 16S rDNA amplicons from TC-surface (a), TC-30M (b), TC-100M (c), and TC-150M (d). Each sample was loaded 10 μl.

For TC-surface, 7 total colonies were observed in aerobic and 4 colonies in anaerobic (Table 4.2). All 11 isolates were 16S rDNA sequenced, and were all annotated different species, except *Micrococcus* sp. TVS41 and *Micrococcus* sp. APP-12 could belong clonal variants of each other. 4 isolates matched strains from uncultured experiments. 1 isolate had no significant hit (Table 4.3a).

For TC-30M, 9 colonies were observed in aerobic, and 4 colonies were observed in anaerobic (Table 4.2). These 13 isolates colonies contained 2 belonged the same species (*Micrococcus luteus* B88), and 2 belonged the same species (*Paenibacillus* sp.). 5 isolates belonged uncultured experiments, given 3 isolates the species could not specified (Table 4.3b).
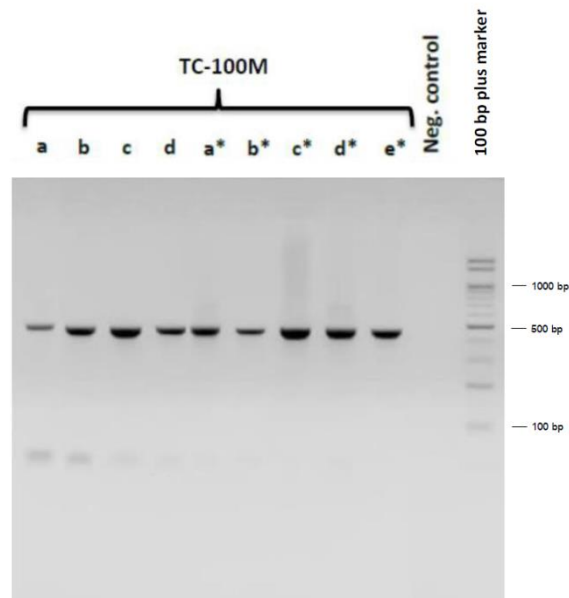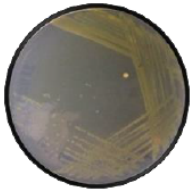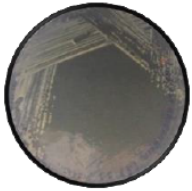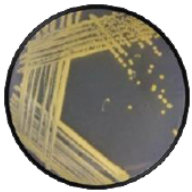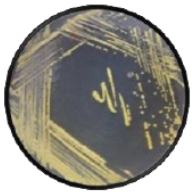
For TC-100M, 4 colonies were observed in aerobic, and 5 colonies were observed in anaerobic (Table 4.2). These 9 isolates colonies contained *Pseudoalteromonas lipolytica* ZR095, *Bacillus cereus* BcTNAU6, and *Pseudomonas fluorescens* SBW25. 3 isolates belonged uncultured experiments, given 3 isolates the species could not specified (Table 4.3c).
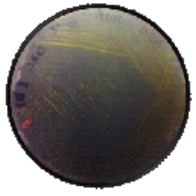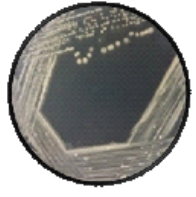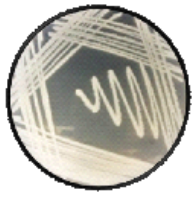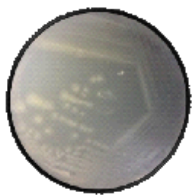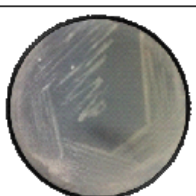
For TC-150M, 4 colonies were observed in aerobic, and 4 colonies were observed in anaerobic (Table 4.2). These 8 isolate colonies contained *Bacillus* sp. Q3 and *Roseinatronobacter* sp. LC05R24. 6 isolates belonged uncultured experiments (Table 4.3d).

Some genus can be found uniquely in some depth. For instances, *Halomonas* sp. and *Sphingomonas* sp. can be found uniquely in TC-surface. *Paenibacillus* sp., *Stenotrophomonas maltophilia*, and *Thermosipho melanesiensis* can be found uniquely in TC-30M. *Corynebacterium argentoratense*, *Idiomarina* sp., *Pseudoalteromonas lipolytica*, and *Pseudomonas fluorescens* can be found uniquely in TC-100M. *Roseinatronobacter* sp., *Thalassospira* sp. can be found uniquely in TC-150M. TC-surface and TC-30M shared same genus, for example, *Erythrobacter* sp., *Micrococcus* sp., *Staphylococcus* sp. can be found in both depths. *Bacillus* sp. can be found in both TC-100M and TC-150M (Table 4.3).
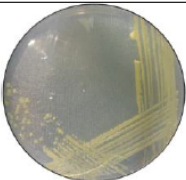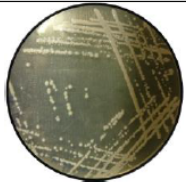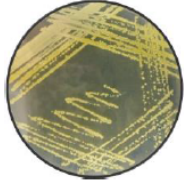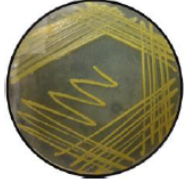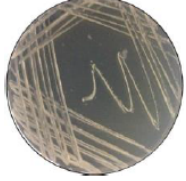
Table 4.3 Colony morphology, gram stain and 16s RNA gene species annotation of each bacteria isolates
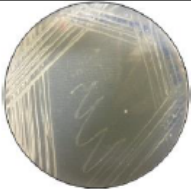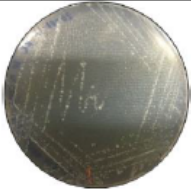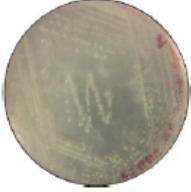
a. TC-surface

Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	yellow, circular, entire	Gram-negative	*Erythrobacter sp.* JC212	HG008905.1
	aerobe	no color, punctiform, entire	Gram-negative	Uncultured bacterium	AB745650.1
	aerobe	dark yellow, circular, entire	Gram-negative	*Sphingomonas sp.*	KF378753.1
	aerobe	light yellow, circular, entire	Gram-positive	*Micrococcus* sp. TVS41	KF142392.1
	aerobe	dark yellow, circular, entire	Gram-negative	*Micrococcus* sp. APP-12	KF705253.1
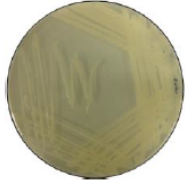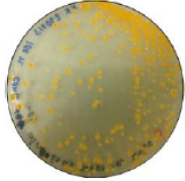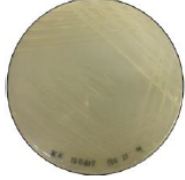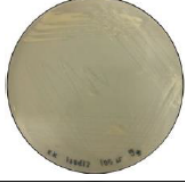
Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	dark yellow, punctiform, entire	Gram-negative	Uncultured marine bacterium	FM211112.1
	aerobe	cream, circular, entire	Gram-positive	no significant hit	-
	anaerobe	no color, circular, entire	Gram-positive	Uncultured *Staphylococcus* sp.	JQ820173.1
	anaerobe	no color, circular, entire	Gram-negative	*Halomonas* sp. LAMA 634	JX860204.1
	anaerobe	white, circular, entire	Gram-positive	*Staphylococcus* sp. BFES95	KC550638.1
	anaerobe	cream, circular, entire	Gram-negative	Uncultured bacterium	JX941176.1
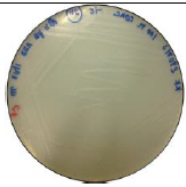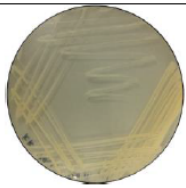
b. TC-30M

Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	dark yellow, circular, entire	Gram-negative	*Erythrobacter* sp. IC114	AB196251.1
	aerobe	no color, circular, entire	Gram-negative	*Stenotrophomonas maltophilia* PISmvs5	KC896401.1
	aerobe	dark yellow, circular, entire	Gram-positive	Uncultured bacterium SPU	KF500796.1
	aerobe	light yellow, circular, entire	Gram-positive	*Micrococcus luteus* B88	EU240406.1
	aerobe	cream, circular, entire	Gram-negative	Uncultured bacterium clone E71-231	DQ638472.1
	aerobe	cream, circular, entire	Gram-positive	Bacterium JANV-8	KF453791.1
	aerobe	dark yellow, circular, entire	Gram-positive	*Micrococcineae bacterium* ST5-4	AM500680.1
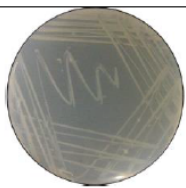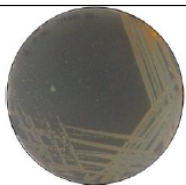
Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	no color, circular, entire	Gram-positive	Uncultured *Staphylococcus* sp.	JQ820176.1
	aerobe	no color, circular, entire	Gram-positive	Uncultured *Paenibacillus* sp.	AM489498.1
	anaerobe	beige, circular, entire	Gram-positive	*Micrococcus luteus* B88	EU240406.1
	anaerobe	no color, circular, entire	Gram-negative	*Paenibacillus* sp. HGH0034	JX520001.1
	anaerobe	yellow, circular, entire	Gram-negative	*Thermosipho melanesiensis* BI429	CP000716.1
	anaerobe	white, circular, entire	Gram-positive	Uncultured bacterium clone R4J3T4_A12	GQ467672.1
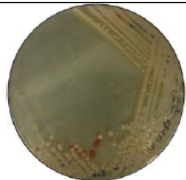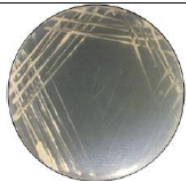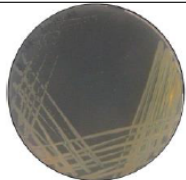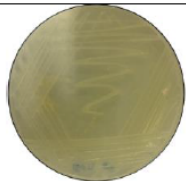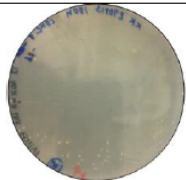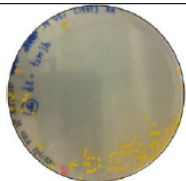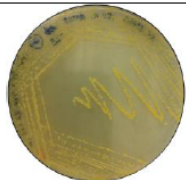
c. TC-100M

Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	no color, circular, entire	Gram-negative	*Pseudoalteromonas lipolytica* strain ZR095	JX173531.1
	aerobe	light brown, circular, entire	Gram-negative	Uncultured bacterium clone A165_BATS	HM032303.1
	aerobe	no color, circular, entire	Gram-positive	*Bacillus cereus* BcTNAU6	KC540842.1
	aerobe	no color, circular, entire	Gram-negative	Uncultured *Idiomarina sp.* clone C146100034	JX530208.1
	anaerobe	dark yellow, circular, entire	Gram-positive	no significant hit	-
	anaerobe	dark yellow, circular, entire	Gram-positive	Uncultured bacterium clone SPU	KF500904.1

Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	anaerobe	cream, circular, entire	Gram-negative	no significant hit	-
	anaerobe	dark yellow, circular, entire	Gram-negative	*Pseudomonas fluorescens* SBW25	AM181176.4
	anaerobe	no color, circular, entire	Gram-negative	no significant hit	-

d. TC-150M

Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	no color, circular, entire	Gram-negative	Uncultured gamma proteobacterium clone J60-41 16S	KC603422.1
	aerobe	yellow, circular, entire	Gram-negative	Uncultured gamma proteobacterium clone J60-41 16S	KC603422.1

Isolate	Cultured oxygen condition	Colony morphology	Gram strain	BLASTN identification	GenBank accession no.
	aerobe	white, circular, entire	Gram-negative	*Bacillus* sp. Q3	JX143762.1
	aerobe	cream, circular, entire	Gram-negative	Uncultured *Thalassospira* sp. clone C146100353	JX530527.1
	aerobe	white, circular, entire	Gram-negative	Uncultured bacterium clone SPU	KF500904.1
	anaerobe	dark yellow, circular, entire	Gram-negative	*Roseinatronobacter* sp. LC05R24	JX945831.1
	anaerobe	cream, circular, entire	Gram-negative	Uncultured bacterium clone SPU	KF500904.1
	anaerobe	dark yellow, circular, entire	Gram-negative	Uncultured bacterium clone BF5_1295	KC307096.1
	anaerobe	cream, circular, entire	Gram-negative	Uncultured alpha proteobacterium	EF614431.1

4.3 Metagenomic DNA isolations

Figure 4.2 displayed the filtrated membranes of PT and TC water samples. Note TC-surface filter showed the most debris and particle on.

