Biodegradation of hydrocarbons in marine sediment from Bangkhuntien, Bangkok, Thailand

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การย่อยสลายทางชีวภาพของสารประกอบไฮโดรคาร์บอนในตะกอนดินทะเล จากบางขุนเทียน กรุงเทพมหานคร ประเทศไทย

นางสาวปริชญา ถิระเลิศพานิชย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปริชญา ถิระเลิศพานิชย์ : การย่อยสลายทางชีวภาพของสารประกอบไฮโดรคาร์บอนในตะกอนดินทะเลจากบางขุนเทียน กรุงเทพมหานคร ประเทศไทย (Biodegradation of hydrocarbons in marine sediment from Bangkhuntien, Bangkok, Thailand) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. พรินท์ พิดา สนธิพันธ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. เอกวัล ลือพร้อมชัย, 112 หน้า.

การย่อยสลายน้ำมันปิโตรเลียมโดยวิธีทางชีวภาพเป็นทางเลือกที่สำคัญในการนำมาประยุกต์ใช้บำบัดสิ่งแวดล้อมปนเปื้อน เนื่องจากเป็นมิตรต่อสิ่งแวดล้อมค่าใช้จ่ายต่ำ และคาดว่าสามารถย่อยสลายน้ำมันได้อย่างมีประสิทธิภาพ วัตถุประสงค์ของงานวิจัยนี้ คือ 1) เพื่อประมาณอัตราการย่อยสลายทางชีวภาพของสารประกอบไฮโดรคาร์บอนที่สนใจในดินตะกอนน้ำเค็ม โดยวัดปริมาณสารประกอบไฮโดรคาร์บอนที่เหลืออยู่ด้วยวิธี Gas Chromatography ที่ต่อกับ Flame Ionization Detector แล้วสร้างกราฟแสดงปริมาณไฮโดรคาร์บอนแต่ละชนิดที่ลดลงต่อเวลา เพื่อคำนวณอัตราการย่อยสลายของแต่ละสาร 2) เพื่อศึกษากลุ่มประชากรแบคทีเรียที่มีบทบาทในการย่อยสลายสารไฮโดรคาร์บอนที่สนใจโดยวิธี Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) และ 3) เพื่อคัดแยกแบคทีเรียที่มีประสิทธิภาพในการย่อยสลายสารประกอบไฮโดรคาร์บอนที่สนใจ โดยการเพาะในอาหารเลี้ยงเชื้อที่มีสารประกอบไฮโดรคาร์บอนแต่ละชนิดเป็นแหล่งคาร์บอนเพียงชนิดเดียว การทดลองครั้งนี้มีสารประกอบไฮโดรคาร์บอนที่สนใจ 3 ชนิด ได้แก่ น้ำมันดีเซล เฮคซะเดคเคน และฟีแนนทรีน ทั้งนี้ใช้ดินตะกอนน้ำเค็มจากบางขุนเทียน ประเทศไทย เป็นแบบจำลองของตัวอย่างจากระบบนิเวศทางทะเล เนื่องจากเป็นบริเวณที่มีการปนเปื้อนสารประกอบไฮโดรคาร์บอนเป็นระยะเวลานาน ผลการทดลองจากระบบนิเวศน์จำลองแสดงให้เห็นว่า แบคทีเรียในดินตะกอนตัวอย่างสามารถย่อยน้ำมันดีเซลความเข้มข้น 500 มิลลิกรัมต่อกิโลกรัมดินตะกอน ได้ 84% ด้วยอัตรา 81.96 มิลลิกรัมต่อกิโลกรัมดินตะกอนต่อวัน สามารถย่อยเฮคซะเดคเคนความเข้มข้น 250 มิลลิกรัมต่อกิโลกรัมดินตะกอน ได้ 99% ด้วยอัตรา 86.43 มิลลิกรัมต่อกิโลกรัมดินตะกอนต่อวัน และสามารถย่อยฟีแนนทรีนความเข้มข้น 125 มิลลิกรัมต่อกิโลกรัมดินตะกอน ได้ 96% ด้วยอัตรา 9.03 มิลลิกรัมต่อกิโลกรัมดินตะกอนต่อวัน จากการวิเคราะห์กลุ่มประชากรแบคทีเรียหลักด้วยเทคนิค PCR-DGGE พบว่าส่วนใหญ่เป็นแบคทีเรียย่อยสลายน้ำมันที่เคยมีการค้นพบในแหล่งน้ำเค็ม จากการทดลองคัดแยกแบคทีเรียจากดินตะกอนพบแบคทีเรียในกลุ่ม *Acinobacter* sp. *Bacillus* sp. และ *Staphylococcus* sp. จากอาหารเลี้ยงเชื้อที่มีเฮคซะเดคเคนเป็นแหล่งคาร์บอน นอกจากนี้ยังสามารถคัดแยก *Pseudomonas* sp. ได้จากอาหารเลี้ยงเชื้อที่มีฟีแนนทรีนเป็นแหล่งคาร์บอน และยังสามารถคัดแยก *Bacillus* sp. และ *Pseudomonas* sp. ได้จากอาหารเลี้ยงเชื้อที่มีน้ำมันดีเซลเป็นแหล่งคาร์บอนด้วย แม้ว่าชนิดของแบคทีเรียที่คัดแยกได้จะต่างจากที่พบด้วยเทคนิค PCR-DGGE แต่ผลการทดลองทั้งหมดแสดงให้เห็นว่าสามารถใช้วิธีทางชีวภาพและแบคทีเรียท้องถิ่นของดินตะกอนน้ำเค็ม เพื่อบำบัดสารประกอบไฮโดรคาร์บอนที่ปนเปื้อนได้

คำสำคัญ : ดินตะกอนน้ำเค็ม, การย่อยสลายทางชีวภาพ, น้ำมันดีเซล, ฟีแนนทรีน, เฮคซะเดคเคน

สาขาวิชา การจัดการสารอันตรายและสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5787524020 : MAJOR HAZARDOUS SUBSTANCE AND ENVIRONMENTAL MANAGEMENT
KEYWORDS: BIODEGRADATION / DIESEL / PHENANTHRENE / HEXADECANE / MARINE SEDIMENT

PARICHAYA TIRALERDPANICH: Biodegradation of hydrocarbons in marine sediment from Bangkhuntien, Bangkok, Thailand. ADVISOR: PRINPIDA SONTHIPHAND, Ph.D., CO-ADVISOR: ASSOC. PROF. EKAWAN LUEPROMCHAI, Ph.D., 112 pp.

Biological method is an important alternative for solving petroleum hydrocarbon contamination issue because it is environmental friendly, low cost and has a potential to effectively remediate the oil. The objectives of this study were; 1) to estimate the biodegradation rate of diesel, hexadecane, and phenanthrene in marine sediment by using Gas Chromatography with Flame Ionization Detector (GC-FID) to monitor the remaining amount of hydrocarbons and constructing the degradation plot of each hydrocarbon per time for calculation of the degradation rate, 2) to investigate the hydrocarbon-degrading bacterial community in marine sediment by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) technique and 3) to isolate diesel-, hexadecane- and phenanthrene-degrading bacteria using media with each hydrocarbon as a sole carbon source. In this study, there were 3 interesting hydrocarbons including diesel, hexadecane and phenanthrene. The marine sediments were collected from Bangkhuntien, Thailand to use as a surrogate of a chronically hydrocarbon contaminated bay marine ecosystem. The results from hydrocarbon-contaminated microcosms showed that the removal percentage and the degradation rates for 500 mg kg sediment^{-1} diesel were 84% and 81.96 mg kg sediment^{-1} day^{-1}, for 250 mg kg sediment^{-1} hexadecane were 99% and 86.43 mg kg sediment $^{-1}$ day^{-1} and for 125 mg kg sediment^{-1} phenanthrene were 96% and 9.03 mg kg sediment^{-1} day^{-1}, respectively. The dominant bacterial populations analyzed by PCR-DGGE technique were mostly recognized as halophilic-hydrocarbon degrading bacteria. The isolated strains from enrichment technique were belonged to *Acinobacter* sp., *Bacillus* sp. and *Staphylococcus* sp. from hexadecane supplemented broth. We also isolated *Pseudomonas* sp. from phenanthrene supplemented broth and *Bacillus* sp. and *Pseudomonas* sp. from diesel supplemented broth. Although, the species of isolated bacteria were different from those detected by PCR-DGGE technique. The overall results suggested that bioremediation and indigenous bacteria in marine sediments could be applied to treat the contaminated hydrocarbons.

Keywords: Marine sediment, Biodegradation, Diesel, Phenanthrene, Hexadecane

Field of Study: Hazardous Substance and Environmental Management
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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Chapter I
Introduction

1.1 Introduction

Petroleum oil contamination in marine ecosystem is considered one of the major causes that harms the environment (Hara et al., 2003). Although there are both natural and man-made sources of oil contamination in the environment, human activities such as accidentally spill, discharging from land and offshore manufacture are the major source of petroleum oil contamination (Parker, 2008). Petroleum contamination in the environment affect human and other living organisms in different way, either direct or indirect way such as health effect and economical effect. The biodegradation of hydrocarbons by local microorganisms is considered as a potential removal process. However, the study of petroleum biodegradation in the marine sediment collected in Thailand, especially around Bangkok area is limited.

There were several oil spill events in the Gulf of Thailand, for example, crude oil spill from the crude oil flow line leakage of PTTGC Co. Ltd. in 2013. The oil spill occurrence data provided by the Marine Department of Thailand indicated the continuous oil spill in the marine environment which affect the adverse chronic consequence to living organism at that area. The spills still remain in the nature even after a clean-up process had been done, which require further clean-up process (Marine Department website, cited 2016).

There are three main methods that have been used to remediate petroleum hydrocarbon contamination in nature (i.e. physical method, chemical method and biological method) (Humbert et al., 2010). In this study, biological method is believed to be the most interesting method to manage the oil contamination problem.

In this study, the main sampling station is in Bangkhuntien district, Bangkok, Thailand (Figure 1). Bangkhuntien is the only area in Bangkok that connected to the Gulf of Thailand. Bangkhuntien is one of the locations for ecotourism attraction,

including mangrove forests and sightseeing by boats along the canal. This place is also famous for seafood restaurants because the canal is connected to the Gulf of Thailand where people are able to find fresh and good quality seafood. The sampling station is a petrol station located along Sahakorn canal at Bangkhuntien – Chaitalae road. This canal is a main transportation route where local people use their boats to transfer/exchange their products (floating market) and to commute. Boats around this area stop at this station to fill their fuel tanks or rest and fix their boats. So it is possible for oil leakage or oil contamination at this sampling site. It is an interesting place to assess microbial community and expect for the novel hydrocarbon-degrading bacterial strains. The site is considered a marine-related environment because of seawater intrusion into the canal (Figure 1).

Figure 1 Sampling station at Sahakorn Canal, Bangkhungtien which is located at 438 km away from the Gulf of Thailand (Created by google maps).

This study investigated hydrocarbon degradation rates of diesel, hexadecane, and phenanthrene in marine sediment. In addition, the community of hydrocarbon-degrading bacteria that potentially played an important role in degrading diesel, hexadecane, and phenanthrene was monitored. Hence the indigenous bacteria that capable to degrade the interesting hydrocarbons had been expected to be appeared in this sediment sample and could be analyzed by PCR-DGGE technique which was a conventional technique used for bacterial community studying. We also expected to be able to isolate the hydrocarbon degrading bacteria by the media enriched with the interesting hydrocarbon.

1.2 Hypothesis:

1.2.1 Indigenous bacterial consortium in marine sediment are able to significantly degrade diesel, hexadecane, and phenanthrene.

1.2.2 The hydrocarbon- degrading bacteria which potentially play a key role in remediating hydrocarbons of interest can be revealed by Denaturing Gradient Gel Electrophoresis (DGGE).