a   b

c   d   e
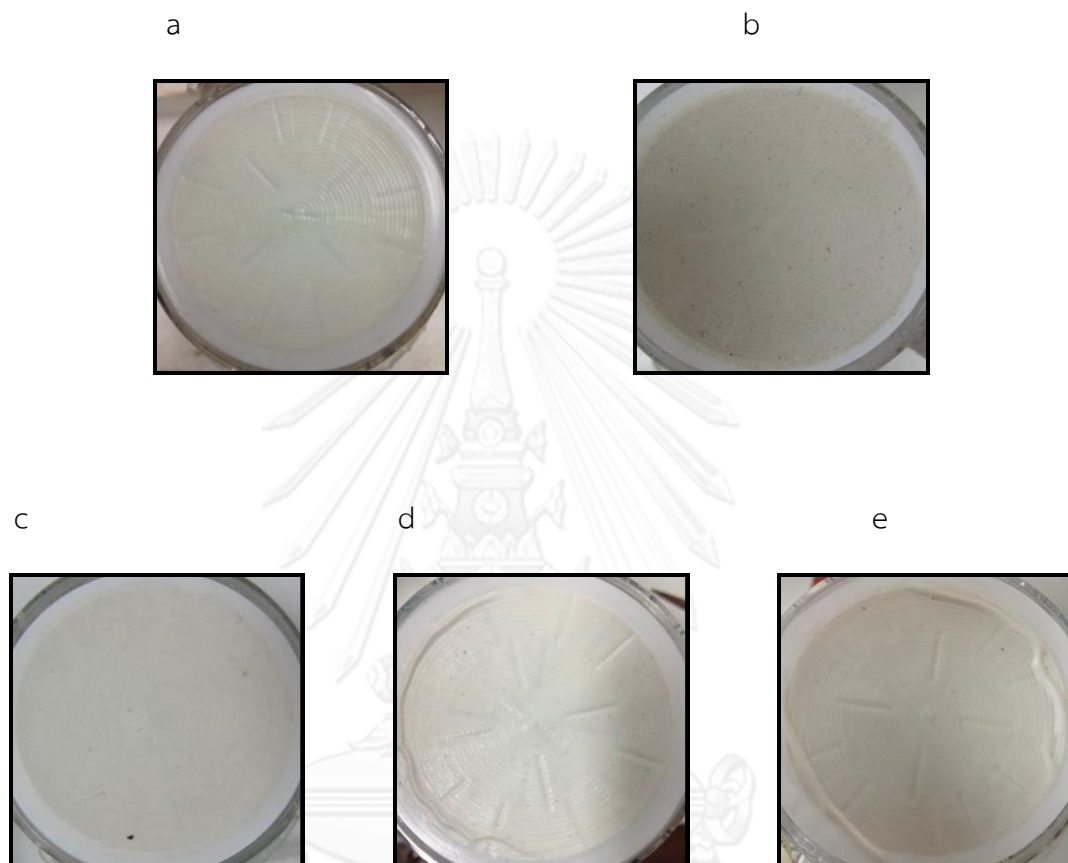
Figure 4.2 Filtered 0.22 μm membranes of PT-30M (a), TC-surface (b), TC-30M (c), TC-100M (d) and TC-150M (e) water samples.

Following a minimum of three independently metagenomic DNA extractions, the quality and concentration of the metagenomic DNA were determined by agarose gel electrophoresis (Figures 4.3 and 4.4). The average metagenomic DNA concentrations in nanograms per liter of seawater for PT-30M was 23.99; and TC-surface 23.99, TC-30M 14.01, TC-100M 72.01, and TC-150M 111.99, respectively.
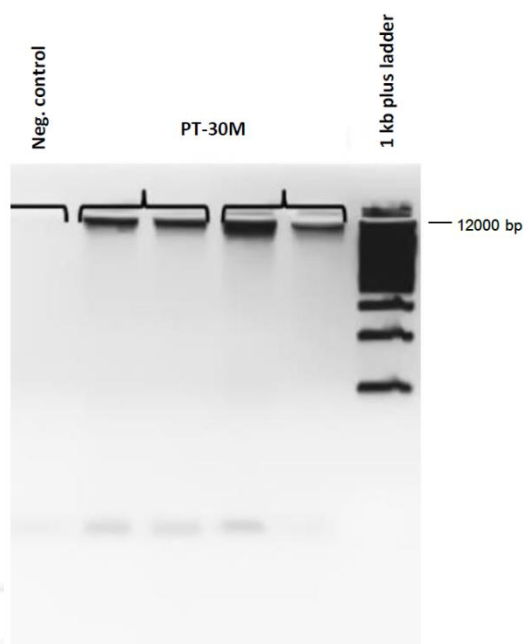
Figure 4.3 Metagenomic DNA of PT-30M visualized by 0.55% agarose gel electrophoresis

a. TC-surface
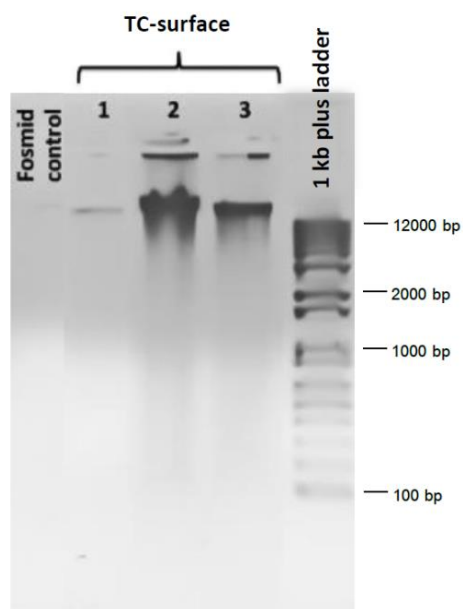
c. TC-100M
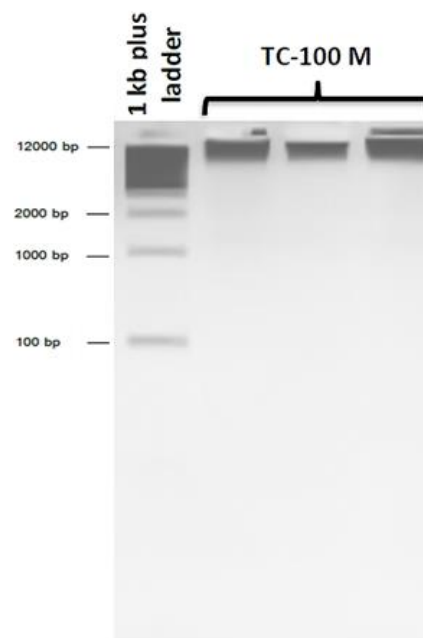
b. TC-30M
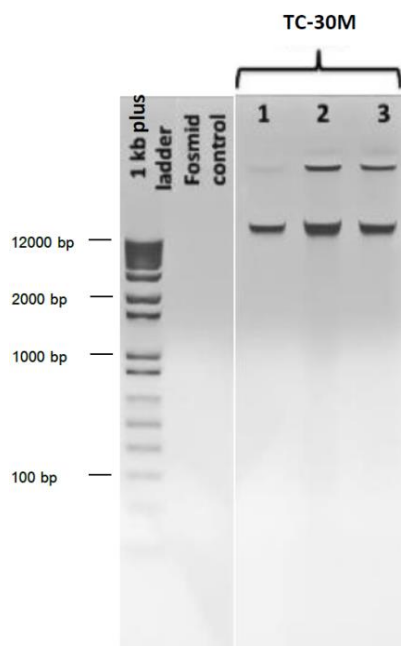
d. TC-150M
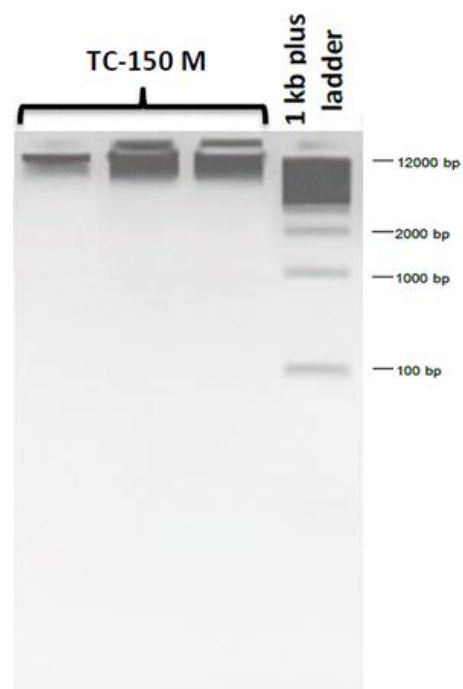
Figure 4.4 Metagenomic DNA of TC-surface (a), TC-30M (b), TC-100M (c), and TC-150M (d), visualized by 0.55% agarose gel electrophoresis

4.4 Construction of pyrotagged 16S rDNA libraries

The PT-30M, TC-surface, TC-30M, TC-100M, and TC-150M 16S rDNA libraries each representing bacteria and archaea biodiversity of the site were constructed. The pyrotagged amplicons of each sample library were estimated 466 nucleotides based on 1.75% agarose gel electrophoresis (Figure 4.5). Nanodrop spectrophotometer was used to quantify the DNA concentrations of the PT and TC libraries: PT-30M 3.87 ng/µl, TC-surface 3.87 ng/µl, TC-30M 4.52 ng/µl, TC-100M 5.81 ng/µl, and TC-150M 6.91 ng/µl respectively.
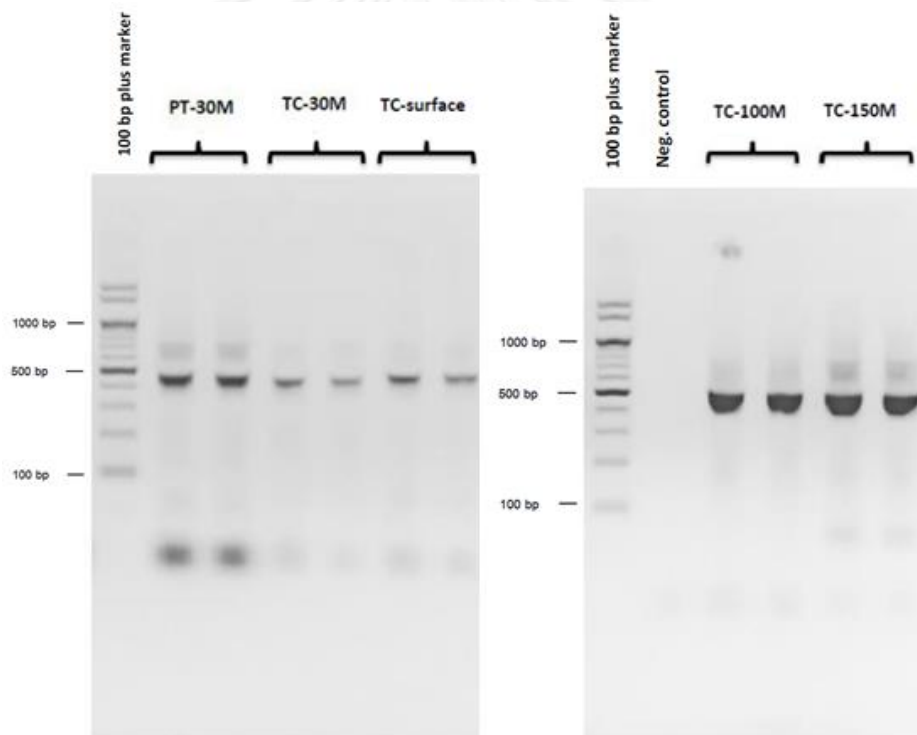
Figure 4.5 Pyrotagged 16S rDNA amplicons of PT and TC samples. Each sample was loaded 10 µl.

4.5 Pyrosequencing and bioinformatic analysis

We obtained over 10,000 pyrosequencing reads of the V3-V4 amplicons for the PT-30M, TC-30M and TC-150M libraries. For TC-surface and TC-100M only 4 and 189 reads were obtained. After discard of less than 50 nucleotides length sequence, PT-30M contained 13,693 reads with average sequence length of 288 bp, and 13,607 reads (99.37%) could be annotated by BLASTN with significant E-values. TC-surface contained 4 with average sequence length of 307 bp and 4 reads (100%) could be annotated. TC-30M contained 10,808 with average sequence length of 275 bp and 10,701 reads (99.01%) could be annotated. TC-100M contained 189 with average sequence length of 298 bp and 189 reads (100%) could be annotated. TC-150M contained 39,086 with average sequence length of 262 nucleotide and 38,762 reads (99.17%) could be annotated (Table 4.4). An unannotated read infers the read that yielded no significant hit by BLASTN against NCBI non-redundant, RDP and Greeegenes databases.

Table 4.4 Number of reads from pyrosequencing

Sample	Reads before quality cutoff	Reads after quality cutoff	Average sequence length (bp)	Reads annotated (%) with significant E-values
PT-30 M	13693	13693	288	13607 (99.37%)
TC-surface	4	4	307	4 (100%)
TC-30 M	11138	10808	275	10701 (99.01%)
TC-100 M	190	189	298	189 (100%)
TC-150 M	39094	39086	262	38762 (99.17%)

a. PT-30M

Proteobacteria	Bacteroidetes	Actinobacteria	Nitrospirae
Methylococcales	Deferribacteres	Gemmatimonadetes	Firmicutes
Verrucomicrobia	Nitrospira	Caldithrix	GN04
KSB1	OP3	Planctomycetes	Tenericutes
MVP-15	Deltaproteobacteria	Chloroflexi	Betaproteobacteria
Fusobacteria	Cyanobacteria	LCP-89	Chlorobi
Dehalococcoidetes	Caldiserica	OP8	Spirochaetes
NC10	OP10	Lentisphaerae	Chlamydiae
ZB2	Deinococcus-Thermus	Rhodocyclales	Gammaproteobacteria
OP11	Rhodocyclaceae	TG3	GN02
unculturedbacterium	49S1_2B	Euryarchaeota	Thermotogae
		SAR406	Alteromonadaceae

b. TC-surface

Proteobacteria
100%

c. TC-30M

Proteobacteria
Chlorobi
Chloroflexi
Elusimicrobia
TM6
unculturedbacterium
Flavobacteria
Euryarchaeota (Archaea)
Cyanobacteria
Vibrio
GOUTA4

Bacteroidetes
Firmicutes
Caldithrix
OP3
Chlamydiae
GN02
OP9
LCP-89
Paracoccus
Caldiserica
HDBW-WB69

Nitrospirae
Gemmatimonadetes
Spirochaetes
WS3
MVP-15
SAR406
O1aA90
Thermotogae
Pseudomonas
Cellvibrio
Rhodobacteraceae

Acidobacteria
Verrucomicrobia
Fusobacteria
GN04
SBR1093
Tenericutes
AC1
WS6
SPAM
Deinococcus-Thermus
Syntrophobacter

Actinobacteria
Deferribacteres
KSB1
OP8
Planctomycetes
TG3
BRC1
GN06
Synergistetes
Deltaproteobacteria
Thermoanaerobacter

Bacteroidetes 3.72%

Nitrospirae 2.87%

Proteobacteria 84.99%

d. TC-100M

Proteobacteria 60.54%

Nitrospirae 16.76%

Bacteroidetes 7.03%

Verrucomicrobia 4.87%

Firmicutes 3.24%

Acidobacteria 2.70%

Proteobacteria ■ Nitrospirae ■ Bacteroidetes ■ Verrucomicrobia ■ Firmicutes ■ Acidobacteria ■ Chloroflexi ■ GN02 ■ Gemmatimonadetes

e. TC-150M

Figure 4.6 Percent phyla compositions of PT-30M (a), TC-surface (b), TC-30M (c), TC-100M (d), and TC-150M (e). Phyla with less than 2% composition were not shown the percentages.

At phylum level, the majority of the sequences fall within a phylum Proteobacteria for both PT and TC samples: PT-30M 75.25%, TC-surface 100%, TC-30M 84.99%, TC-100M 60.54%, and TC-150M 26.09%. Further, phyla Proteobacteria and Bacteroidetes were more proportionated at PT than TC. For TC site, the deeper sea level showed even fewer Proteobacteria (Figure 4.6).

However, classification into genus and species levels showed some distinguishes. Table 4.5 compared and contrasted the genus compositions of these sites and depths. TC-30M (394 genera) included more genera than in PT-30M (274 genera) supported TC site is more suitability for bacteria in PT site. Both sites share 172 genera together such as *Tranquillimonas* (PT-30M 0.01%, TC-30M 7.99%), *Opitutus* (PT-30M 0.01%, TC-30M 4.38%), *Acetivibrio* (PT-30M 0.11%, TC-30M 4.10%), *Spirochaeta* (PT-30M 0.18%, TC-30M 4.03%), *Ignavibacterium* (PT-30M 0.42%, TC-30M 1.88%), *Gemmatimonas* (PT-30M 0.87%, TC-30M 0.97%), *Nitrosospira* (PT-30M 1.29%, TC-30M 0.21%), and *Desulfobacterium* (PT-30M 0.98%, TC-30M 0.14%). For TC sites, TC-30M showed the most number of genus. Consistently, compositions by species were correlated with those by genera. For example, uniquely dominated species at PT-30M were *Alkaliphilus transvaalensis, Bacillus flexus, Congregibacter litoralis, Desulfatibacillum alkenivorans,* and *Desulfomicrobium terraneus*. 306 genera found in TC-30M only for example Gp23 (7.99%), *Tranquillimonas* (7.99%), *Opitutus* (4.38%), *Acetivibrio* (4.10%), *Spirochaeta* (4.03%). 3 genera can be found in TC-100M only which were *Chitinilyticum* (0.54%), *Ramlibacter* (0.54%), and *Thiobacter* (0.54%). While 31 genera can be found in TC-150M, for example *Hyphomonas* (0.13%), *Methylosphaera* (0.13%), and *Nitrincola* (0.13%). Only one genus can be found in all depth from TC site was *Methylosarcina*, gram negative, aerobic, obligate methanotrophic (TC-surface 50%, TC-30M 0.14%, TC-100M 0.54%, and TC-150M 0.71%) This is an aerobic, Gram-negative, non-motile bacterium capable of growth on methane, grows within a pH range of 4-8, with the optimum between pH 5.5 and 6.5. (Kalyuzhnaya et al., 2005; Rahalkar et al., 2007).

Table 4.5 Prokaryotic diversity classified in genera percentages.