1.2.3 Different hydrocarbon-degrading bacterial species can be isolated from marine sediment enriched with diesel, hexadecane and phenanthrene.

1.3 Objectives:

1.3.1 To estimate the biodegradation rates of diesel, hexadecane and phenanthrene in marine sediment

1.3.2 To investigate the hydrocarbon-degrading bacterial community in marine sediment

1.3.3 To isolate diesel-, hexadecane- and phenanthrene-degrading bacteria from marine sediment

1.4 Scope of Study:

1.4.1 This study focused on biodegradation rates and hydrocarbon-degrading bacterial community in marine sediment collected from Sahakorn canal, Bangkhuntien, Bangkok, Thailand. The sample was collected in two time points, representing winter and summer seasons.

1.4.2 Conductivity, pH, and temperature were measured on site. Salinity, total phosphorus (TP), total nitrogen (TN), and total carbon (TC) were analyzed in the laboratory.

1.4.3 Diesel was purchased from the petrol station, located on Sahakorn canal, Bangkhuntein, Bangkok. Hexadecane and phenanthrene were purchased from Sigma Company.

1.4.4 Microcosms were set up to determine the degradation rates of diesel, hexadecane, and phenanthrene. The degradations of diesel, hexadecane, and phenanthrene were measured by GC-FID. Hydrocarbon-degrading bacterial community was monitored during an incubation period, using PCR-DGGE analysis.

1.4.5 The enrichment and isolation experiment of diesel-, hexadecane- and phenanthrene-degrading bacteria were conducted using marine sediment as a seeding.

Chapter II

Theoretical Background and Literature Review

2.1 Hydrocarbon classification

2.1.1 Hydrocarbon is a chemical compound, mainly composing of hydrogen and carbon atoms.

Saturated hydrocarbon – The structure that hydrogen atoms are attaching to every carbon atom and the carbon atoms are connecting to each other by single bond (Figure 2) (Gauthier, cited 2016). Aromatic hydrocarbon – the aromatic hydrocarbon are considered as any hydrocarbon containing unsaturated cyclic hydrocarbon. The double bonds in the hydrocarbon are in resonance form, which allow electron to delocalize or move from one atom to another atom. The most famous aromatic hydrocarbon is benzene (Wyman, cited 2016).

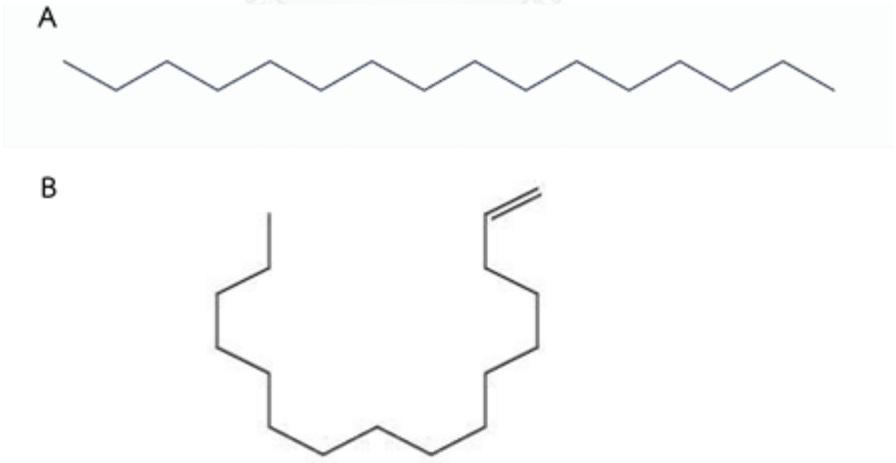

Figure 2 Examples of saturated and unsaturated hydrocarbon structure
A; Hexadecane (saturated) and B; Hexadecene (unsaturated) *(Gauthier, cited 2016)*

Unsaturated – The hydrocarbon that have double or single bond in it (Figure 2).

Alkanes – the hydrocarbon that are all saturated. Alkanes can be forming in linear structure or branched structure. The formula for an alkane is $C_nH_{(2n+2)}$ where n is a number of atom of the element. (Figure 3). Sometimes alkanes are found in ring form. The ring form structure called 'Cycloalkanes', still being saturated because the ring form is also containing single bond (Figure 4) (Gauthier, cited 2016).

Alkenes – Carbon atoms in alkenes are connected to each other with the double bond, inducing this type being unsaturated. The formula for this type of hydrocarbon maybe tricky because of number of double bond in the compound can vary. Generally, the formula for alkenes with 1 double bond are written as C_nH_{2n} (Gauthier, cited 2016).

Alkynes – The hydrocarbon compound containing triple bond in it. As same as alkenes, they are unsaturated hydrocarbon. (Gauthier, cited 2016).

.1.2 Composition of hydrocarbon compounds in petroleum hydrocarbon

Focusing on petroleum products, the hydrocarbon components are commonly divided in to two parts as following; oil part (saturated and aromatic) and polar part (resin and asphaltene; Harayama et al., 1999).

1.) Oil part

- Saturated hydrocarbon

Including alkanes (with C_5 to C_{40}), branched alkanes, cycloalkanes or naphthalene, terpenes and steranes (cycloalkanes with multiple rings), and alkenes (Figures 3 and 4)

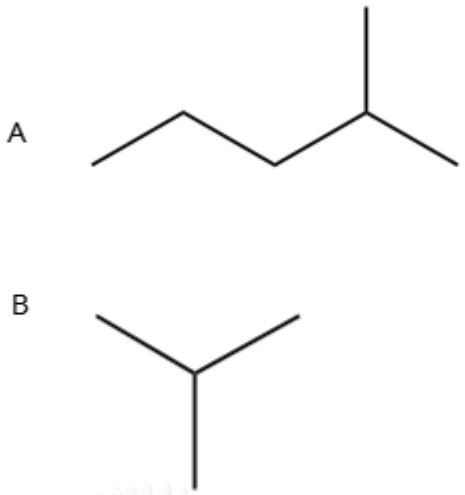

Figure 3 Example of saturated hydrocarbon found in petroleum product
A = 2,3-Dimethylbutane and (B) = Isobutane (Testa and Winegardner, 2000)

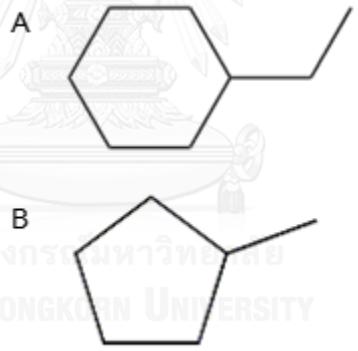

Figure 4 Examples of cycloalkane found in petroleum product
A; Ethylcyclohexane B; Methylcyclopentane *(Testa and Winegardner, 2000)*

- Aromatic hydrocarbon

The unsaturated hydrocarbon which contains at least one benzene ring in it. Benzene ring is a six carbon atom ring that holding shared hydrogen inside (Figure 5). Sometimes the ring may connects to other alkyl group that leads to other type of aromatic compound (Figure 5)

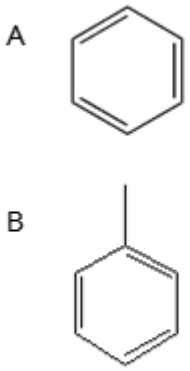

Figure 5 Examples of aromatic hydrocarbon found in petroleum product
A; Benzene B; Toluene (Testa and Winegardner, 2000)

The ring may connected to other ring(s) that cause a structure called polycyclic aromatic hydrocarbon (PAH) (Figure 6)

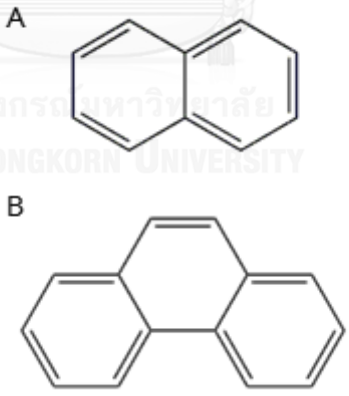

Figure 6 Example of PAH found in petroleum product
A; Naphthalene B; Phenanthrene (Testa and Winegardner, 2000)

This structure is considering as environmental toxic, the more ring connected, the harder degrading form.

2.) Polar part

- Resin

The resin is having a key role in stability of petroleum. The resin is usually found in dark brown, shiny and sticky.

- Asphaltene

Asphaltene is the constituent that has the largest and heaviest part of petroleum. The exact component for asphaltene cannot be identified. The characteristic of asphaltene is usually black, crumby and polished.

2.2 The properties of diesel, hexadecane, and phenanthrene

2.2.1 Hexadecane

Hexadecane is the aliphatic hydrocarbon consist of 16 carbon atoms and 34 hydrogen atoms. The structure of hexadecane is showing in Figure 7 and the properties are describing in table 1.

Figure 7 2D structure of hexadecane
(http://www.chemicalbook.com/ProductChemicalPropertiesCB8854465_EN.htm)

Table 1 Properties of hexadecane

Property	Information	Reference
IUPAC name	Hexadecane	NCBI[2] (2016)
Formula	$C_{16}H_{34}$	NCBI[2] (2016)
Molecular weight	226.44116 g/mol	NCBI[2] (2016)
Melting point	18.14 °C	NCBI[2] (2016)
Boiling point	286.5 °C(lit.)	NCBI[2] (2016)
Density	0.77335 g/mL at 20 °C	NCBI[2] (2016)
Vapor density	7.8	NCBI[2] (2016)

Property	Information	Reference
Vapor pressure	1 mm Hg at 105.3 °C	NCBI[2] (2016)
Volatility	Non-volatile	NCBI[2] (2016)

In addition, hexadecane is usually employed as a representative alkane in petroleum hydrocarbon (Schurig et al., 2014).

2.2.2 Phenanthrene

Phenanthrene is a polycyclic aromatic hydrocarbon consist of 3 benzene rings that attached to each other as showed in Figure 8. It is used as the representative low molecular weight PAH existing in petroleum product. The properties of phenanthrene are describing in Table 2

Figure 8 2D structure of phenanthrene
(http://www.chemicalbook.com/ProductChemicalPropertiesCB8854465_EN.htm)

Table 2 Properties of phenanthrene

Property	Information	Reference
IUPAC name	Phenanthrene	OSHA (2006)
Formula	$C_{14}H_{10}$	OSHA (2006)
Molecular weight	178.23 g/mol	OSHA (2006)
Melting point	100 °C	OSHA (2006)
Boiling point	340 °C	OSHA (2006)
Density	1.179 g/mL at 25 °C	NCBI[1] (2016)
Vapor density	6.15	NCBI[1] (2016)

Property	Information	Reference
Vapor pressure	1 mm Hg @ 118.2°C	OSHA (2006)
Volatility	Semi-volatile	NCBI[1] (2016)

.2.3 Diesel

Diesel is widely known as an engine fuel that human apply in daily life such as shipping trucks, construction equipment, trains and cargo and cruise ships. Typical hydrocarbon compound in some petroleum product are describing in Table 3.

Table 3 Typical hydrocarbon compound in some petroleum product (%) (Wang et al., 2006)

Group	Compound Class	Gasoline	Diesel	Light Crude oil	Heavy crude oil
Saturates	Alkanes Cyclo-alkanes Waxes	50-60 45-55 ~5	65-95 35-45 30-50 0-1	55-90	25-80
Olefins		5-10	0-10		
Aromatics	BTEX PAHs	25-40 15-35	5-25 0.5-2 0.5-5	10-35 0.1-2.5 0.5-3	15-40 0.01-2 1-4
Polar compounds	Resins Asphaltenes		0-2	0-10 0-10	2-25 0-20
Sulfur		<0.05	0.05-0.5	0-2	0-5
Metal (ppm)				30-250	100-500

According to Table 3, it shows that diesel is rich in broad range of hydrocarbon (alkane, cycloalkane and PAHs).

2.3 Petroleum hydrocarbon contamination in marine environment of Thailand

The Department of Marine and Coastal Resource (DMCR website, cited 2016), Thailand revealed that the 50,000 L of crude oil leaked from the crude oil flow line PTTGC Co. Ltd. in 2013 which covered the seawater surface around 600 m^2. The water parameters (e.g. temperature, salinity, dissolved oxygen, heavy metals and polycyclic aromatic hydrocarbons) were higher than the standard level 4 which human cannot perform a recreation activity around this contaminated area. Due to the DMCR report, even the situation seemed to be better but the parameters which indicate environmental health still remained above level after 1 year later.

The Department of Marine and Coastal Resource (DMCR), Thailand has reported petroleum oil contamination events around Thailand's coastal from 2004 to 2011. The petroleum oil contamination level in Thailand varied from 0.16 μg l^{-1} to 41 μg l^{-1} (DMCR, 2015). The DMCR has categorized the source of petroleum oil contamination in shoreline into two major sources. The first source is from inland contamination, caused by industrial activities such as petroleum oil contaminated wastewater effluent. The second source is from off land contamination, cause by transportation accidents (e.g. shipwreck), leakage of oil containers or pipes, illegal oil dumping and oil leakage during ship fixation. All petroleum oil residues are potentially carried out by wind and water currents then become tar ball along shoreline.

For petroleum oil contamination survey in Thailand, Wattayakorn and Rungsupa (2012) reported the detection of petroleum oil in seawater, sediment, and mussels from samples collected from Koh Srichang-Sriracha, Chonburi, Thailand. The petroleum oil concentration was measured by UV fluorescence spectroscopy (UVF), using chrysene as a standard reference compound. They found that the petroleum hydrocarbon concentrations in inshore coastal water, seawater, sediment, and mussel tissue were 1.65±2.14 μg l^{-1}, 0.40±0.29 μg g^{-1}, 29.4±30 μg g^{-1}, and 43.7±55.5 μg g^{-1}, respectively. The main sources of petroleum oil contamination were human activities, including oil contaminated waste and lubricant from shipping and water transportation around the studied area (Wattayakorn and Rungsupa, 2012).

2.4 Effects of petroleum contamination

Oil spill along shoreline affects living organisms, natural resources, and economic (Zhu et al., 2010). Firstly, in oil spill event, hydrocarbon concentration in seawater becomes higher, which is toxic to animal's health. The spill can also directly harm the health of marine organisms such as oil slick covering on their body which affect their living lifestyle. Secondly, a vision of oil floating on seawater and attaching to the beach reduces an intrinsic value of the environment and destroys natural resource. Consequently, people are not able to access the full use of this area. Finally, the effect on economic approach is that there will be fewer tourists in the oil spill impacted environment and resulted in reduced income for local people who run tourism business.

ITOPF (2011) provided some mechanisms of the petroleum hydrocarbon that affect the living organism. Firstly, the hydrocarbon might disfunction the organism organs. Secondly, it might harm the organism by its concentration. Thirdly, we might lost the important species due to the environmental change and the dominant species might success by other species. Finally, the petroleum might affect organism indirectly by takeover their living place or shelter.

For economic approach, the trend had divided into two. Firstly, the money people lost due to the contamination, for example, the room reservation of the hotel were cancelled because the presence of oil in the environment and the reduction fishery product supply which less income. Secondly, the money people needed to spend more for the additional process.

For the hydrocarbon of interest in this study, hexadecane causes eye, skin and respiratory tract irritation. Hexadecane that enter to human body via ingestion route could damage lung. There is no carcinogenic to human report for this substance. Phenanthrene also causes eye, skin and respiratory tract irritation. Higher amount of phenanthrene with skin contact can cause allergic symptom. The chronic effect such as carcinogenic report of phenanthrene was not obvious. The diesel, which is used as fuel for marine engine, is classified as the possible carcinogenic to human (BP diesel

MSDS). It causes eye, skin and respiratory tract irritation. Inhalation of large amount causes drowsiness and dizziness which may leads to the central nervous system depression as a worst case. However, these exposure routes could avoid by personal protection equipment during the experiment. Some toxicology of these hydrocarbons were shown in Table 4.

Table 4 Toxicities of hexadecane, phenanthrene, and diesel

	Hexadecane	**Phenanthrene**	**Diesel**
Oral LD_{50} (mg L^{-1})	5,000	700	7,600
Inhalation LD_{50} (mg L^{-1})	N/A	N/A	4.1
Dermal LD_{50} (mg L^{-1})	3,160	N/A	>4,300
Persistence	Readily biodegradable	Partially biodegradable	Partially biodegradable
Reference	Sigma MSDS	Sigma MSDS	BP MSDS

2.5 Bioremediation of petroleum hydrocarbon

The current oil spill treatment methods are listed mechanical, chemical, and biological methods (Zhu et al., 2010). Booms and skimmer is an example of mechanical method. Boom is an equipment that is used to control surface oil spreading and known as the first response of oil spill method in US. Then the trapped oil was removed by the device called skimmers. Another example of mechanical method is Absorption by sorbent (oleophilic and hydrophobic materials) such as peat moss. However this technique is only suitable for small oil spill event. Chemical method is commonly used for cleaning up oil spill from contaminated shorelines. Dispersant is the chemical which is able to break down oil droplet into small molecules, enhancing the rates of evaporation and biodegradation process. Another chemical that have been used to eliminate oil spill Gelling agent. Gelling agent is the chemical that helps oil solidify. Therefore, the rubber-like form of oil was recovered easily.

The main focus of this study is biological method or bioremediation. Biological method relies on natural mechanism, especially, by microorganisms (U.S. congress,

1991). Natural hydrocarbon-degrading bacterial species are ubiquitous in the environments; however, they take time to degrade the oil. Enhancing factors i.e. fertilizer and surfactant are commonly applied to facilitate the natural biodegradation. However, mechanical and chemical methods are not able to completely remove oil spills from the contaminated site. Another disadvantage is that the byproducts obtained from mechanical and chemical methods are weathering oil which is hard to remove from the contaminated site. Installation a physical equipment or applying high chemical dosage into the site can impact the natural environment; those methods cannot completely remove total amount of the petroleum products (Humbert et al., 2010). Therefore, biological method has advantages over mechanical and chemical methods because of its complete mineralization of oil (Wang et al., 2013). In addition, biological method is cost effective and environmental friendly (ASTM, 2011). Moreover, microorganisms are ubiquitous, numerous, and believed to be able to adapt to the environment rather than the mechanical and chemical methods (Stevens and Aurand, 2008). Biological method focuses on natural removal of pollutant by microorganisms, this also known as 'Biodegradation'. U.S. congress (1991) described biodegradation as an ability of living microorganisms in breaking down the pollutant into other molecules in less toxic form. Bioremediation is a term for any technique for improving the biodegradation such as modifying or adding some nutrients to the contaminated site. Adams et al. (2015) reviewed that microorganisms are able to transform hydrocarbon molecules using various mechanisms such as transform a pollutant into less toxic form and reduce the size of molecules with the pollutant inside the molecule. These activities are related to energy production in microbial cells. Bioremediation can be divided into two processes: bioaugmentation and biostimulation.

2.5.1 Bioaugmentation

Bioaugmentation is the process that introduces microorganisms with ability in degrading pollutants of interest into a contaminated site. For example, a study of Ueno et al. (2006) introducing *Pseudomonas aeruginosa* strain WatG into 0.3% diesel contaminated soil. The result indicated that the existing of this strain helps diesel

degradation in soil. Moreover, the WatG produced dirhamnolipid which may enhance biodegradation in soil by stimulating soil indigenous strain.

2.5.2 Biostimulation

Biostimulation is the process that adds an enhancing factor to modify an environmental condition to favor indigenous microbial ability in degrading pollutants of interest. The enhancing factors commonly applied into the contaminated sites are nutrient and electron acceptor. Atagana (2004) had successfully remediate 38,000 mg kg^{-1} of 2-3-ring polycyclic aromatic hydrocarbons (PAHs) within 19 months by adding organic nutrient from wood chips and sewage sludge. The result showed that it was 100% removal of the pollutants. Inorganic nutrient can also be added to increase biodegradation rate. Chorom (2010) employed inorganic fertilizer (Nitrogen, Phosphorus, and Potassium; NPK) and found 40-60% reduction of paraffin and isoprenoid within 10 weeks. The addition of nutrients optimized the carbon and nitrogen ratio in the contaminated site. This helps enhance microbial ability in degrading the pollutants. In addition, the combination of adding hydrocarbon-degrading bacteria with enhancing factors has been studied. Abdulsalam (2009) reported 65% removal of 40,000 mg kg^{-1} of motor oil by introducing Bacillus sp., and Pseudomonas sp. with NPK fertilizers into the contaminated site.

2.6 Biodegradation rates of hydrocarbon

Das and Chandran (2011) showed that the biodegradation of hydrocarbons by marine bacteria was in the range of 0.003% to 100%. They reported that temperature and nutrients were the two factors that most affect the petroleum hydrocarbon degradation. The temperature affects the viscosity of the petroleum hydrocarbons as well as the biodegradation rate. Their study suggested the highest degradation rate in soil, freshwater and marine environment were occurred at 30°C-40°C, 20°C-30°C and 35°C-40°C, respectively. The nutrients, especially nitrogen and phosphorus, usually became limiting factors in the environment during petroleum hydrocarbon

contamination. The contaminant supply carbon into the environment which the bacteria also need other nutrient in order to survive in the hydrocarbon contaminated environment. Muangchinda et al., (2013) used sediment sample from Don Hoi Lot, Samut Songkram Province, Thailand for estimation of polycyclic aromatic hydrocarbon degradation (PAH). The PAHs was believed to be the toxic and persist hydrocarbon in the environment. This aromatic compound also used as aromatic compound model in the petroleum hydrocarbon. They investigated the degradation rate of acenaphthene and phenanthrene were ranging from $3.1x10^3$ to $1.3x10^5$ MPN g $sediment^{-1}$ and $2.8x10^3$ to $5.3x10^4$ MPN g $sediment^{-1}$. Woolfenden et al., (2011) spiked diesel 4,000 mg kg $sediment^{-1}$ and reported the diesel degradation rate of 245 mg kg $sediment^{-1}$ per year using the sediment from Antarctica. They also predicted the contamination at this concentration would persist in the environment for at least 5 years at this biodegradation rate.

2.7 Biodegradation pathways of petroleum hydrocarbons

2.7.1 Alkane biodegradation

The alkane biodegradation can occur by terminal oxidation and subterminal oxidation.

1.) Terminal oxidation

The *n*-alkanes biodegradation usually begin with oxidation at the terminal methyl group, converting the alkane into primary alcohol by alkane hydroxylase (AH) as shown in Figure 9. The primary alcohol then was transformed into aldehyde by alcohol dehydrogenase. Finally, the aldehyde ends up with fatty acid which may conjugate with CoA to be used for acetyl-CoA production under β-oxidation (Rojo, 2009)

Moreover, both alkane ends can be reacted with **ω**-hydroxylase as illustrated in Figure 9, the enzyme converts a methyl group at **ω** position of the fatty acid to generate **ω**-hydroxy fatty acid.

2.) Subterminal oxidation

The oxidation can alsooccurrs at subterminal position, generating a secondary alcohol, which then being converted to ketone. The ketone then reacts with Baeyer–Villiger monooxygenase to forman ester. The ester was hydrolysed by esterase, forming alcohol and fatty acid as shown in Figure 9 (Rojo, 2009).