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Acidobacteria	Acanthopleuribacter	0.01	--	0.42	--	--
Acidobacteria	Acidimicrobineae	0.04	--	--	--	--
Acidobacteria	Acidobacterium	--	--	0.07	--	--
Acidobacteria	Actinobacteria	0.01	--	--	--	--
Acidobacteria	Bifidobacterium	0.02	--	--	--	--
Acidobacteria	Conexibacteraceae	0.02	--	--	--	--
Acidobacteria	Coriobacterineae	0.01	--	--	--	--
Acidobacteria	Frankineae	0.01	--	--	--	--
Acidobacteria	Geothrix	0.03	--	1.04	1.08	--
Acidobacteria	Gp10	--	--	1.18	--	--
Acidobacteria	Gp17	--	--	0.07	--	--
Acidobacteria	Gp22	--	--	1.81	--	--
Acidobacteria	Gp23	--	--	7.99	--	--
Acidobacteria	Gp26	--	--	0.14	--	--
Acidobacteria	Gp3	--	--	0.76	--	--
Acidobacteria	Gp7	--	--	0.35	--	--
Acidobacteria	Gp9	--	--	0.69	--	--
Acidobacteria	Holophaga	0.01	--	0.21	--	--
Acidobacteria	Ilumatobacter	0.01	--	--	--	--
Acidobacteria	Propionibacterineae	0.01	--	--	--	--
Acidobacteria	Streptosporangineae	0.03	--	--	--	--
Actinobacteria	Acidothermus	--	--	0.21	--	--
Actinobacteria	Actinospica	--	--	0.49	--	--
Actinobacteria	Conexibacter	--	--	0.28	--	--
Actinobacteria	Iamia	--	--	0.07	--	--
Actinobacteria	Micrococcineae	0.09	--	0.07	--	--
Actinobacteria	Nitriliruptor	--	--	0.07	--	--
Actinobacteria	Pseudonocardia	0.02	--	0.07	--	--
Actinobacteria	Saccharopolyspora	--	--	0.21	--	--
Actinobacteria	Thermobifida	--	--	0.07	--	--
Bacteroidetes	Actibacter	0.03	--	1.04	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Bacteroidetes	Adhaeribacter	--	--	0.56	--	--
Bacteroidetes	Aequorivita	--	--	0.07	--	0.25
Bacteroidetes	Aestuariicola	--	--	0.14	--	--
Bacteroidetes	Algoriphagus	0.03	--	0.21	--	--
Bacteroidetes	Aureibactercoralii	--	--	0.07	--	--
Bacteroidetes	Chitinophaga	0.01	--	--	--	--
Bacteroidetes	Croceibacter	--	--	0.07	--	--
Bacteroidetes	Croceibacteratlanticus	--	--	0.07	--	--
Bacteroidetes	Ferruginibacter	0.01	--	0.07	--	--
Bacteroidetes	Flavisolibacter	0.01	--	--	--	0.03
Bacteroidetes	Flavobacteriales	0.01	--	--	--	--
Bacteroidetes	Flavobacterium	0.01	--	--	--	--
Bacteroidetes	Fluviicola	0.01	--	0.07	--	0.05
Bacteroidetes	Formosa	0.02	--	--	--	--
Bacteroidetes	Fulvivirga	--	--	0.07	--	0.03
Bacteroidetes	Haliscomenobacter	0.47	--	0.07	1.62	0.03
Bacteroidetes	Hymenobacter	--	--	0.56	--	--
Bacteroidetes	Lacinutrix	0.01	--	--	--	--
Bacteroidetes	Leeuwenhoekiella	--	--	0.07	--	--
Bacteroidetes	Lewinella	0.08	--	0.07	--	0.10
Bacteroidetes	Lishizhenia	0.01	--	--	--	--
Bacteroidetes	Lutibacter	0.01	--	0.07	--	--
Bacteroidetes	Marinoscillum	0.01	--	--	--	--
Bacteroidetes	Mesoflavibacter	0.01	--	0.07	--	--
Bacteroidetes	Niastella	0.01	--	--	--	--
Bacteroidetes	Nubsella	--	--	0.14	--	--
Bacteroidetes	Owenweeksia	0.02	--	--	--	--
Bacteroidetes	Paludibacter	--	--	0.42	--	--
Bacteroidetes	Pedobacter	0.01	--	--	--	--
Bacteroidetes	Persicobacter	--	--	0.07	--	--
Bacteroidetes	Pontibacter	--	--	--	--	0.03
Bacteroidetes	Prolixibacter	--	--	0.14	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Bacteroidetes	Rhodothermaceae	0.01	--	--	--	--
Bacteroidetes	Rhodothermus	0.01	--	--	--	--
Bacteroidetes	Rikenella	0.01	--	--	--	--
Bacteroidetes	Robiginitalea	0.08	--	0.07	--	--
Bacteroidetes	Salinibacter	0.01	--	--	--	--
Bacteroidetes	Salisaeta	0.01	--	0.07	--	--
Bacteroidetes	Saprospiraceae	0.01	--	--	--	--
Bacteroidetes	Sediminibacter	0.07	--	0.07	--	--
Bacteroidetes	Solitalea	0.02	--	--	--	--
Bacteroidetes	Sphingobacteriales	0.07	--	--	--	--
Bacteroidetes	Sphingobacterium	0.01	--	--	--	--
Bacteroidetes	Tenacibaculum	--	--	--	--	0.03
Bacteroidetes	Terrimonas	0.07	--	0.07	--	--
Bacteroidetes	Ulvibacter	0.01	--	--	--	--
Bacteroidetes	Ulvibacter	--	--	0.07	--	--
Bacteroidetes	Vitellibacter	--	--	0.07	--	--
BRC1	BRC1	--	--	0.07	--	--
Chlamydiae	Neochlamydia	0.01	--	--	--	--
Chlamydiae	Parachlamydia	0.07	--	0.07	--	0.05
Chlamydiae	Simkania	0.07	--	0.07	--	--
Chlorobi	Chlorobiaceae	0.01	--	--	--	--
Chlorobi	Chlorobium	--	--	0.07	--	--
Chlorobi	Ignavibacterium	0.42	--	1.88	--	3.80
Chlorobi	Prosthecochloris	0.01	--	0.07	--	0.61
Chloroflexi	Bellilinea	0.01	--	0.14	--	--
Chloroflexi	Caldilinea	0.04	--	0.07	--	--
Chloroflexi	Dehalogenimonas	--	--	0.07	--	--
Chloroflexi	Longilinea	0.01	--	--	--	--
Chloroflexi	Sphaerobacter	--	--	0.07	--	--
Deferribacteres	Caldithrix	0.92	--	0.07	--	--
Deferribacteres	Mucispirillum	--	--	0.14	--	--
Deinococcus-Thermus	Deinococcus	--	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Deinococcus-Thermus	Truepera	0.01	--	--	--	--
Euryarchaeota	Methanolinea	0.01	--	--	--	--
Fibrobacteres	Fibrobacter	--	--	--	--	0.03
Firmicutes	A55_D21	--	--	--	--	0.03
Firmicutes	Acetivibrio	0.11	--	4.10	--	--
Firmicutes	Acidaminobacter	0.01	--	--	--	--
Firmicutes	Alicyclobacillus	--	--	0.07	--	--
Firmicutes	Alkaliphilus	0.01	--	0.07	--	--
Firmicutes	Anaerobacter	--	--	0.07	--	--
Firmicutes	Anaerovorax	--	--	--	--	0.03
Firmicutes	Caloramator	--	--	0.14	--	--
Firmicutes	Clostridiales	0.01	--	--	--	--
Firmicutes	Clostridium	0.03	--	0.07	--	--
Firmicutes	Cohnella	--	--	0.07	--	--
Firmicutes	Coprobacillus	0.01	--	--	--	--
Firmicutes	Coprothermobacter	--	--	0.07	--	--
Firmicutes	Dehalobacterium	0.01	--	--	--	--
Firmicutes	Desulfitobacter	--	--	--	--	0.03
Firmicutes	Dialister	--	--	0.07	--	--
Firmicutes	Eubacterium	0.04	--	0.07	--	--
Firmicutes	Fusibacter	0.01	--	--	--	0.18
Firmicutes	Geobacillus	--	--	0.07	--	--
Firmicutes	Geosporobacter	--	--	0.07	--	--
Firmicutes	Lactobacillus	0.01	--	--	--	--
Firmicutes	Mahella	--	--	0.07	--	--
Firmicutes	Oscillibacter	0.01	--	0.07	--	--
Firmicutes	Paenibacillus	--	--	0.07	--	--
Firmicutes	Parvimonas	--	--	0.07	--	--
Firmicutes	Peptococcus	0.01	--	--	--	--
Firmicutes	Ruminococcus	0.01	--	--	--	--
Firmicutes	Sarcina	--	--	0.07	--	--
Firmicutes	Sedimentibacter	0.01	--	--	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Firmicutes	Sedis	0.01	--	--	--	--
Firmicutes	Sporacetigenium	0.01	--	0.07	1.08	--
Firmicutes	Sporanaerobacter	--	--	0.07	--	--
Firmicutes	Sporobacter	--	--	0.07	--	--
Firmicutes	Staphylococcus	--	--	0.07	--	--
Firmicutes	Thermicanus	--	--	0.07	--	--
Firmicutes	Thermincola	--	--	0.07	--	--
Firmicutes	Thermoanaerobacter	--	--	0.07	--	--
Firmicutes	Thermodesulfobium	0.03	--	--	--	--
Firmicutes	Turicibacter	--	--	0.07	--	--
Fusobacteria	Cetobacterium	--	--	0.07	--	--
Fusobacteria	Fusobacterium	--	--	0.07	--	--
Fusobacteria	Ilyobacter	--	--	0.14	--	--
Fusobacteria	Propionigenium	0.01	--	0.07	--	0.05
Gemmatimona-detes	Gemmatimonas	0.87	--	0.97	1.08	0.10
Lentisphaerae	Victivallis	0.01	--	--	--	--
Nitrospirae	4-29	--	--	0.49	14.59	1.63
Nitrospirae	BD2-6	--	--	1.67	--	10.53
Nitrospirae	GOUTA19	--	--	0.83	1.08	4.49
Nitrospirae	GOUTA7	--	--	--	--	0.13
Nitrospirae	HB118	--	--	--	--	0.05
Nitrospirae	LCP-6	--	--	0.28	0.54	1.99
Nitrospirae	Nitrospira	0.49	--	0.14	0.54	--
Nitrospirae	Thermodesulfovibrio	0.04	--	0.07	--	--
Planctomycetes	Gemmata	0.01	--	--	--	--
Proteobacteria	Acetobacter	--	--	0.07	--	--
Proteobacteria	Achromobacter	0.02	--	0.14	--	--
Proteobacteria	Acidisphaera	0.01	--	0.07	--	--
Proteobacteria	Acidovorax	0.01	--	--	--	--
Proteobacteria	Acinetobacter	--	--	0.07	--	0.03
Proteobacteria	Aeromonas	0.01	--	0.07	--	--
Proteobacteria	Agrobacterium	--	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Alcanivorax	0.06	--	0.07	--	--
Proteobacteria	Alkalilimnicola	--	--	0.56	--	--
Proteobacteria	Alkalimonas	--	--	0.07	--	--
Proteobacteria	Alkalispirillum	--	--	--	--	0.05
Proteobacteria	Allochromatium	0.04	--	--	--	0.08
Proteobacteria	Altererythrobacter	--	--	0.14	--	--
Proteobacteria	Alteromonas	0.02	--	--	--	--
Proteobacteria	Amaricoccus	--	--	0.07	--	0.05
Proteobacteria	Amoebobacter	--	--	0.07	--	--
Proteobacteria	Anaeromyxobacter	--	--	0.07	0.54	--
Proteobacteria	Anderseniella	0.03	--	0.28	--	--
Proteobacteria	Angiococcus	--	--	0.14	--	--
Proteobacteria	Aquabacterium	--	--	0.07	--	0.15
Proteobacteria	Aquamicrobium	--	--	0.07	--	--
Proteobacteria	Aquimonas	--	--	0.14	--	--
Proteobacteria	Archangium	--	--	0.14	--	--
Proteobacteria	Arenimonas	--	--	0.14	--	0.05
Proteobacteria	Aromatoleum	--	--	0.07	--	--
Proteobacteria	Aspromonas	--	--	0.07	--	--
Proteobacteria	Azoarcus	0.06	--	0.07	--	0.03
Proteobacteria	Azohydromonas	0.01	--	0.07	0.54	0.13
Proteobacteria	Azomonas	0.03	--	--	--	--
Proteobacteria	Azonexus	0.06	--	0.07	--	--
Proteobacteria	Azospira	0.01	--	0.07	0.54	--
Proteobacteria	Azotobacter	--	--	0.14	--	--
Proteobacteria	Bacteriovorax	0.13	--	0.07	--	0.15
Proteobacteria	BD2-13	--	--	--	--	0.03
Proteobacteria	Bdellovibrio	0.07	--	0.07	--	--
Proteobacteria	Bordetellahinzii	--	--	0.14	--	--
Proteobacteria	Burkholderia	0.03	--	0.14	0.54	--
Proteobacteria	Byssovorax	--	--	0.07	--	--
Proteobacteria	Caedibacter	--	--	--	--	0.03
Proteobacteria	Caenibacterium	--	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Caldimonas	--	--	--	--	0.03
Proteobacteria	Caminibacter	--	--	0.07	--	--
Proteobacteria	Candidatus	0.1	--	0.90	--	0.76
Proteobacteria	Cardiobacterium	0.01	--	0.07	--	--
Proteobacteria	Catellibacterium	--	--	0.14	--	--
Proteobacteria	Caulobacter	0.01	--	0.07	--	--
Proteobacteria	Chitinilyticum	--	--	--	0.54	--
Proteobacteria	Chondromyces	0.02	--	0.07	--	0.03
Proteobacteria	Chromobacterium	--	--	0.07	--	--
Proteobacteria	Chromohalobacter	--	--	0.07	--	--
Proteobacteria	Citrobacter	--	--	0.07	--	--
Proteobacteria	Citrobacterfarmeri	--	--	0.07	--	--
Proteobacteria	Codakia	--	--	0.35	--	--
Proteobacteria	Comamonas	--	--	0.07	--	--
Proteobacteria	Congregibacter	0.01	--	0.21	--	0.03
Proteobacteria	Corallococcus	--	--	0.07	--	--
Proteobacteria	Coxiella	--	--	0.14	--	--
Proteobacteria	Crenothrix	--	--	--	--	0.03
Proteobacteria	Croceicoccus	0.02	--	--	--	--
Proteobacteria	Cycloclasticus	--	--	0.14	--	--
Proteobacteria	Cystobacteraceae	0.14	--	--	2.70	--
Proteobacteria	Dasania	0.02	--	0.42	--	--
Proteobacteria	Dechloromonas	0.23	--	0.14	--	--
Proteobacteria	Denitratisoma	0.03	--	0.14	--	--
Proteobacteria	Derxia	0.01	--	0.35	--	--
Proteobacteria	Desulfacinum	0.01	--	--	--	--
Proteobacteria	Desulfarculus	0.03	--	--	--	--
Proteobacteria	Desulfatibacillum	0.02	--	0.56	--	--
Proteobacteria	Desulfatiferula	0.02	--	0.21	--	--
Proteobacteria	Desulfatirhabdium	0.01	--	0.14	--	--
Proteobacteria	Desulfobacca	0.29	--	0.69	--	1.45
Proteobacteria	Desulfobacterales	0.01	--	--	--	--
Proteobacteria	Desulfobacterium	0.98	--	0.14	--	0.03

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Desulfobacula	--	--	0.07	--	--
Proteobacteria	Desulfobotulus	0.07	--	0.07	--	--
Proteobacteria	Desulfobulbus	0.57	--	0.21	--	0.03
Proteobacteria	Desulfocapsa	0.01	--	0.07	--	0.08
Proteobacteria	Desulfococcus	0.02	--	0.07	--	0.03
Proteobacteria	Desulfocurvus	0.02	--	--	--	--
Proteobacteria	Desulfofaba	0.03	--	0.07	--	0.03
Proteobacteria	Desulfofrigus	0.08	--	0.07	--	0.15
Proteobacteria	Desulfomicrobium	0.01	--	0.07	0.54	--
Proteobacteria	Desulfomonile	0.10	--	0.07	--	0.25
Proteobacteria	Desulfonatronum	0.04	--	0.07	--	0.13
Proteobacteria	Desulfonema	0.21	--	0.83	--	--
Proteobacteria	Desulfopila	--	--	0.07	--	--
Proteobacteria	Desulforegula	0.01	--	--	--	--
Proteobacteria	Desulforhopalus	0.03	--	0.14	--	--
Proteobacteria	Desulfosalina	--	--	0.07	--	--
Proteobacteria	Desulfosarcina	0.46	--	0.14	--	0.28
Proteobacteria	Desulfotalea	0.01	--	0.07	--	--
Proteobacteria	Desulfotignum	0.01	--	0.35	--	--
Proteobacteria	Desulfovibrio	0.07	--	0.14	--	0.08
Proteobacteria	Desulfovirga	0.02	--	0.07	--	--
Proteobacteria	Desulfurivibrio	0.07	--	0.63	--	--
Proteobacteria	Desulfuromonas	0.40	--	0.07	0.54	--
Proteobacteria	Desulfuromusa	0.02	--	--	--	--
Proteobacteria	Diaphorobacter	--	--	0.07	--	0.03
Proteobacteria	Dinoroseobacter	--	--	0.07	--	--
Proteobacteria	Dokdonella	0.01	--	0.07	--	--
Proteobacteria	Dyella	0.01	--	0.83	--	--
Proteobacteria	Ectothiorhodosinus	0.04	--	1.74	--	--
Proteobacteria	Ectothiorhodospira	0.04	--	0.56	--	0.03
Proteobacteria	Enhydrobacter	--	--	0.07	--	--
Proteobacteria	Enhygromyxa	--	--	0.07	--	--
Proteobacteria	Enterobacter	--	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Erythrobacter	--	--	0.07	--	--
Proteobacteria	Escherichia/Shigella	--	--	0.07	--	--
Proteobacteria	Ferribacterium	0.01	--	0.07	--	--
Proteobacteria	Ferrimonas	0.01	--	--	--	--
Proteobacteria	Ferrocurvibacter	--	--	0.14	--	--
Proteobacteria	Filomicrobium	--	--	0.07	--	--
Proteobacteria	Fulvimonas	--	--	0.07	--	--
Proteobacteria	Gallionella	--	--	0.07	--	0.64
Proteobacteria	Geobacter	0.11	--	0.14	2.70	0.03
Proteobacteria	Geobacterpelophilus	--	--	0.07	--	--
Proteobacteria	Geopsychrobacter	0.01	--	0.07	--	--
Proteobacteria	Geothermobacter	0.02	--	0.63	--	--
Proteobacteria	Gibbsiellaquercinecans	--	--	0.14	--	--
Proteobacteria	Gluconacetobacter	--	--	0.42	--	--
Proteobacteria	Gluconobacter	--	--	0.21	--	--
Proteobacteria	Granulibacter	--	--	0.42	--	--
Proteobacteria	Granulosicoccus	0.01	--	0.14	--	--
Proteobacteria	Haematobacter	--	--	0.07	--	--
Proteobacteria	Hahella	--	--	0.14	--	--
Proteobacteria	Haliangiaceae	0.21	--	--	--	--
Proteobacteria	Haliangium	--	--	0.14	--	0.10
Proteobacteria	Haliclona	--	--	0.07	--	--
Proteobacteria	Haliea	1.21	--	--	--	--
Proteobacteria	Haliea	--	--	0.90	--	--
Proteobacteria	Halochromatium	0.10	--	0.14	--	0.20
Proteobacteria	Halomonas	0.04	--	0.07	--	--
Proteobacteria	Halomonasaquamarina	--	--	0.07	--	--
Proteobacteria	Halomonasventosae	--	--	0.07	--	--
Proteobacteria	Halorhodospira	0.01	--	0.42	--	--
Proteobacteria	Halothiobacillus	--	--	0.07	--	--
Proteobacteria	Halovibrio	--	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	HB2-32-21	--	--	0.07	--	--
Proteobacteria	Herbaspirillum	--	--	0.07	--	--
Proteobacteria	HTCC	--	--	0.83	--	0.41
Proteobacteria	HTCC2207	--	--	--	--	0.05
Proteobacteria	Hydrogenophaga	0.01	--	0.07	--	--
Proteobacteria	Hydrogenovibrio	0.01	--	0.14	--	--
Proteobacteria	Hyphomicrobium	0.04	--	0.07	--	--
Proteobacteria	Hyphomonas	--	--	--	--	0.13
Proteobacteria	Ideonella	0.03	--	0.14	0.54	0.03
Proteobacteria	Idiomarina	--	--	0.07	--	--
Proteobacteria	Ignatzschineria	0.10	--	0.07	--	--
Proteobacteria	Ignatzschinerialarvae	--	--	0.07	--	--
Proteobacteria	Inquilinus	--	--	0.07	--	--
Proteobacteria	Jannaschia	--	--	0.07	--	--
Proteobacteria	Janthinobacterium	--	--	--	--	0.03
Proteobacteria	JTB255	--	--	0.42	--	--
Proteobacteria	Kangiella	--	--	0.07	--	0.03
Proteobacteria	Kofleria	--	--	0.14	--	--
Proteobacteria	Kofleriaceae	0.11	--	--	--	--
Proteobacteria	Lacibacter	0.01	--	--	--	--
Proteobacteria	Laribacter	--	--	0.07	--	--
Proteobacteria	Legionella	0.01	--	0.07	--	--
Proteobacteria	Leucothrix	--	--	0.07	--	--
Proteobacteria	Limnobacter	0.01	--	0.28	--	0.03
Proteobacteria	Listonella	--	--	0.07	--	--
Proteobacteria	Loktanella	0.01	--	0.07	--	--
Proteobacteria	Lucinanassula	--	--	0.07	--	--
Proteobacteria	Luteibacter	--	--	0.35	--	--
Proteobacteria	Luteimonas	0.02	--	--	--	--
Proteobacteria	Lysobacter	--	--	0.07	--	--
Proteobacteria	Malikia	--	--	--	--	0.03
Proteobacteria	Marichromatium	0.01	--	0.07	--	0.03
Proteobacteria	Marinimicrobium	0.04	--	--	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Marinobacter	--	--	0.07	--	0.08
Proteobacteria	Marinobacteralgicola	--	--	0.07	--	--
Proteobacteria	Marinobacterium	0.33	--	0.42	--	3.29
Proteobacteria	Massilia	0.01	--	--	--	--
Proteobacteria	Melitea	--	--	0.56	--	--
Proteobacteria	Mesorhizobium	0.01	--	0.07	--	--
Proteobacteria	Methylibium	0.01	--	0.07	--	--
Proteobacteria	Methylobacillus	0.01	--	0.07	--	--
Proteobacteria	Methylobacte	--	--	0.14	--	--
Proteobacteria	Methylobacter	0.84	--	0.14	--	--
Proteobacteria	Methylobacterium	--	--	0.07	--	--
Proteobacteria	Methylocaldum	0.16	--	0.14	--	--
Proteobacteria	Methylococcaceae	0.01	--	--	--	--
Proteobacteria	Methylococcus	0.01	--	0.14	0.54	--
Proteobacteria	Methylocystis	0.01	--	--	--	--
Proteobacteria	Methylohalomonas	--	--	0.07	--	--
Proteobacteria	Methylomicrobium	0.10	--	0.07	--	--
Proteobacteria	Methylomonas	0.15	--	0.07	--	0.33
Proteobacteria	Methylophaga	0.01	--	--	--	0.03
Proteobacteria	Methylophilus	0.12	--	0.14	--	--
Proteobacteria	Methylosarcina	0.10	50	0.14	0.54	0.71
Proteobacteria	Methylosoma	0.12	--	--	--	--
Proteobacteria	Methylosphaera	--	--	--	--	0.13
Proteobacteria	Methylotenera	0.04	--	0.07	--	0.10
Proteobacteria	Methyloversatilis	0.04	--	0.14	--	0.03
Proteobacteria	Microbulbifer	--	--	0.07	--	0.10
Proteobacteria	Myrtea	--	--	0.07	--	--
Proteobacteria	Myxococcus	--	--	0.14	--	0.03
Proteobacteria	Nannocystaceae	0.05	--	--	--	--
Proteobacteria	Nannocystis	--	--	0.07	--	--
Proteobacteria	Nevskia	--	--	0.07	--	--
Proteobacteria	Nitratireductor	--	--	0.07	--	--
Proteobacteria	Nitrincola	--	--	--	--	0.13

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Nitrosococcus	0.01	--	--	--	0.03
Proteobacteria	Nitrosomonas	0.02	--	0.07	--	--
Proteobacteria	Nitrosospira	1.29	--	0.21	2.16	--
Proteobacteria	Nitrospina	0.02	--	--	--	--
Proteobacteria	Novosphingobium	0.04	--	0.07	--	0.03
Proteobacteria	nsmpVI18	--	--	--	--	0.03
Proteobacteria	Oceanibaculum	0.04	--	0.28	--	--
Proteobacteria	Oceanicola	0.01	--	0.70	--	--
Proteobacteria	Oceanospirillum	--	--	0.07	--	--
Proteobacteria	Osedax	--	--	0.07	--	--
Proteobacteria	Pandoraea	--	--	--	--	0.03
Proteobacteria	Paracoccus	--	--	0.14	--	0.03
Proteobacteria	Paracoccusdenitrificans	--	--	0.07	--	--
Proteobacteria	Parvularcula	--	--	0.07	--	--
Proteobacteria	Pedomicrobium	--	--	0.07	--	0.03
Proteobacteria	Pelagibacter	0.01	--	0.07	--	--
Proteobacteria	Pelagibius	0.01	--	0.07	--	--
Proteobacteria	Pelobacter	0.28	--	0.63	--	0.15
Proteobacteria	Peredibacter	--	--	0.07	--	--
Proteobacteria	Phaeobacter	0.02	--	--	--	--
Proteobacteria	Phaselicystidaceae	0.01	--	--	--	--
Proteobacteria	Phaselicystis	--	--	0.07	--	--
Proteobacteria	Phenylobacterium	0.01	--	0.07	--	--
Proteobacteria	Photobacterium	0.01	--	0.07	--	0.10
Proteobacteria	Phototrophicbacterium	--	--	0.07	--	--
Proteobacteria	Phyllobacterium	0.04	--	--	--	--
Proteobacteria	Plesiomonas	--	--	--	--	0.03
Proteobacteria	Polyangiaceae	0.15	--	--	1.08	--
Proteobacteria	Polynucleobacter	--	--	--	--	0.05
Proteobacteria	Porphyrobacter	0.02	--	0.07	--	--
Proteobacteria	Propionivibrio	0.02	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Prosthecomicrobium	0.07	--	0.14	--	--
Proteobacteria	Proteobacteria	--	--	0.35	--	--
Proteobacteria	Pseudidiomarina	--	--	0.07	--	--
Proteobacteria	Pseudomonas	0.08	--	0.14	--	0.03
Proteobacteria	Pseudoruegeria	--	--	0.28	--	0.03
Proteobacteria	Pseudoxanthomonas	0.01	--	--	--	--
Proteobacteria	Psychrobacter	--	--	0.07	--	--
Proteobacteria	Ramlibacter	--	--	--	0.54	--
Proteobacteria	Raoultella	0.01	--	0.07	--	--
Proteobacteria	Rheinheimera	--	--	0.07	--	--
Proteobacteria	Rhizobacter	0.02	--	0.07	--	--
Proteobacteria	Rhizobium	0.01	--	0.07	--	--
Proteobacteria	Rhizobiumundicola	--	--	0.07	--	--
Proteobacteria	Rhodanobacter	--	--	0.14	--	--
Proteobacteria	Rhodobacter	0.02	--	0.07	--	--
Proteobacteria	Rhodobiaceae	0.12	--	--	--	--
Proteobacteria	Rhodocyclales	0.01	--	--	--	--
Proteobacteria	Rhodocyclus	0.21	--	0.07	--	0.25
Proteobacteria	Rhodoferax	--	--	0.07	--	--
Proteobacteria	Rhodovibrio	0.03	--	0.07	--	--
Proteobacteria	Rhodovulum	--	--	0.14	--	--
Proteobacteria	Rickettsia	--	--	--	--	0.03
Proteobacteria	Riftia	--	--	0.14	--	--
Proteobacteria	Roseateles	--	--	0.07	--	--
Proteobacteria	Roseibacterium	--	--	0.07	--	--
Proteobacteria	Roseicyclus	0.01	--	0.07	--	--
Proteobacteria	Roseobacter	--	--	0.07	--	--
Proteobacteria	Roseomonas	--	--	0.07	--	--
Proteobacteria	Roseovarius	0.01	--	--	--	--
Proteobacteria	Rubellimicrobium	0.01	--	--	--	--
Proteobacteria	Rubrivivax	0.01	--	0.14	--	--
Proteobacteria	Ruegeria	0.01	--	--	--	--
Proteobacteria	Ruegeria	--	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Saccharospirillum	0.01	--	0.07	--	--
Proteobacteria	Salicola	0.01	--	0.07	--	--
Proteobacteria	Salinimonas	--	--	--	--	0.03
Proteobacteria	Salinisphaera	0.02	--	--	--	--
Proteobacteria	Sedimenticola	--	--	0.07	--	--
Proteobacteria	Serratia	--	--	0.14	--	--
Proteobacteria	Shewanella	0.01	--	--	--	--
Proteobacteria	Shinella	0.04	--	--	--	--
Proteobacteria	Silicibacter	--	--	0.07	--	--
Proteobacteria	Skermanella	--	--	0.07	--	--
Proteobacteria	Smithella	0.06	--	0.35	--	--
Proteobacteria	Solimonas	--	--	0.83	--	--
Proteobacteria	Sorangium	--	--	0.35	--	--
Proteobacteria	Sphingomonas	--	--	0.07	--	0.03
Proteobacteria	Sphingopyxis	0.10	--	0.07	--	--
Proteobacteria	Spongiibacter	--	--	0.56	--	--
Proteobacteria	Steroidobacter	0.40	--	1.60	2.16	--
Proteobacteria	Sterolibacterium	--	--	0.14	--	--
Proteobacteria	Stigmatella	--	--	0.28	--	--
Proteobacteria	Sulfuricurvum	0.02	--	0.07	--	--
Proteobacteria	Sulfurimonas	0.01	--	0.21	--	--
Proteobacteria	Sulfurivirga	--	--	0.14	--	--
Proteobacteria	Sulfurospirillum	0.01	--	0.07	--	--
Proteobacteria	Sulfurovum	0.31	--	0.07	--	--
Proteobacteria	Sutterella	0.01	--	0.14	--	--
Proteobacteria	Sva0091	--	--	0.21	--	--
Proteobacteria	Sva0120	--	--	0.28	--	--
Proteobacteria	Sva0318	--	--	0.14	--	--
Proteobacteria	Sva0864	--	--	0.14	--	--
Proteobacteria	Syntrophobacter	0.21	--	0.07	--	--
Proteobacteria	Syntrophorhabdus	--	--	0.07	--	--
Proteobacteria	Syntrophus	0.32	--	0.14	0.54	0.18
Proteobacteria	Tatlockia	0.01	--	0.07	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Tepidimonas	0.01	--	--	--	--
Proteobacteria	Tepidiphilus	--	--	0.07	--	--
Proteobacteria	Thalassobacter	--	--	0.07	--	--
Proteobacteria	Thalassobius	0.02	--	0.07	--	--
Proteobacteria	Thauera	0.08	--	0.07	--	--
Proteobacteria	Thermomonas	0.01	--	--	--	--
Proteobacteria	Thialkalivibrionitratireducens	--	--	0.07	--	--
Proteobacteria	Thioalbusdenitrificans	--	--	0.14	--	--
Proteobacteria	Thioalkalispira	0.26	--	0.07	--	--
Proteobacteria	Thioalkalivibrio	0.10	--	0.69	--	--
Proteobacteria	Thiobacillus	0.15	--	0.07	--	--
Proteobacteria	Thiobacter	--	--	--	0.54	--
Proteobacteria	Thiocapsa	--	--	0.07	--	0.03
Proteobacteria	Thioclava	0.02	--	0.21	--	0.20
Proteobacteria	Thiococcus	0.01	--	--	--	--
Proteobacteria	Thiocystis	0.01	--	--	--	--
Proteobacteria	Thiodictyon	--	--	0.14	--	--
Proteobacteria	Thiofaba	0.12	--	0.56	--	--
Proteobacteria	Thiohalobacter	--	--	0.14	--	--
Proteobacteria	Thiohalocapsa	0.01	--	0.28	--	--
Proteobacteria	Thiohalomonas	0.01	--	0.21	--	0.08
Proteobacteria	Thiohalophilus	--	--	1.11	--	--
Proteobacteria	Thiohalospira	--	--	0.07	--	--
Proteobacteria	Thiolamprovum	0.01	--	0.14	--	--
Proteobacteria	Thiomonas	--	--	0.07	--	--
Proteobacteria	Thiorhodococcus	--	--	0.07	--	--
Proteobacteria	Thiorhodospira	--	--	0.07	--	0.05
Proteobacteria	Thiorhodovibrio	0.01	--	0.07	--	0.05
Proteobacteria	Thiothrix	0.11	--	0.07	--	--
Proteobacteria	Tolumonas	0.26	--	0.07	--	0.03
Proteobacteria	Tranquillimonas	0.01	--	7.99	--	--