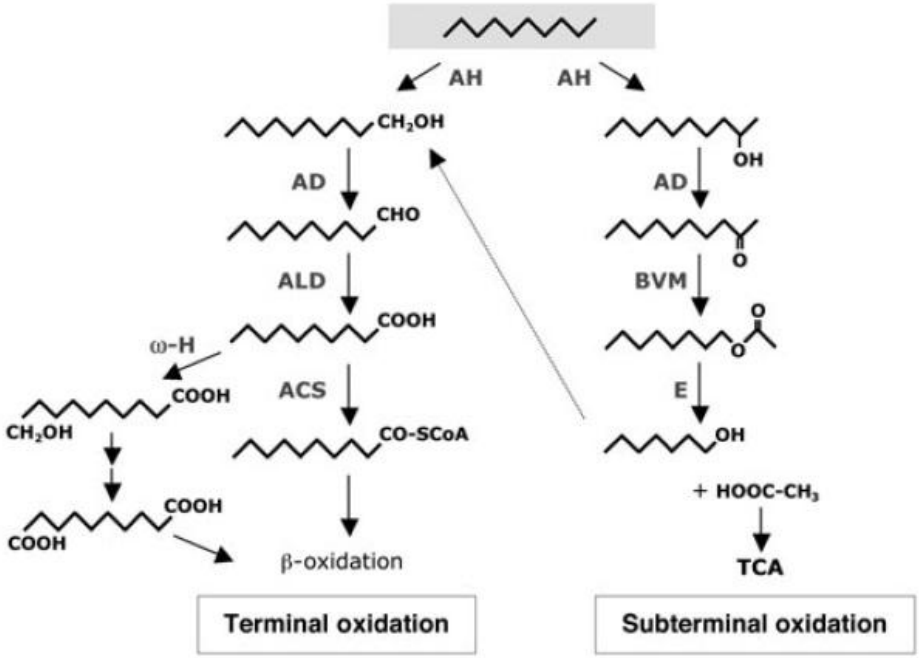

Figure 9 Degradation of n-alkane

AH, alkane hydroxylase; AD, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; ACS, acyl-CoA synthetase; w-H, w-hydroxylase; BVM, Baeyer–Villiger monooxygenase; E, esterase; TCA, tricarboxylic acids cycle. (Rojo, 2009).

2.7.2 Aromatics biodegradation

The aromatics hydrocarbons commonly degrades by dihydroxylation at one aromatic ring by multicomponent dioxygenase as the first step (Harayama et al., 1999: Fritsche and Hofrichter, 2008). This process is followed by ring cleavage which is catalyzed by dioxygenase. The cleavage can occur at *ortho* position (*o*-cleavage) or *meta* position (*m*-cleavage). The product from *o*-cleavage will finally transformed into dicarboxylic acid which is going to attach to CoA, follow by thiolytic cleavage forming

acetyl- CoA and succinate. While the product from *m*-cleavage was metabolized into formate, acetaldehyde and pyruvate. The final products from both cleavage reactions will further enter to the tricarboxylic acids (TCA) cycle or central metabolism as shown in Figure 10 (Fritsche and Hofrichter, 2008). The degradation pathway of phenanthrene is shown in Figure 10 (Harayama et al., 1999). Phenanthrene can be degraded by dihydroxylation and *meta* cleavage, being catalyzed by multicomponent dioxygenase containing reductase and *meta* cleavage enzyme containing iron, respectively.

Several intermediates (i.e., 3,4-Dihydroxyphenanthrene, *o*-Phthalate, Acetyl CoA and succinate CoA) are generated during the reaction to eventually being transformed to CO_2 and H_2O as the end products (Harayama et al., 1999).

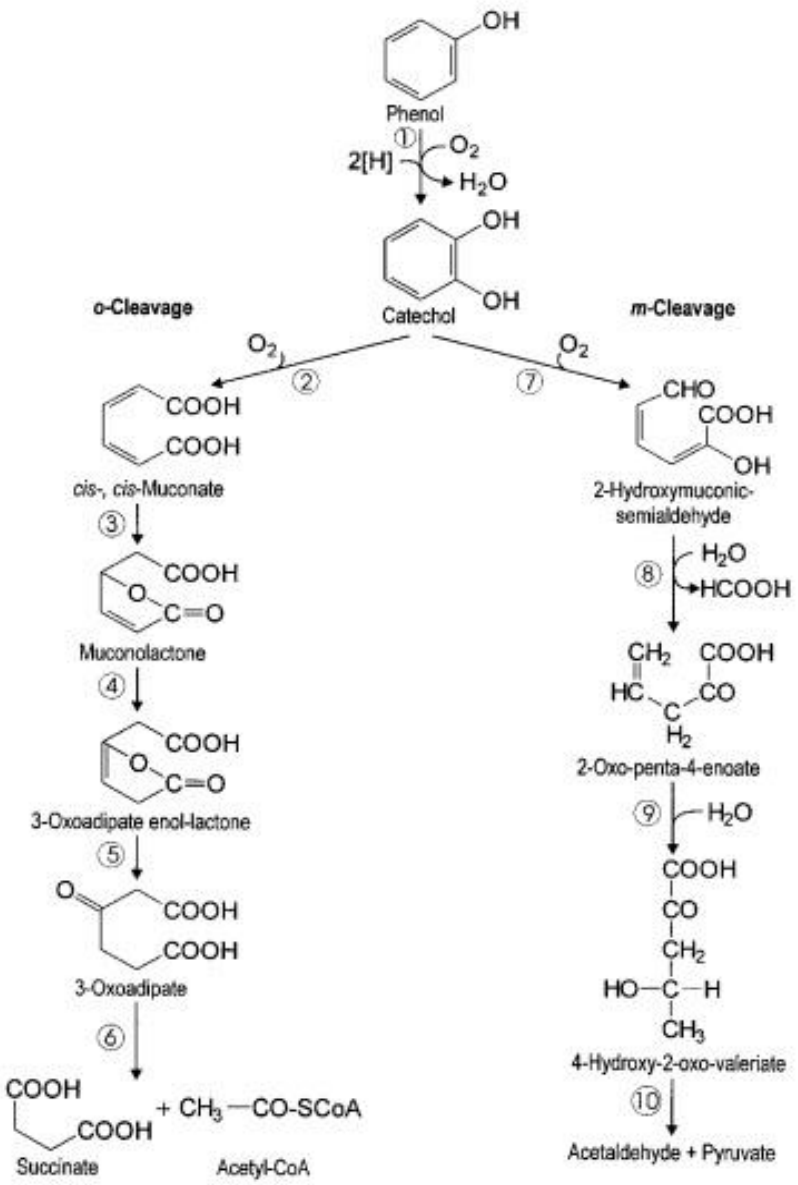

Figure 10 o-cleavage, m-cleavage and end products of aromatics compound (Fritsche and Hofrichter, 2008)

2.8 Hydrocarbon-degrading bacteria in marine environments

Marine environment is considered the major water resource on earth. Marine environment contains high amount of nutrients, water and energy for supporting living organisms. Therefore, marine environment is an interesting ecosystem to investigate the diversity and abundance of microorganisms of interest (Dash et al., 2013).

Hydrocarbon-degrading bacteria can be found in natural environments (Head et al., 2006).

Prince et al. (2003) reported one of hydrocarbon contamination source comes from nature, this supporting hydrocarbon-degrading bacteria were potentially ubiquitous in nature. Marisa et al (2013) reported a significant amount of crude oil was biodegraded by beach sand after 15-day incubation, especially when the beach and was amended with biostimulation or bioaugmentaion method.

Dash et al. (2013) has mentioned about marine bacterial genera that holding hydrocarbon degradability. The genera are *Acinetobacter*, *Marinococcus, Methylobacterium, Micrococcus, Nocardia, Planococcus,* and *Rhodococcus.* Yakimov et al. (2007) also mentioned about 'obligate hydrocarbonoclastic bacteria' or OHCB. They believe that the marine OHCB played a major role in cleaning up oil that contaminating the marine ecology. Several species that have been mentioned in their research but the most functionable is the specie named *Marinobacter* which contains a larger number of unrevealed gene compare to *Alcanivorax*, the well-known hydrocarbon- degrading bacterial species that has been previously discovered. Therefore, there are a number of species that has been discovered and having abilities to degrade various compound. Hydrocarbon-degrading bacteria that can utilize a specific hydrocarbon substrate are shown in Table 5.

Table 5 Summary of hydrocarbon-degrading bacteria with a specific substrate

Substrate	Microorganism	Reference
Benzene	*Acinetobacter*, *Delftia*, *Pseudomonas putida*, *Shewanella* and *Stenotrophomonas*	Sheppard et al. (2013)
Hexadecane	*Alcanivorax*, *Bacillus, Brevimonas*, *Chryseobacterium*, *Pseudomonas*, *Rhodococcus* and *Roseobacter*	
Phenanthrene	*Cycloclasticus pugetii, Colwellia aestuarii, Olleya marilimosa, Neptuniibacter caesariensis, Pseudoalteromonas tetraodonis* and *Halomonas axialensis*	Gutierrez et al. (2013)
Hexadecane	*Alcanivorax borkumensis, Marinobacter algicola* and *Oleibacter marinus*	
Crude oil	*Pseudomonas stutzeri* strain JMC01, *Microbacterium aquimaris* strain DT27, *Marinobacter hydrocarbonoclasticus* strain P210(9), *Gordonia amicalis* strain CS-12, *Pseudomonas aeruginosa* strain NBAII AFP-4	Hassanshahian et al. (2012)
Crude oil	*Acinetobacter calcoaceticus, Alcanivorax dieselolei* strain PM07, *Halomonas organivorans* strain G-16.1, *Halomonas halodurans* strain DSM 5160 and *Rhodococcus wratislaviensis* isolate FPA1	

Substrate	Microorganism	Reference
Phenanthrene	*Sphingopyxis* sp. PR52-21, *Sphingopyxis* sp. 2PR58-1, *Sphingomonadaceae bacterium* E4A9, *Sphingomonas* sp. 2MPII and *Sphingomonas* sp. Pee Shoal	Pinyakong et al. (2012)