Phylum	Genus	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
Proteobacteria	Tropicimonas	--	--	0.14	--	--
Proteobacteria	Vibrio	--	--	0.42	--	0.05
Proteobacteria	Vogesella	--	--	0.07	--	--
Proteobacteria	Xanthomonas	--	--	0.14	--	--
Proteobacteria	ZD0117	--	--	0.07	--	--
Proteobacteria	Zoogloea	0.03	--	--	--	0.05
Proteobacteria	Zooshikella	0.01	--	0.76	--	0.15
SAR406	SargSea-WGS	--	--	0.42	--	--
SAR407	Arctic95A-2	0.01	--	--	--	--
Spirochaetes	Exilispira	0.08	--	--	--	--
Spirochaetes	IE043	--	--	--	--	0.03
Spirochaetes	KSA2	--	--	0.56	--	2.27
Spirochaetes	Leptonema	0.01	--	--	--	0.03
Spirochaetes	SA-8	--	--	--	--	0.13
Spirochaetes	SJA-88	--	--	0.07	--	0.74
Spirochaetes	Sphaerochaeta	--	--	--	--	0.03
Spirochaetes	Spirochaeta	0.18	--	4.03	--	--
Spirochaetes	TM3	--	--	0.14	--	2.29
Spirochaetes	Treponema	0.07	--	--	--	--
Spirochaetes	Turneriella	--	--	0.07	--	--
Synergistetes	Thermovirga	--	--	0.07	--	--
Tenericutes	Acholeplasma	0.01	--	--	--	--
Tenericutes	Haloplasma	0.20	--	--	--	--
Tenericutes	Mycoplasma	0.01	--	0.14	--	--
Thermotogae	Thermosipho	0.05	--	0.28	--	--
uncultured clone		79.70	50	0.35	60.54	57.58

"--" represents 0.00 percent.

The differences among prokaryotic community structures of PT and TC, without and with 75 other global ocean sampling (GOS) sites were compared using Yue & Clayton theta similarity coefficients named Thetayc and Bray-Curtis indices, in mothur (Yue et al., 2005; Chao et al., 2006; Schloss et al., 2009). The computed data were displayed in Tables 4.6a and 4.6b, respectively. Overall, PT and TC prokaryotic community structures were found relatively closet among one another and with those of Tha Wang and Tham Phang: Thetayc ranged 0.367095- 0.068415. With the other GS profiles belonging to the other countries's maritime zones, Thetayc were higher. The lower the Thetayc to 1.000 infers the closer the community structure between the pair is (Yue et al., 2005). Figure 4.7 represented a principle component analysis (PCoA) in three-dimension format, and Figure 4.8 was a dendrogram constructed using Table 4.6 data.

Table 4.6 Thetayc of PT and TC sites, without (a) and with 75 other GOS sites (b)

a

Site	PT-30M	TC-surface	TC-30M	TC-100M	TC-150M
PT-30M	0.0000	N/A	0.0636	N/A	N/A
TC-surface	N/A	0.0000	0.9906	0.9744	0.9957
TC-30M	0.0636	0.9906	0.0000	0.9807	0.7732
TC-100M	N/A	0.9744	0.9807	0.0000	0.9937
TC150M	N/A	0.9957	0.7732	0.9937	0.0000

b

Pair		Thetayc	Bray-Curtis
Site 1 (PT-30M)	Site 2		
PT-30M	TC-30M	0.0684	0.0588
PT-30M	Tha Wang	0.5850	0.9647
PT-30M	Tham Phang	0.7224	0.9919
PT-30M	GS012	0.9783	0.9997
PT-30M	GS110b	0.9840	1.0000
PT-30M	GS049	0.9976	1.0000

Pair		Thetayc	Bray-Curtis
Site 1 (TC-surface)	Site 2		
TC-surface	GS110b	0.9444	0.8947
TC-surface	TC-30M	0.9825	0.9987
TC-surface	TC-100M	0.9845	0.9643
TC-surface	TC-150M	0.9915	0.9998
TC-surface	Tha Wang	0.9944	0.9997
TC-surface	Tham Phang	0.9948	0.9995
TC-surface	GS012	1.0000	1.0000
TC-surface	GS049	1.0000	1.0000

Pair		Thetayc	Bray-Curtis
Site 1 (TC-30M)	Site 2		
TC-30M	TC-150M	0.6513	0.8951
TC-30M	Tha Wang	0.8800	0.9274
TC-30M	TC-100M	0.9732	0.9883
TC-30M	Tham Phang	0.9734	0.9405
TC-30M	TC-surface	0.9825	0.9987
TC-30M	GS110b	0.9851	0.9994
TC-30M	GS012	0.9946	0.9963
TC-30M	GS049	0.9946	0.9988

Pair		Thetayc	Bray-Curtis
Site 1 (TC-100M)	Site 2		
TC-100M	TC-30M	0.9732	0.9883
TC-100M	TC-150M	0.9892	0.9981
TC-100M	TC-surface	0.9845	0.9643
TC-100M	GS110b	0.9901	0.9701
TC-100M	Tha Wang	0.9939	0.9975
TC-100M	Tham Phang	0.9973	0.9970
TC-100M	GS012	1.0000	1.0000
TC-100M	GS049	1.0000	1.0000

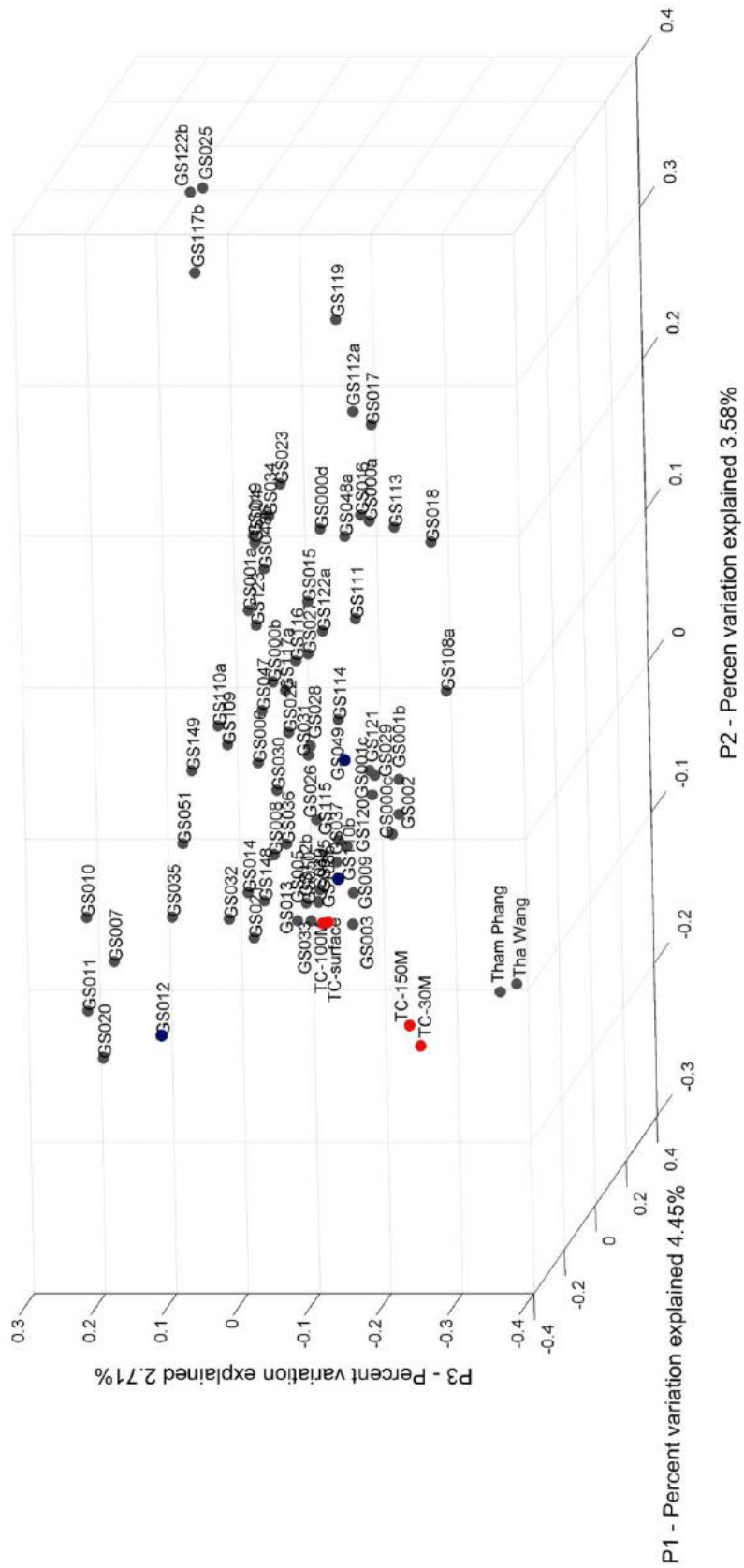| Pair		Thetayc	Bray-Curtis
Site 1 (TC-150M)	Site 2		
TC-150M	TC-30M	0.6513	0.8951
TC-150M	Tha Wang	0.9304	0.9186
TC-150M	Tham Phang	0.9859	0.9544
TC-150M	TC-100M	0.9892	0.9981
TC-150M	TC-surface	0.9915	0.9998
TC-150M	GS110b	0.9930	0.9998
TC-150M	GS049	0.9961	0.9997
TC-150M	GS012	0.9966	0.9991

Figure 4.7 PCoA diagram showing prokaryotic population structures relatedness among PT and TC samples with other 75 GOS profile
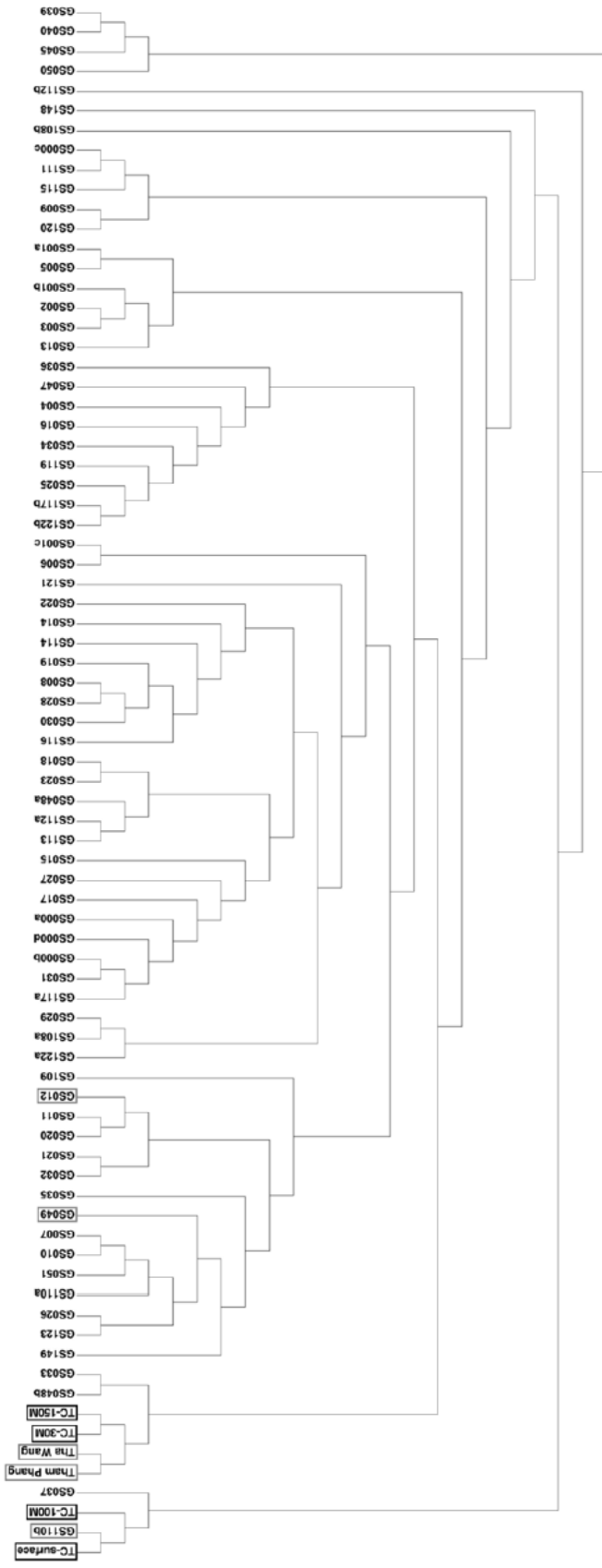
Figure 4.8 Dendrogram of PT and TC samples with other 75 GOS profile