Chapter III

Methodology

3.1 Experimental framework

The experimental framework of this study was described in figure 11

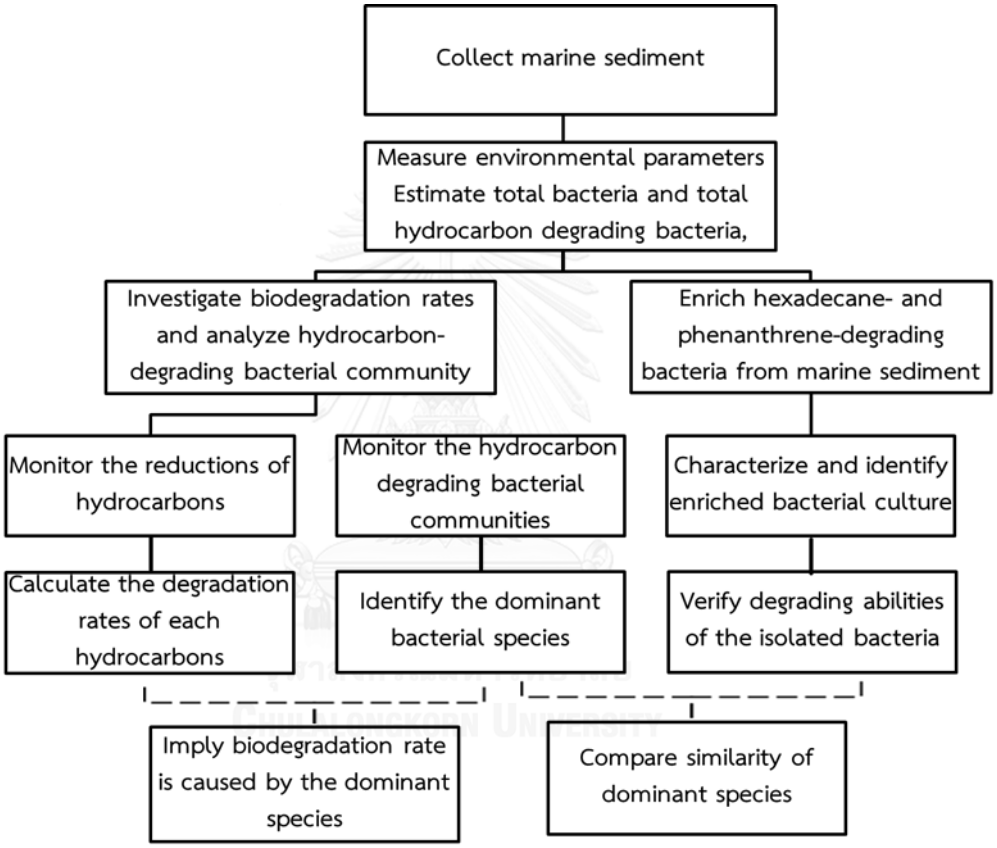

Figure 11 Experimental frame work of the study

3.2 Sampling site characteristic

The sampling station was located at a wood port connected to a miscellaneous shop nearby Sahakorn canal, Takaam district, Bangkhuntien, Bangkok, Thailand (13°32'22.2"N 100°26'36.5"E). The front of the shop was facing the canal, the houses in this area were also located next to canal, and therefore, local people likely use boats as one of the main route for transportation and selling their fishery goods. The shop sold ice, drink and fuel for those people. During the sample collection of 4 hours, there were at least 2 boats passed by in every 5 minutes and people stopped by to fuel their boats approximately every 30 minutes. There were two types of fuels selling at this station, gasoline and diesel. The diesel was more common for fueling boats (communication with the owner of the petroleum station). The motor boat stopped at the port and shopkeeper brought the fuel nozzle into people's motor boat. Sometimes the fuel was bought as a bottle, without any awareness, the fuel spill during selling either directly into the environment or on the port. Consequently, there were chance of oil contamination in this area. Moreover, oil spill could be observed as a film floating on the surface water at the sampling station (Figure 12 and Figure 13). Conductivity, pH, temperature, and dissolved oxygen were measured on site using the portable probe. Salinity was analyzed in laboratory using refractometer. Total phosphorus (TP), total nitrogen (TN), and total carbon (TC) were analyzed in the laboratory.

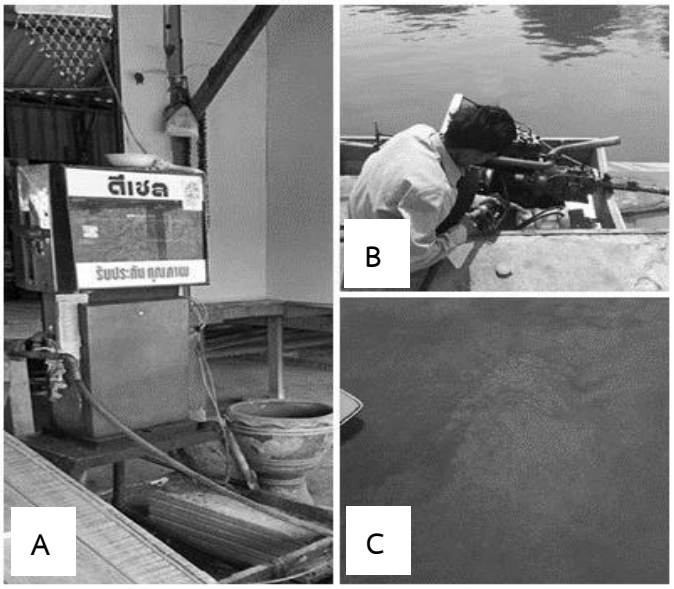

Figure 12 Sampling site area

A) The petroleum station at the sampling station, B) People's activity at the sampling station (fuel a boat) and C) Oil floating film on the surface of seawater

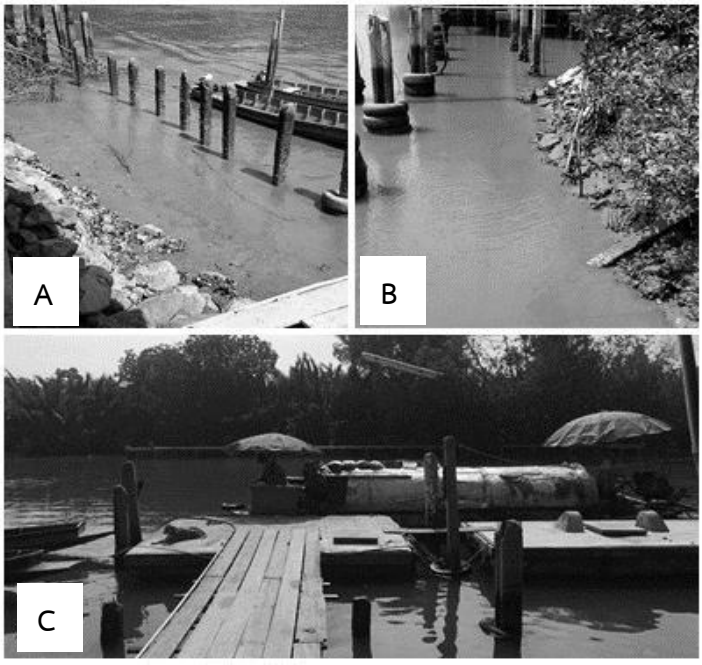

Figure 13 An overview of the sampling site

A) Left side of the sampling station, B) Right side of the sampling station and C) The overall sight of sampling station

3.3 Sample collection

Sediment samples were randomly collected from five spots, using a modified PVC pipe equipment. The sediment samples were kept in a sterile 2L-PP bottle. Approximately 10 cm depth of surface sediment were collected for microcosm construction. Seawater samples were randomly collected from five spots around the sampling station, using a plastic bottle. All samples were kept on ice during transportation. The samples were stored at 4°C until they were analyzed. The sediment samples from five spots were pooled before being used for microcosm construction. The water level was approximately 50 cm depth in wet season and 10 cm in dry season.

3.4 Total bacterial count

A 96-well plate was filled with 180 µL of sterile marine broth. One gram of the sediment was diluted in the BHMS broth 9 mL and 10 µL of nystatin, while one mL of seawater sample and 9 mL of sterile Marine broth (Difco) were mixed. Then, a multi-channel pipette was used to inoculate 20 µL diluted seawater sample into the first roll of pre-filled well. After the diluted seawater and the broth were well mixed, 20 µL of sample were transferred into the next roll for higher dilution. The 96-well plate was incubated at room temperature for 3-5 days. The results were interpreted by measuring media turbidity that caused by bacterial growth compared with the negative control which was the Marine broth without inoculum. The plate was exposed under 540 nm, using a microplate reader. The positive result was counted when the absorbance of the inoculated well was higher than the negative control. The analysis was performed in triplicates. Then the final result was compared to 3-tube MPN table (Haines et al., 1996) to estimate the statistic value of the total bacteria.

3.5 Total hydrocarbon-degrading bacterial count

The method was similar to section 3.4. To determine the number of total hydrocarbon-degrading bacteria, Bushnell-Haas broth together with 2 µL hydrocarbon L hexane^{-1} was added into each well of a 96-well plate. After 1-2 week(s) incubation, 50 µL of filter sterile iodonitrotetrazolium chloride (INT dye) from 3 g L^{-1} DMSO was added to the cultured and shaken for 30 minutes. The red precipitate appeared, indicating a positive bacterial activity result. In order to interpret the result, the plate was exposed under 450 nm.

3.6 Microcosm construction

The experiment was performed in 40 mL glass vial. Sediment microcosms was consisted of 5 g (wet weight) of sediment mixed with 2 mL of sterile seawater sample. In each microcosm, 2.5 mg of diesel (100 µL of 25,000 mg L hexane^{-1} stock solution) was added into the vial resulting 500 mg kg sediment^{-1}, 0.75 mg of phenanthrene (60 µL of 12,500 mg L hexane^{-1} stock solution) was added into each vial, resulted in the final concentration of 150 mg kg sediment^{-1} (Figure 14). While 1.25 mg (50 µL of 25,000 mgL hexane^{-1} of stock solution) hexadecane was added separately into another set, resulting in the final concentration of 250 mg kg sediment^{-1}. Hexane was used to dissolve the hydrocarbons; it had evaporated before closed the vials. A sterile sediment sample was used as a negative control; the samples was autoclaved three times to kill all living microorganisms. The negative control was conducted to estimate abiotic degradation of each hydrocarbon. The microcosms were statically incubated in rack throughout an incubation period, at room temperature. However, the microcosms were manually shaken once a day throughout the incubation period. The samples from each incubation set were collected during an incubation period to determine the biodegradation rates. The samples from microcosm were collected at days 0, 1, 3, 5, 7, 14, 21 and 28 to monitor the degradation of each hydrocarbon.

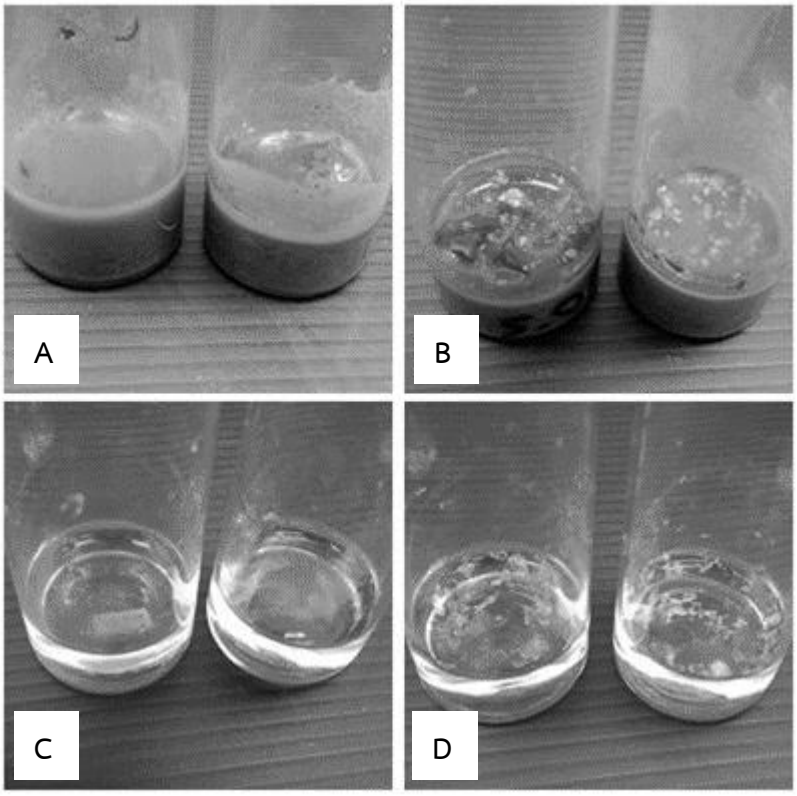

Figure 14 Microcosm construction
A and C represent microcosms with hexadecane or diesel additions (hydrocarbon disappeared); B and D represent microcosms with
phenanthrene addition (hydrocarbon appeared in a crystalized form)

3.7 Biodegradation rates of diesel, hexadecane and phenanthrene

3.7.1 Extraction of hydrocarbon from sediment sample

Ten milliliters of solvent (hexane) was used to extract hydrocarbon from sediment sample with 2:1 ratio. The sample was mixed by vortex for 1 minute and had been shaken at 200 rpm for 30 minutes. The hexane part from each sample was mixed with dehydrated Na_2SO_4 to remove the remaining water. Transfered 2.0 mL of hexane solution into amber GC vials. The filled GC vials are kept at 4^{o}C until analysis.

3.7.2 Measurements of diesel, hexadecane and phenanthrene concentrations by GC-FID

Each extracted hydrocarbon was analyzed in a liquid form (dissolved in hexane as previously described in section 3.6.1), using gas chromatography (GC; Agilent 6890N), with HP5 column and auto sampling injector. The analysis conditions for each hydrocarbon were described as following. For diesel measurement condition, the GC oven was programmed from 40°C (1 min) to 320°C at a rate of 20°C min_{-1}. Helium was used at a constant flow of 2 mL min^{-1}. Two µL of sample was injected in splitless mode with a splitless time of 1 min. For hexadecane measurement condition, injector temperature is 100°C, detector temperature is 300°C, and initial column temperature is 100°C hold time 1 minute, then programmed at 250°C at a rate of 25°C min^{-1}, hold for 3 min. Nitrogen will used as a carrier gas, the injection volume is two µL, and a splitless injection mode was employed. For phenanthrene measurement condition, injector temperature is 280°C, detector temperature is 250°C, and initial column temperature is 80 °C hold for 1 min, then programmed at 80°C to 160°C at a rate of 25°C min^{-1}, hold for 3 min and then the program was set to 160 °C to 220°C at a rate of 3°Cmin^{-1}, hold for 2 min, followed by 220°C to 300 °C at a rate of 40 °C min^{-1}, and hold for 7 min. As each microcosm experiment were constructed in triplicates, each sampling-point sample from each microcosm experiment was measured twice. The standard deviations were calculated using the function in Microsoft excel. Raw data of each microcosm experiment were provided in the appendix C.

3.7.3 Calculation of the hydrocarbon degradation rate

All hydrocarbon concentration data detected by GC-FID were plotted as an x, y graph using Microsoft excel to observe the trend of degradation rate within incubation time. The sampling time point that gave the sharpest decreasing of hydrocarbon concentration was chosen, therefore the rate would be calculate using the data at the beginning of the incubation time. The slope of the chosen sampling time was considered as degradation rate of that environmental sample and the lag phase did not include in this study.

The hydrocarbon mean's difference between sediment set and sterile sediment set was compared. Using one tail t-test paired two sample for mean of Microsoft excel to confirm the significant difference between two set (Control and experimental set). The null hypothesis (H_0) in this experiment is set as the hydrocarbon concentration mean between control set and experimental are not significantly different. The alternative hypothesis is the hydrocarbon concentration mean of control set is significantly higher than in experimental set. The significant level ($P_{\boldsymbol{\alpha}}$) is 0.05. The null hypothesis was accepted when $P_{\boldsymbol{\alpha}}$ is greater than P_{cal} (Obtained from statistic calculation).

3.8 Bacterial community analysis

3.8.1 DNA extraction

The DNA sample from each set was extracted using a FastDNA® SPIN Kit for soil. About 0.5 g of sediment was collected separately from microcosm vial. Enriched culture was collected from colony on a purified agar plate. All collected samples was kept in 1.5 mL labeled microcentrifuge tubes and stored at -20°C until analysis. DNA extraction method were performed according to a modified protocol from FastDNA® SPIN Kit for soil. Firstly, 987 µL of sodium Phosphate Buffer was added to the sample tube, then the MT buffer 122 µL was added. FastPrep® instrument was used to homogenize sample and buffer at the speed of 6.0 for 40 seconds. After that the homogenized sample was centrifuged at 14,000 g for 5 minutes. The supernatant was transferred to 2.0 mL microcentrifuge tube, 250 µL of Protein Precipitation Solution

(PPS) was added, followed by mixing manually for 10 times. The sample was centrifuged again at 14,000 g for 5 minutes for pellet precipitation. The supernatant was transferred to a new clean 1.5 mL microcentrifuge tube. The Binding Matrix of one mL was added to the supernatant. To bind DNA with the matrix solution, the sample tube was mixed by hand for 2 minutes and kept at room temperature for 3 minutes, allowing the silica matrix to precipitate. 500 µL of the supernatant was discarded. The matrix was resuspended in the remaining amount of supernatant and approximately 600 µL of the mixture was transferred into a SPIN^{TM} Filter. Then the mixture was centrifuged at 14,000 g for 1 minute, empty the catch tube and repeat this step until there is no supernatant in the catch tube. 500 µL of prepared SEWS-M were added to the tube and mix by the force if the pipette tube, followed by centrifuge at 14,000 g for 1 minute, empty the catch tube and replace. Drying the matrix by centrifuge at 14,000 g for 2 minutes without additional liquid fill, replace the catch tube with the new one. Air dry the matrix at room temperature for 5 minutes. Gently mix the matrix with 70 µL of DNase/Pyrogen-Freewater (DES). Centrifuge at 14,000 g for 1 minute. After discarded the filter, the extracted DNA is now ready for the downstream process. The extracted DNA was store at -20°C until analysis.

3.8.2 PCR-DGGE analysis of hydrocarbon- degrading bacterial community

The hydrocarbon- degrading bacterial community was determined by PCR-DGGE approach, followed by an identification step using 16S rRNA sequencing. The DNA *Tag* polymerase is purchased from BioLabs® inc., therefore, PCR temperature and time was set according to BioLabs® inc. protocol; initial denaturation step as 95°C for 30 seconds follow by 30 cycles of the 2nd denaturation at 95°C for 30 seconds, annealing 55°C for 30 seconds, extension 68°C for 5 minutes and holding at 12°C. The potential hydrocarbon- degrading bacterial community was monitored according to previous published protocol (Muyzer et al., 1993). The hydrocarbon-degrading community was analyzed by DGGE with 10% gel and 30%-70% denaturing gradient. The electrophoresis was operated under 60°C, 85 V, 840 minutes (14 hours).

3.9 Enrichment of the diesel-, hexadecane- and phenanthrene- degrading bacteria

A hundred milliliters of BHMS broth (Appendix A1) was added with 2% NaCl was used to stimulate the hydrocarbon-degrading bacterial strain from the sediment samples. After filter sterilization, 2 mL stock solution of hydrocarbon 25,000 ppm was added separately. The solvent was evaporated before the sample inoculation. One gram of sediment sample was used as inoculum. Then hydrocarbon concentration was measured by GC-FID. The hydrocarbon was extracted using 1:1 with hexane and shaking for 30 min before analyzed to ensure the biodegradation process. After the growth is observed, 2.5 mL of culture was transferred into 50 mL BHMS broth with 500 mg L^{-1} of filter sterile hydrocarbon. Media supplemented with hydrocarbon without inoculum was used as negative control to confirm that the hydrocarbons were reduced by a biotic process.

3.10 Isolation and characterization of diesel-, phenanthrene- and hexadecane-degrading bacteria

One hundred µL of enriched culture were spread on BHMS agar sprayed with 20,000 mg L^{-1} hydrocarbon (diesel, phenanthrene or hexadecane), incubated at room temperature until the growth observe. Different colony morphology such as size, shape, margin and color was selected. The selected colony was further purified on BHMS agar sprayed with 20,000 mg L^{-1} hydrocarbon (diesel, phenanthrene or hexadecane) until the we get a single colony. The single colony was confirmed for their hydrocarbon- degrading activity by BHMS media supplemented with each hydrocarbon concentration of 500 mg L^{-1}, shaken at 200 rpm at room temperature under natural light. The degradation of each hydrocarbon was determined by comparing the amount of remaining hydrocarbon with the supplemented media without inoculum. Only the isolates that showed the degrading ability would sent for sequencing the selected islolates was streaked on LB agar before being identified using 16S rRNA sequencing. The glycerol stock of each isolate were sequenced at Macrogen Inc., Korea. The purified colony was kept at – 20°C on BHMS media with glycerol in the

laboratory. The stored colonies have been labeled as ERH-002, ERH-003, ERH-006, ERH-007, ERP-002, ERP-003, ERP-007, ERD-002, ERD-004 and ERD-005, respectively.

Chapter IV
Results and Discussion

4.1 Preliminary experiment

Preliminary experiment was conducted to evaluate the possibility of hydrocarbon biodegradation in analyzed samples and to evaluate all analytical methods that had been conducted for this study. The first time point samples were collected in December 2015. Note that the samples were collected after it was raining on the sampling date. This could affect water level and water chemistry in water column. Generally, there were lots of motor boats traveling along the canal. Approximately 5 boat per hour stopped at the sampling station for refuel. The water level was not cover the bank, the sediment bank was observed.

Water chemistry results were shown in Table 6. These water chemistry results implied the condition for natural attenuation. The pH indicated the neutral condition. Salinity indicated that the samples were from saline environment.

The excess amount of water because of the rain might dilute the chemical concentrations in water column. This could be the reason for the much different water chemistry results between wet and dry seasons (Tables 6 and 8).

Table 6 Water chemistry analysis of wet season (December 2015)

Analysis	Result
Salinity (mg L^{-1})	30.6*
Total N (mg L^{-1})	0.5*
Total P (mg L^{-1})	0.43*
Total C (mg L^{-1})	25.96*
pH	7.60±0.10
Conduct (mS cm^{-1})	40.85±5.74
Temp (°C)	33±0.61

*SD data was not available because it was from composite sample

4.1.1 Hydrocarbons detection limit of GC-FID

Hydrocarbon concentrations in the range of 0.1 – 1,000 mg L hydrocarbon^{-1} had been analyzed in order to find the detection limit of the GC-FID. The results indicated that the detection limit for diesel, hexadecane and phenanthrene were 5 mg L hexane^{-1}, 0.1 mg L hexane^{-1} and 1 mg L hexane^{-1}, respectively. Therefore, the standard curve of diesel was performed with concentrations of 5, 50, 100, 250, 500 and 1000 mg L hexane^{-1}. The standard curve of hexadecane was performed with concentrations of 0.1, 1, 5, 10, 50, 125, 250 and 500 mg L hexane^{-1} and the standard curve of phenanthrene was performed with concentrations of 1, 5, 10, 75, 150 and 250 mg L hexane^{-1} (all standard curves were shown in an appendix B).

4.1.2 Hydrocarbon recovery percentage

A hundred mg L hexane^{-1} stock solution of each hydrocarbon was injected into each type of microcosm. They were extracted immediately using hexane to find the recovery amount. The results showed that the recovery percentages of diesel, hexadecane and phenanthrene were 85%, 95% and 90% respectively. The recovery amount of these three hydrocarbons was in acceptable range (more than 80% of injected concentration), showing a good efficiency of the extraction method.

4.1.3 Total bacterial and total hydrocarbon-degrading bacterial analyses

Total bacterial analysis was conducted to estimate the original amount of bacteria in the sediment and water samples. The results showed that total bacteria in sediment and water samples were 3.28±0.23 x 10^8 MPN g^{-1} and 4.50±0.16 x 10^6 MPN mL^{-1}, respectively (Table 7). The number of total bacteria in sediment was around 100 times greater than those in seawater. The reasons for these findings were that sediment contained higher organic matter for the bacteria to attach and form biofilm. Bacteria were also able to use organic matter as nutrients for their growth. Total diesel, hexadecane- and phenanthrene- degrading bacteria were analyzed in order to estimate the number of bacteria capable of degrading diesel, hexadecane, and phenanthrene, respectively. In sediment samples, the numbers of the total diesel and hexadecane-

degrading bacteria were quite similar, but they were approximately 10 times higher than those of phenanthrene-degrading bacteria (Table 6). For total diesel-, hexadecane- and phenanthrene-degrading bacteria were 3.6%, 30% and 0.7% of total bacteria, respectively. However, none of them was detected in seawater samples. This might be due to the limitation of culture technique. Nonetheless, the results were corresponded with the data from section 4.1.5, where hydrocarbon-degradation activity in seawater microcosms was not observed.