4.6 culture-dependent and culture-independent bacterial diversity of PT and TC site

The cultured and uncultured data were combined, and expressed in Table 4.7. The total numbers of phyla upon combining data were not changed as the cultured data contained species in the phyla already included by the uncultured data. Yet, cultured experiment discovered many new genera and species that were missed by the uncultured experiment: for examples, *Erythrobacter* sp. JC212, *Sphingomonas* sp., and *Staphylococcus* sp. for TC-surface; and *Stenotrophomonas maltophilia*, *Paenibacillus* sp., and *Thermosipho melanesiensis* for TC-30M (Table 4.7). Moreover, both cultured and uncultured experiments found many clones that BLASTN indicated no significant hit to the clones in the NCBI, RDP and Greengenes databases; and thus these cultured clones and uncultured reads belonged "clone with no species assigned by BLASTN" group (Table 4.7: the last column).

Table 4.7 Combined cultured and uncultured aquatic bacteria diversity of PT and TC sites

Water sample	Number of phyla			Number of genera			Number of species			Number of clone or read assigned "no specie name" by BLASTN		
	cultured	uncultured	total	cultured	uncultured	total	cultured	uncultured	total	cultured	uncultured	total
PT-30M	N/A	63 (*13607)	63	N/A	164 (1784)	164	N/A	12 (24)	12	N/A	13583	13583
TC-surface	3 (*6)	1 (2)	3	5 (6)	1 (1)	6	0 (6)	0	0	5 (6)	2	7
TC-30M	5 (6)	50 (35755)	50	6 (9)	399 (8957)	399	4 (5)	156 (544)	156	4	35211	35215
TC-100M	3 (5)	12 (185)	12	5 (5)	27 (73)	32	4 (4)	1	5	4	184	188
TC-150M	2 (6)	54 (3922)	54	3 (3)	120 (1663)	120	0	55 (154)	55	3	3768	3771

N/A represents data not available, since no bacterial culture was performed on PT-30M.

* Number in parenthesis represents number of total isolates from cultured experiment, or total reads from uncultured experiment. Some isolates or reads could come from the same clone, so the number in parenthesis is always equal or greater than the number above the parenthesis.

CHAPER V

DISCUSSION

5.1 Water characteristics of PT and TC samples

PT and TC samples were collected from the same ocean around the same GPS latitude but longitude and sea depth, so the differences observed might involve these factors. TC had higher dissolved oxygen and alkalinity, suggesting the more living-friendly for marine organisms at the farther offshore site (Table 4.1).

5.2 Cultured isolates of bacteria from TC site

Direct and concentrate plate count and isolation methods showed TC-surface and TC-30M the greater number of aerobic bacterial abundance. On the other hand, the number of anaerobic bacterial abundance became more equally present at the deeper sea level (Table 4.2). All the colonies with distinct morphologies were isolated, and successfully sequenced for species identification (Figure 4.1). Although distinct colony morphological appearances, some species within the same marine sample were denoted the same species, for instances, *Micrococcus luteus* B88 (GenBank Accession no. EU240406.1) in TC-30M; and uncultured gamma proteobacterium clone J60-41 16S (KC603422.1) (Table 4.2). This was possible, because differences in colony morphology is useful when trying to isolate bacterial strains, species, or genera and also when plates of pure culture have become contaminated. If the majority of colonies in what it supposed to be a pure culture are look similar in appearance, but there are a couple of colonies, these colonies may arise from contamination of the plate. However, colony morphology is not a reliable way to identify bacteria, as many different types of bacteria can have similar colony morphology.

Further, species discovered by cultured but uncultured experiments, or vice versa, were possible (Na et al., 2011; Auld et al., 2013). The results from cultured experiment did not represent the true bacteria diversity because several limiting factors, such as nutrient medium recipe and incubation condition. This method merely showed microorganisms that could grow or outgrow the others under this sample transport and cultivation conditions (Auld et al., 2013). Examples of the species only found in cultured experiment included, for TC-30M: *Micrococcus luteus,* found in soil, water, air, and could live in low nutrient environment (Casida, 1980), *Paenibacillus* sp., an endospore-forming bacteria that could live in diverse environments, including soil, water, vegetable and insect larvae (Cheong et al., 2005; Thombre et al., 2013). *Thermosipho melanesiensis* which is common in high-temperature seawater (Antoine et al., 1977). For TC-100M, they were *Pseudoalteromonas lipolytica*, *Bacillus cereus* and *Pseudomonas fluorescens*. For TC-150M, they were *Bacillus* sp. and *Roseinatronobacter* sp. (Table 4.3).

5.3 Metagenomic DNA isolations

Following the quality and concentration of the metagenomic DNA determination, the total nucleic acid yield in nanograms of metagenomic DNA per liter of seawater was for TC-150M, followed by TC-100M, TC-surface and PT-30M, and TC-30M, respectively (Figures 4.3 and 4.4). The data were corresponded to the intensity of the filtrated membranes colors (Figure 4.2). TC-150M, representing the deepest sea level, showed the highest total nucleic acid yield likely because the organic matters were deposited mainly at the sediments and the deeper sea see level.

5.4 Construction of pyrotagged 16S rDNA libraries

Construction of pyrotagged 16S rRNA gene libraries for pyrosequencing using universal primers were success for all the samples. All the samples showed proper amplicon length, and weighted 3.87 ng/μl for PT-30M, TC-surface 3.87 ng/μl, TC-30M

4.52 ng/µl, TC-100M 5.81 ng/µl, and TC-150M 6.91 ng/µl (Figure 4.5). Note all the samples were also verified for concentrations and purity by Bioanalyzer (in-house BIOTEC facility).

5.5 Pyrosequencing and bioinformatic analysis

After pyrosequencing, most reads passed the read length cutoff. Read length ranged from 262 to 307 bp. More than 99% of the reads could also species identified by BLASTN. Nonetheless, TC-surface and TC-100M yielded very few numbers of sequencing reads than the other samples (Table 4.4). This was unexplained, as all the steps during the pyrosequencing passed the quality check. One possible reason suggested by Dr. Sitthichoke Tangphatsornrueng (personal communication) was that the machine might not be operated for more than a month prior to this pyrosequencing run, causing the machine inconsistent; however, the samples were re-run again and similar results were found.

Overall, archaea were minor present, consistent with previous studies that reported 0-0.4% of archaea in general marine environments (Biers et al., 2009). While Proteobacteria was the predominated phylum in both PT and TC sites: PT-30M 75.25%, TC-surface 100%, TC-30M 84.99%, TC-100M 60.54%, and TC-150M 26.09%. In addition, most members of the phylum were common inhabitants of seawater (Tables 4.5 and 4.6a) (Hugenholtz et al., 2002; Cottrell et al., 2005). Phylum Bacteroidetes were more proportionated at PT than TC. PT and TC showed more differences at genus and species levels. TC-30M, and especially the deeper sea level TC-150M, was more species diverse than PT-30M (Table 4.5).

Prokaryotic communities comparison of PT and TC sites with 75 GOS sites pointed PT and TC communities were closely related to each other, and to the marine sites of comparative GPS coordinates (2-8°N/S differences) and climates (±1-2°C differences): Tha Wang, Tham Phang, GS110b and GS049, in orderly. On the other hand, GS012 is located in a very far latitude (~30°N difference) where the climate is very cold (seawater temperature 1°C), so its community structure was found far

related to PT and TC communities (Table 4.6b). Analyses via PCoA (Figure 4.7) and clustering algorithm (Figure 4.8) suggested the computed similarity coefficient indices. The results supported the different bacteria population structures in associated with their latitudes from an equator and relative climate.

5.6 Culture-dependent and culture-independent bacterial diversity of PT and TC site

This study shows the culture-independent method the more powerful tool in identifying bacterial diversity than the culture-dependent method. Although some similar and different findings upon culture-dependent and culture-independent experiments were found, many species were detected specially by the culture-independent technique (Table 4.7). This highlighted the limitation of the current laboratory culture technique (Stressmann et al., 2011; Boase et al., 2013). The high number of clones whose significant BLASTN hits assigned "no specie name" (Table 4.7) supported previous literatures that the natural marine microbiota remains many to be discovered (Simon et al., 2011; Felczykowska et al., 2012).

Still, traditional bacteria culture method that was performed on TC samples helped fulfill the findings by culture-independent technique. A total of 42 clones were successfully isolated, given some belonged the same species; thus in summary 24 species identifiable by BLASTN , 15 species non-identifiable by BLASTN, and 3 species no significant hit by BLASTN were revealed (Table 4.7).

The cultured and uncultured data were combined (Table 4.6). The small number of cultured bacteria compared with numerous species diversity revealed by pyrosequencing supported the culture-independent technique as the more advanced approach to reveal natural microbiota. It is clear that the vast majority of bacteria in the ocean still could not be culture by traditional culture method. In general, the cultivated species were mostly Proteobacteria, supporting the data by 16S rRNA tagged pyrosequencing. One reason why isolation of the true abundant bacteria in the sea has been difficult is that we have an insufficient knowledge of the

microbes and their appropriate culture media and culture conditions. For examples, some organic or inorganic substrates, or co-growth with other organisms might be required to grow some microbes. We need cultured clones to be able to investigate the physiology of bacteria. However, culture-independent techniques allowed us to obtain the more complete prokaryotic profiles of PT and TC sites in the Andaman Sea, helped better understanding of the marine microbial ecosystems in Andaman Sea, as well as global ocean ecosystems. Most of the sea bacteria belonged to gram-negative, consistent with previous study that reported less than 10% of gram-positive bacteria in the sea (Zobell and Upham, 1944). The previous study suggested that the more lipid membranes of gram-negative bacteria make them become more resistant to environments than those of gram-positives. Similarities and differences in biodiversity, ranging from phylum to genus and species distribution levels were detected. These results supported different oceanographies affected marine biodiversity.

TC site had the higher dissolved oxygen and alkalinity than PT, suggesting the more living-friendly for marine organisms. Supportively, the data showed its greater prokaryotic species diversity. Archaea were minor present in PT and TC, consistent with previous studies that reported 0-0.4% of archaea in general marine environments (cite). Although phylum Proteobacteria was prevalent in PT and TC, the species biodiversity and distribution patterns were different (Figure 4.6). For example, genus *Sedimenticola* was observed uniquely in TC-30M (Table 4.5).

As discussed above, it is likely that bacteria may not grow well in laboratory culture because growth of a single bacterial clade in pure cultures destroys the complex relationships that occur in the natural environment. Comparative global analysis for prokaryotic structure helped disclose how the marine microbiota in this Southeast Andaman Sea water off the west coast of Thailand after the 2004 tsunami disaster were in relative to the others. Development of biodiscovery research will require innovative approaches to be developed to significantly improve culture methods. These should be based on the growing cultured dependent method, data produced by molecular and metagenomic approaches together. This knowledge would improve our understanding of the microbial diversity and ecosystem services

for which bacteria are critical components (Martiny et al, 2006). Bacterial diversity may also be useful for informing ecosystem-level conservation and management decisions (Richardson and Whittaker, 2010).

CHAPER VI

CONCLUSION

This study show different and additional findings upon culture-dependent and culture-independent techniques, and thus both might be used in complementary to yield a complete picture of the true microbiota of the environment. Moreover, the study showed metagenomic combined with NGS a high-throughput and rapid method to allow accessing the natural microbial diversity in a very powerful way.

Though the instrument, reagent and maintenance costs for NGS are high, this method became inexpensive when compared with the huge number of sequencing reads it provide (DeLong and Karl, 2005; Sogin et al., 2006). The cultured and uncultured databases of bacteria and archaea of these sites and depths worthed invaluable to marine scientist, microbiologist, and the others, helping to understand the true marine microbiota and their population structures representing the southeast Andaman See off the west coast of Thai shores.