Table 7 Total bacteria and total diesel-, hexadecane and phenanthrene- degrading bacteria in sediment and seawater samples

Total bacteria in sediment sample (MPN g^{-1})	**Hydrocarbon-degrading bacteria in sediment (MPN g^{-1})**		
$3.28\pm0.23 \times 10^{8}$	Diesel	Hexadecane	Phenanthrene
	$1.2\pm0.22 \times 10^{7}$	$9.5\pm0.17 \times 10^{7}$	$2.2\pm0.30 \times 10^{6}$
Total bacteria in water sample (MPN mL^{-1})	**Hydrocarbon-degrading bacteria in water sample (MPN mL^{-1})**		
$4.50\pm0.16 \times 10^{6}$	Diesel	Hexadecane	Phenanthrene
	ND	ND	ND

*ND = Not detected

4.1.4 Hexadecane and phenanthrene degradation rates

Microcosms for both sediment and water samples were constructed to evaluate the potential of biological process within an incubation period. Hexadecane and phenanthrene concentrations of 250 and 125 mg kg $sediment^{-1}$, respectively, were added to each set of microcosms. The samples were collected across the incubation period for monitoring the degradations of hexadecane and phenanthrene. The results showed that sediment samples had a potential to biodegrade hexadecane and phenanthrene and these hydrocarbons were able to be degraded within the incubation times of 14 and 28 days, respectively (Figure 15).

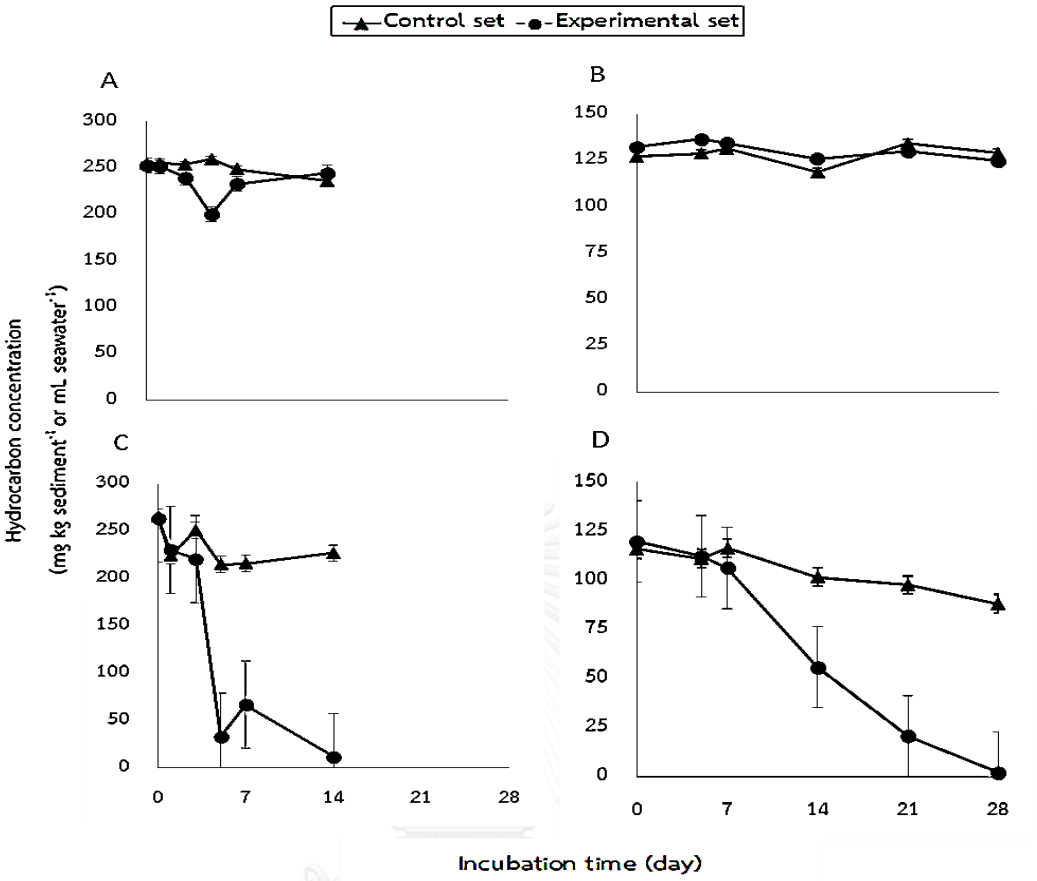

Figure 15 Hydrocarbon degradations in sediment and water samples
A) Hexadecane degradation in seawater sample, B) Phenanthrene degradation in seawater sample, C) Hexadecane degradation in sediment sample
and D) Phenanthrene degradation in sediment sample

The concentrations of both hydrocarbons were significantly decreased when compare to the control set (P_{cal} = 0.03 for hexadecane and P_{cal} = 0.04 for phenanthrene). However, the degradation results in seawater sets (Figure 18A and B) were not significantly different (P_{cal} = 0.158 for hexadecane and P_{cal} = 0.160 for phenanthrene). The total bacteria in seawater sample was probably too low and total hydrocarbon-degrading bacteria were undetectable in the seawater (Table 7). In order to improve the hydrocarbon degradation in seawater, the seawater might need to be concentrated to increase a total amount of bacteria per milliliter or other

supplementary (i.e. nutrient) should be added to ensure trace element sufficiency in the microcosm.

In sediment sample set, the results showed that the biodegradations of hexadecane and phenanthrene were complete within 14-day and 28-day incubation, respectively (Figures 15C and D). In hexadecane experimental set (Figure 15C), the hexadecane concentration was rapidly decreased within day 3 to day 5 of incubation time (From 250 mg kg sediment^{-1} to below 10 mg kg sediment^{-1}). After 5-day incubation, the results showed that hexadecane concentration became steady and no significant degradation was occurred after this period. The slightly increased hexadacane at day 7 time might be due to the variable of sediment sample with different microbial community. At the end of the incubation time, hexadecane had been degraded up to 96% of the injected concentration in the experimental set and it was 14% in the control set. The rate of hexadecane degradation was 52.03 mg kg sediment^{-1} day^{-1} (R^2=0.78) in experimental set and it was 2.29 mg kg sediment^{-1} day^{-1} (R^2=0.06) in control set.

Phenanthrene concentration was continuously declined in sediment microcosms after 7-day incubation time until 28-day incubation (Figure 15C). The phenanthrene degradation was gradually reduced during day 7 to day 24 in the experimental set while it was slightly change in control set during incubation time. At the end of the incubation time, phenanthrene had been degraded up to 97% of the injected concentration in the experimental set and it was 24% in the control set. Finally, in phenanthrene set, the rate of sediment samples was 6.54 mg kg sediment^{-1} day^{-1} (R^2=0.99) and 1.32 mg kg sediment $^{-1}$ day^{-1} (R^2=0.70) in the sterile sample.

The results indicated that hexadecane and phenanthrene were biodegradable only in the marine sediment. Therefore, the follow-up study focused mainly on the biodegradation of hydrocarbons in the sediment sample.

4.2 Second time point experiment

According to the preliminary experiment, it was assume that it took around 14 and 28 days for the sediment samples to degrade hexadecane and phenanthrene,

respectively. The results from preliminary experiment confirmed that the incubation condition (i.e., static with manual shaken daily, room temperature and under natural light) was appropriate for biodegradation process. In this experiment, diesel contaminated microcosm was added as a representative of petroleum hydrocarbon contamination in a real situation. The hydrocarbon- degrading community analysis by PCR-DGGE was also conducted. Moreover, hydrocarbon-degrading bacteria were also isolated using culture-dependent technique. For the second time point experiment, the samples were collected in March 2016. The main difference between the first and second time point samples was that the first time point samples were collected after raining while the second time point samples were collected in dry season. All water chemistry results of the second time point sampling were relatively different from the previous sampling due to the effect of season change. The results of water chemistry in dry season are shown in Table 8. The salinity showed that water in dry season contained higher salt compare to those in dry season. The total nitrogen, phosphorus and carbon concentration were also higher. This might be due to the evaporation of water in summer, thus the concentration of chemical dissolved in water become higher.

Table 8 Water chemistry analysis of dry season (March 2016)

Analysis	Result
Salinity (mg L^{-1})	33*
Total N (mg L^{-1})	0.8*
Total P (mg L^{-1})	1.18*
Total C (mg L^{-1})	36.18*
pH	7.33±0.18
Conduct (mS cm^{-1})	46.60±0.92
DO (mg L^{-1})	6.38±3.34
Temp (^{o}C)	33±0.92

*SD data not available because it was from composite sample

4.2.2 Total bacteria and total hydrocarbon- degrading bacteria analyses

The total number of bacteria in the second time point (Table 9) was two time higher than those in the first time point (Table 7). Total diesel-degrading bacteria, which was 5.3±1.2x10^8 MPN g^{-1}, was increase from the first time point (3.6%) into 5.5% of total bacteria. For hexdecane-degrading bacteria, which was 1.7±0.14 x10^8 MPN g^{-1}, it was 32%, a bit increased from the first time point (30%). Phenanthrene-degrading bacteria, which was 4.2±0.23 x 10^5 MPN g^{-1}, was 0.8% of the total bacteria while it was 0.7% in the first time point. This might be the result from weather change, in summer the water evaporated, less precipitation, therefore, higher concentration of chemicals dissolved in seawater. The higher amount of petroleum hydrocarbons might increase a number of hydrocarbon-degrading bacteria. Muangchinda et al (2013) enumerated the total bacteria in mangrove sediment from Don Hoi Lot, Samut Songkram Province, Thailand, it was ranging from 9.2x10^7 to 5.0x10^7 MPN g^{-1} and the total phenanthrene-degrading bacteria was ranging from 2.8x10^3 to 5.3x10^4 MPN g^{-1}. The results were less than what had shown in this study (Table 9), this might be the effect of chronic petroleum contamination in this sampling site.

Table 9 Total bacteria and total diesel-, hexadecane and phenanthrene- degrading bacteria analysis in sediment sample

Total bacteria in sediment sample (MPN g^{-1})	**Total hydrocarbon-degrading bacteria in sediment (MPN g^{-1})**		
5.3±1.2 x 10^8	Diesel	Hexadecane	Phenanthrene
	2.9±0.36x 10^7	1.7±0.14x10^8	4.2±0.23 x 10^5

4.2.2 Biodegradation rates of diesel, hexadecane and phenanthrene

In hexadecane experimental set (Figure 16A), the hexadecane concentration dropped rapidly within day 3 of incubation time (From 250 mg kg sediment^{-1} to below 10 mg kg sediment^{-1}). After 3-day incubation, the results showed that hexadecane concentration was under the detection limit. At the end of the incubation time,

hexadecane had been degraded up to 99% of the injected concentration in the experimental set, while the degradation efficiency was 13% in the control set. The rate of hexadecane degradation was 86.43 mg kg sediment^{-1} day^{-1} (R^2=0.97) in experimental set and it was 9.1 mg kg sediment^{-1} day^{-1} (R^2=0.67) in control set. The degraded hexadecane concentration between experimental set and control set were significantly difference (P_{cal} = 0.006). This indicates that the hexadecane degradation was mainly performed by biological process. Schurig et al (2014) reported that hexadecane degradation rate in water saturated soil was 6.4 mg kg sediment $^{-1}$ day^{-1}, which was lower than the hexadecane degradation rate in this study. There is no previous hydrocarbon exposure in the sediment of Schurig et al. (2014). Consequently, their hydrocarbon-degrading community need time to be adapted to the contaminated environment in order to remove the contaminant.