1. By culture-dependent method, we obtained 11, 13, 9, and 9 morphologically different bacterial isolates for TC-surface, TC-30M, TC-100M, and TC-150M, respectively.

2. By culture-dependent method, we obtained 13607, 4, 10701, 189, and 38762 reads for PT-30M, TC-surface, TC-30M, TC-100M, and TC-150M, respectively.

3. The highest yield of ng metagenomic DNA per liter was recovered from TC-150M.

4. The uncultured profiles of PT, TC, and 75 GOS showed PT and TC were close to GS110b, GS049, and GS012, respectively.

5. Similarities and differences in biodiversity, ranging from phylum to genus and species distribution levels were detected. The cultured and uncultured results supported different oceanographies marine diversity.

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APPENDIX

APPENDIX A

Stock reagents

Sterile normal saline solution (0.85% NaCl)

NaCl	8.5	g

Dissolved with distilled water to 1 liter.

10x TBE buffer

Tris	54.0	g
Boric acid	27.5	g
0.5 M EDTA	20	ml

Dissolved all compositions with distilled water to 1 liter.

0.5 M EDTA pH 8.0

EDTA	18.6	g

Adjust pH to 8.0 and adjust volume to 100 mL with distilled water.

0.8x TBE buffer for agarose gel

10x TBE buffer	80	mL

Adjust volume to 1 L with double distilled water.

0.5x TBE buffer for electrophoresis

10x TBE buffer	50	mL

Adjust volume to 1 L with double distilled water.

Fosmid control

Fosmid	0.8	µl

Dye	1.0	µl
Autoclaved dH$_2$O	4.2	µl

0.55% agarose gel

Agarose powder	0.28	g
0.8x TBE	50	ml

1.5% agarose gels

Agarose powder	0.75	g
0.8x TBE	50	ml

1.75% agarose gel

Agarose powder	0.88	g
0.8x TBE	50	ml

Gram stain solution

Crystal violet stain

Crystal violet	0.5	g
Distilled Water	100	mL

Decolourizer

95% ethanol	250	mL
Acetone	250	mL

Gram iodine solution

Iodine	1	g
Potassium iodide	2	g

Distilled Water	300	mL

Safranin O solution

Safranin	2.5	g
Ethanol	100	mL

APPENDIX B

Medium

Salt Nutrient medium

NaCl	27	g
Beef extract	3	g
Peptone	5	g
Agar	15	g

Shake until the solutes have dissolved. Adjust the pH to 6.8 with 5N NaOH. Adjust the volume of the solution to 1 liter with deionized H_2O. And sterilize by autoclaving at $121^{\circ}C$ for 15 minutes.

APPENDIX C

Gram Stain protocols

1. Transfer a drop of the water to the surface of a clean glass slide.
2. Transfer 1-2 loops colony sample onto a glass slide and spread over a small area.
3. Heat fix the smear, by quickly passing it two to three times through a flame without exposing the dried film directly to the flame.
4. Flood the smear with crystal violet. Wait thirty seconds. Drain.
5. Flood the smear with Gram's Iodine. Leave for at least three seconds. Gently rinse off the iodine with tap water.
6. Decolorize by adding 95% alcohol for 10 seconds to the smear and gently rinse off excess decoloriser with tap water.
7. Flood the smear with safranin counterstain. Wait thirty seconds. Gently rinse off excess safranin with tap water. Drain slide and allow it to air dry.
8. The Gram stain is done.
9. Examine the slide under the light microscope.

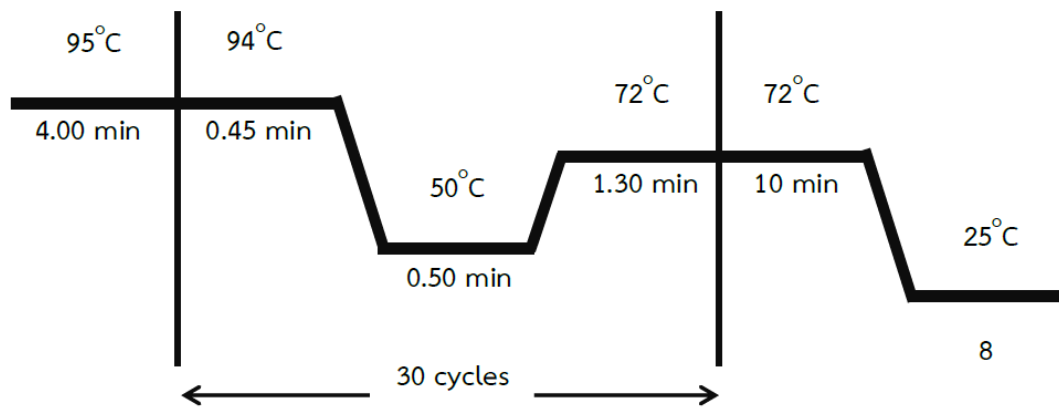APPENDIX D

Oligonucleotide primers

Primer	Forward (5′ ⟶ 3′)	Reverse (3′ ⟶ 5′)
16S rRNA	ACTCCTACGGGAGGCAGCAG	CTACCAGGGTATCTAATC
PT-30M	*ACATCGAG*ACTCCTACGGGAGGCAGCAG	*ACATCGAG*CTACCAGGGTATCTAATC
TC-surface	*TCTCTGTG*ACTCCTACGGGAGGCAGCAG	*TCTCTGTG*CTACCAGGGTATCTAATC
TC-30M	*TCTACTCG*ACTCCTACGGGAGGCAGCAG	*TCTACTCG*CTACCAGGGT ATCTAATC
TC-100M	*TAGTAGCGA*ACTCCTACGGGAGGCAGCAG	*TAGTAGCG*CTACCAGGGTATCTAATC
TC-150M	*AGACGACG*ACTCCTACGGGAGGCAGCAG	*AGACGACG*CTACCAGGGTATCTAATC

* *Italic sequence* represents pyrotagged sequence.

APPENDIX E

Thermalcycling profile

APPENDIX F

Protocol: Metagenomic DNA Isolation Kit for Water

1. To remove debris from the collected water sample, pour the water through sterile cheesecloth.

2. Filter the water (100 ml) through a presterilized 0.22-μm filter to trap cells on the filter.

3. Using forceps and scissors presoaked in 70% ethanol, remove the membrane from the filter apparatus, cut the membrane into four pieces and place them along the side (near the bottom) of a 50-ml sterile conical tube. The upper surface of the filter needs to face the center (not wall) of the tube. Do not allow the filter membrane to dry out.

4. Prepare the Filter Wash Buffer by adding 2 μl of Tween 20 to 1 ml of Filter Wash Buffer immediately before use. Add 1 ml of Filter Wash Buffer containing 0.2% Tween 20 to the filter pieces in the tube to wash off the microbes trapped on the membrane.

5. Vortex the tube at a low setting to rewet the filter pieces, and then increases the setting to the highest speed for ~2 minutes with intermittent breaks.

6. Transfer the cell suspension to a clean microcentrifuge tube, and then centrifuge the tube at 14,000 x g for 2 minutes to pellet the cells. Discard the supernatant.

7. Resuspend the cell pellet in 300 μl of TE Buffer, and then add 2 μl of Ready-Lyse Lysozyme Solution and 1 μl of RNase A to the cell suspension. Mix by vortexing.

8. Incubate at 37°C for 30 minutes.

9. Add 300 μl of Meta-Lysis Solution (2x) and 1 μl of Proteinase K to the tube. Mix by vortexing.

10. Briefly pulse-centrifuge the tube to ensure that all of the solution is in the bottom of the tube.

11. Incubate at 65°C for 15 minutes.

12. Cool to room temperature, and then place on ice for 3-5 minutes.

13. Add 350 μl of MPC Protein Precipitation Reagent to the tube and mix by vortexing vigorously for 10 seconds.

14. Pellet the debris by centrifugation for 10 minutes at 14,000 x g in a microcentrifuge at 4℃.

15. Transfer the supernatant to a clean 1.7 ml microcentrifuge tube and discard the pellet.

16. Add 570 µl of isopropanol to the supernatant. Mix by inverting the tube multiple times.

17. Pellet the DNA by centrifugation for 10 minutes at 14,000 x g in a microcentrifuge at 4℃.

18. Use a pipet tip to remove the isopropanol without dislodging the DNA pellet. Briefly pulse-centrifuge the sample and remove any residual liquid with a pipet tip, without disturbing the pellet.

19. Add 500 µl of 70% ethanol to the pellet without disturbing the pellet. Then centrifuge for 10 minutes at 14,000 x g in a microcentrifuge at 4℃.

20. Use a pipet tip to remove the ethanol without dislodging the DNA pellet. Briefly pulse-centrifuge the sample and remove any residual liquid with a pipet tip, without disturbing the pellet.

21. Air-dry the pellet for 8 minutes at room temperature. Note: Do not over-dry the pellet.

22. Resuspend the DNA pellet in 50 µl of TE Buffer.

23. Validate the size and concentration of the isolated DNA by comparison to the Fosmid Control DNA (40 kb; 100 ng/µl) provided in the kit, via gel electrophoresis on a 0.55% agarose gel.

APPENDIX G

Protocol: GF-1 Bacterial DNA Extraction Kit

1. Pellet 1 - 3 ml of bacteria culture grown overnight.

2. Add 100 μl Resuspension Buffer 1 to the pellet and resuspend the cells completely by pipetting up and down.

3. Add 15 μl lysozyme (50 mg/ml) into the cell suspension. Mix thoroughly and incubate at 37°C for 20 min.

4. Pellet digested cells by centrifugation at 10,000 x g for 3 min. Decant the supernatant completely.

5. Resuspend pellet in 180 μl of Resuspension Buffer 2 and add 20 μl of Proteinase K. Mix thoroughly. Incubate at 65°C for 20 min in a shaking waterbath or with occasional mixing every 5 min.

6. Add 400 μl of Bacterial Genomic Binding Buffer and mix thoroughly by inverting tube several times until a homogeneous solution is obtained. Incubate for 10 min at 65°C.

7. Add 200 μl of absolute ethanol. Mix immediately and thoroughly.

8. Transfer the sample into a column assembled in a clean collection tube. Centrifuge at 10,000 g for 1 min. Discard flow through.

9. Wash the column with 750 μl of Wash Buffer and centrifuge at 10,000 x g for 1 min. Discard flow through.

10. Centrifuge the column at 10,000 x *g* for 1 min to remove residual ethanol.

11. Place the column into a clean microcentrifuge tube. Add 50 - 100 μl of preheated Elution Buffer, TE buffer or sterile water directly onto column membrane and stand for 2 min. Centrifuge at 10,000 x g for 1 min to elute DNA. Store DNA at 4°C or -20°C.

APPENDIX H

Protocol: The PureLink® Quick Gel Extraction Kit

Preparing the Gel Slice

1. Excise the area of the gel containing your desired DNA fragment using a clean, sharp razor blade. Minimize the amount of agarose surrounding the DNA fragment.

2. Weigh the gel slice containing the DNA fragment using a scale sensitive to 0.001 g, and then place the gel into a 1.5- or 5.0 mL microcentrifuge tube.

Proceed to Dissolving the Gel

3. Pipet the dissolved gel piece containing the DNA fragment of interest onto the center of a Quick Gel Extraction Column inside a Wash Tube.

4. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through and replace the Quick Gel Extraction Column into the Wash Tube.

5. Add 500 µl Wash Buffer (W1), containing ethanol to the Quick Gel Extraction Column.

6. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through and replace the column into the Wash Tube.

7. Centrifuge the column again at maximum speed for 1-2 minutes to remove any residual Wash Buffer and ethanol. Discard the Wash Tube and place the Quick Gel Extraction Column into a Recovery Tube.

8. Add 50 µl Elution Buffer (E5) to the center of the Quick Gel Extraction Column.

9. Incubate the column for 1 minute at room temperature.

10. Centrifuge the Column at >12,000 × g for 1 minute. The Recovery Tube contains the purified DNA. Discard the Quick Gel Extraction Column.

11. Store the purified DNA at 4°C for immediate use or aliquot the DNA and store at -20°C for long-term storage. Avoid repeated freezing and thawing of the DNA.

VITA

Miss Khunnalack Khitmoh was born on April 26, 1989 in Songkhla province, Thailand. She graduated with a Bachelor of Science degree in Microbiology from Chulalongkorn University in 2011. She has further studied for the Master of Science degree in Microbiology Department, Chulalongkorn University since 2012. She presented her research proceeding paper in title "Culture-independent prokaryotic diversity in the Andaman Sea, Thailand" at the 25th Annual Meeting of the Thai Society for Biotechnology and International Conference, Bangkok, Thailand in October 2013.