Phenanthrene concentration was continuously declined since the beginning of the incubation time until 21-day incubation (Figure 16B). The phenanthrene degradation was gradually occurred during day 5 to day 14 in the experimental set while it was slightly change in control set during incubation time. Finally, in phenanthrene set, the rate of sediment samples was 9.03 mg kg sediment^{-1} day-^{1} (R^2=0.83) and 1.55 mg kg sediment $^{-1}$ day^{-1} (R^2=0.92) in the sterile sample. The total phenanthrene degradation in sediment and sterile samples were around 96% and 17%, respectively. The degraded phenanthrene concentration between experimental set and control set were significantly different (P_{cal} = 0.03). This indicates that the phenanthrene degradation was mainly performed by biological process. However, microorganisms in the sediment had higher diesel degrading efficiency in this experiment. Muangchinda et al. (2013) conducted the experiment to estimate the biodegradation potential of mangrove sediment from Don Hoi Lot, Samut Songkram province, Thailand to degrade the target polycyclic aromatic hydrocarbon (pyrene, phenanthrene and acenaphthene). The biodegradation rate of phenanthrene in the sediment was 0.549 mg kg sediment $^{-1}$ day^{-1} which was slower than the phenanthrene biodegradation in this study. When compared the numbers of total bacteria and total phenanthrene-degrading bacteria with this study, the results showed 100 times higher bacterial numbers than Muangchinda et al. (2013) (Table 8). This could be the

effect of long term contamination that the local bacterial community were adapted to the petroleum contamination in the environment. In addition, there were two more PAHs available in the Muangchinda et al. (2013)'s microcosm that might competing with phenanthrene. According to the degradation graph in their study, acenaphthene seemed to be more biodegradable. Therefore, phenanthrene might be the second priority to be degraded in that microcosm which lowering its rate. On the other hand, this study used only one hydrocarbon in each microcosm.

Diesel degradation was occurred rapidly since the beginning of the incubation period (Figure 16C). The concentration of diesel dropped sharply within the first three days (approximately 150 mg kg sediment^{-1} of diesel declined). After day 5 of incubation, the diesel concentration was slightly decrease. At the end, 84% of diesel had been degraded in the experimental set and it was 14% in the control set. The rate of diesel degradation was 81.96 mg kg sediment^{-1} day-^{1} (R^2=0.89) in experimental set and it was 55.63 mg kg sediment^{-1} day^{-1} (R^2=0.79) in the control set. The degraded diesel concentrations in experimental set and control set were significantly difference (P_{cal} = 0.003). This indicates that the diesel degradation was mainly performed by biological process. Microorganisms in the sediment played an important role in degrading diesel in the microcosms. Woolfenden et al. (2011) reported the biodegradation rate of the spiked diesel was 4.7 mg kg^{-1} sediment week^{-1}, which was slower than the diesel degradation rate in this study. However, the incubation temperature of the study by Woolfenden et al. (2011) was much lower (0.4 °C) than in this study (31 °C). The difference of temperature during conducting experiment may affect the degradation rate. Das and Chandran (2010) mentioned about the effect of temperature on hydrocarbon degrading bacteria as the biodegradation will become lower when the temperature is getting low. The decreasing of temperature affects the solubility of hydrocarbons as the viscosity become higher and the volatility of the hydrocarbons become lower. This prolong the toxicity of the hydrocarbons to the living organism and lowering the biodegradation rate.

Comparing to the degradation rate of hexadecane and phenanthrene in the first time point experiment (Figures 18C and D), the degradation rate in summer was higher (Figures 19B and C). There was higher concentration of various chemicals in the

summer environment, which was increased due to water evaporation when the temperature became higher. Therefore, the local bacterial community was familiar to this situation and the hydrocarbon degradation became easier than the community in rainy season. This hypothesis was confirmed by Figure 18 c and d, where the bacterial community from rainy season sediment needed longer time to start degrading the interesting hydrocarbon than those from summer season.

The degradation of all hydrocarbons were occurred in the early stage of incubation time. This might be an effect from long term hydrocarbon contamination of the sampling area that could enhance the hydrocarbon- degrading bacterial species to be active in degrading petroleum hydrocarbon in contaminated environment. Microcosms with hexadecane contamination showed the highest degradation rate compare to the other two hydrocarbons. This might be the effect of hydrocarbon structure. Aliphatic hydrocarbons, which is less complicated structure, tended to be easily biodegraded than aromatics (phenanthrene) or a mixture of aliphatic and aromatics (diesel). The degradation rate of diesel was slower than hexadecane but still faster than the rate of phenanthrene. It could be because the alkane part in diesel was degrade first followed by the aromatic. However, the detection method did not present type of hydrocarbon that had been degraded therefore the degradation step was not confirmed. However, the step could be approximated by comparing the rate between alkane representative (hexadecane) and aromatic representative (phenanthrene). Moreover, the trends of alkane and PAHs degradation in this study were similar to Mason et al. (2014), which assessed the succession of sediment community using different hydrocarbon substrates (toluene, dodecane and phenanthrene). At the end they concluded that PAHs seemed to be more persist in the environment.

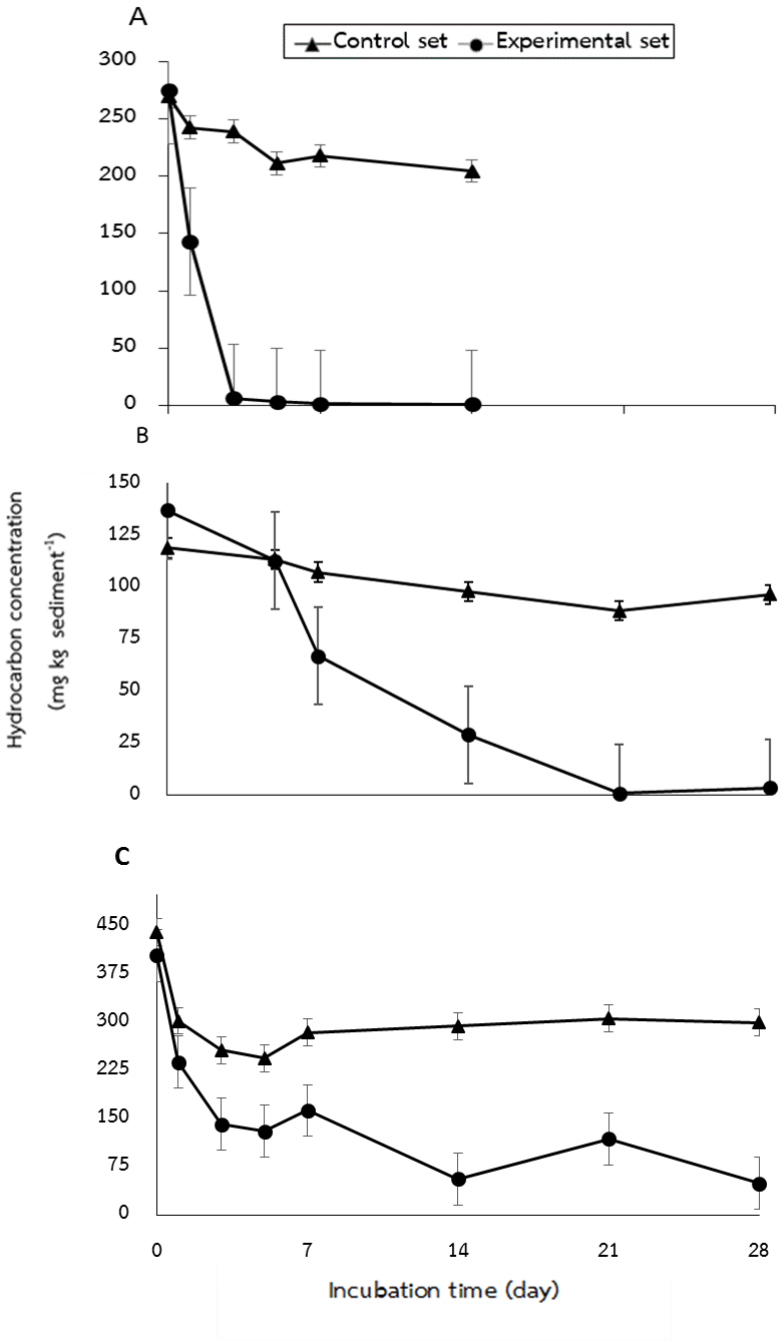

Figure 16 Hydrocarbon degradation in second time point sediment sample after 14- and 28- day incubation

A) Hexadecane degradation in sediment sample,

B) Phenanthrene degradation in sediment sample

and C) Diesel degradation in sediment sample

4.2.3 Hydrocarbon degrading community analysis

The DGGE profile was shown in Figure 17. The DGGE patterns of each microcosm set, including hexadecane (H0, H7 and H14), phenanthrene (P0, P7, P14, P21 and P28), and diesel (D0, D7, D14, D21 and D28) were quite consistent. There were 4 bands representing the original bacterial community in hexadecane contamination (H0; Figure 17). After 7 and 14 days incubation there were only 3 bands shown (H7 and H14; Figure 17). While in the phenanthrene contaminated microcosm, there were 11 bands showed in the original sediment (P0; Figure 17). The band after 7, 14, 21 and 28 day incubation became less than the band appeared on P0. Six different bands were dominant in the DNA from phenanthrene microcosm after 7 and 14 day incubation time. These bands were also appeared in well P0. The band represented in well P21 and P28 were also less than the band in 7 and 14 day (5 bands showed). In diesel contaminated microcosm, there were 4 bands showed in the original sediment (D0; Figure 17). After 7, 14, 21 and 28 day incubation time, only 1 band was appeared. Band number 1* appeared intensively only in the original of every microcosm (H0, P0 and D0; Figure 17), after that the band disappeared until the end of the incubation time (H7, H14, P7, P14, P21, P28, D7, D14, D21 and D28; Figure 20). This might be because of this band was from the dominant indigenous bacteria which was not the hydrocarbon-degrading species, therefore the community was dominated by other bacterial strain then the bacteria of band 1* was undetectable by PCR-DGGE. In contrast, the band 2* (H0, P0 and D0; Figure 17) which was less intensity compare to the band 1*, still appeared in the DNA from the microcosm after 7, 14, 21 and 28 day incubation (H7, H14, P7, P14, P21, P28, D7, D14, D21 and D28; Figure 17). The reason might be the strain representing by this band was the dominant hydrocarbon-degrading bacteria that already existing in this microcosm which dominated the community after band 1*. The appearance of the same band after incubation period might be because of the hydrocarbon-degrading bacteria was already existing in this sediment, might be the effect of chronic contamination at this area that enhance an effective hydrocarbon degrading bacterial community. Band number 1* from D0 (Figure 17) and 2* from D7 (Figure 17) which were classified as following. The sequencing result showed that band

1* is closely related to *Thiomicrospira* sp. Group; it showed 98% identity. *Thiomicrospira* sp. was found in a variety of saline environments such as mud flat and coastal sediment (Brinkhoff et al., 1998). This species was known as sulfur oxidizer which was able to utilize sulfur as an energy source (Brinkhoff and Muyzer, 1997). Head et al (2010) found the 16s rRNA sequences of this bacteria in a sample from oil field. However, a few studies indicated that this bacterial species was related to hydrocarbon-degradation. The study of Head et al (2010) also believed that the presence of this bacteria might be an effect from water flooding before the beginning of their study. So, the *Thiomicrospira* sp. dominated only in the natural situation as its band disappeared after the incubation time began.

The possible dominant species that presented in diesel microcosm after 7-day incubation (band 2*; Figure 17) was closely related to uncultured *Nitrosomonas* sp. (97% identity). The *Nitrosomonas* sp. is usually known as autotrophic nitrifying bacteria, but John and Okpokwasili (2012) are able to isolate the microorganism from mangrove sediment using different hydrocarbons. At the end, they concluded that the isolated *Nitrosomonas* sp. also showed the crude oil degradability in the crude oil contaminated environment. This strain might close to the band 1* because the sediment used in this study was collected from a rich nutrient environment as same as mangrove forest. For another closest strain, Siegert et al (2010) reported a significant growth of *Geobacteraceae* sp. in hexadecane artificially contaminated sediment, which indicated the hydrocarbon-degrading activity of this bacteria. In contrast, Mason et al (2014) investigated a significant of uncultured Gammaproteobacterium and a *Colwellia* species in the sediment core from sediment microcosm artificially contaminated with different hydrocarbon.

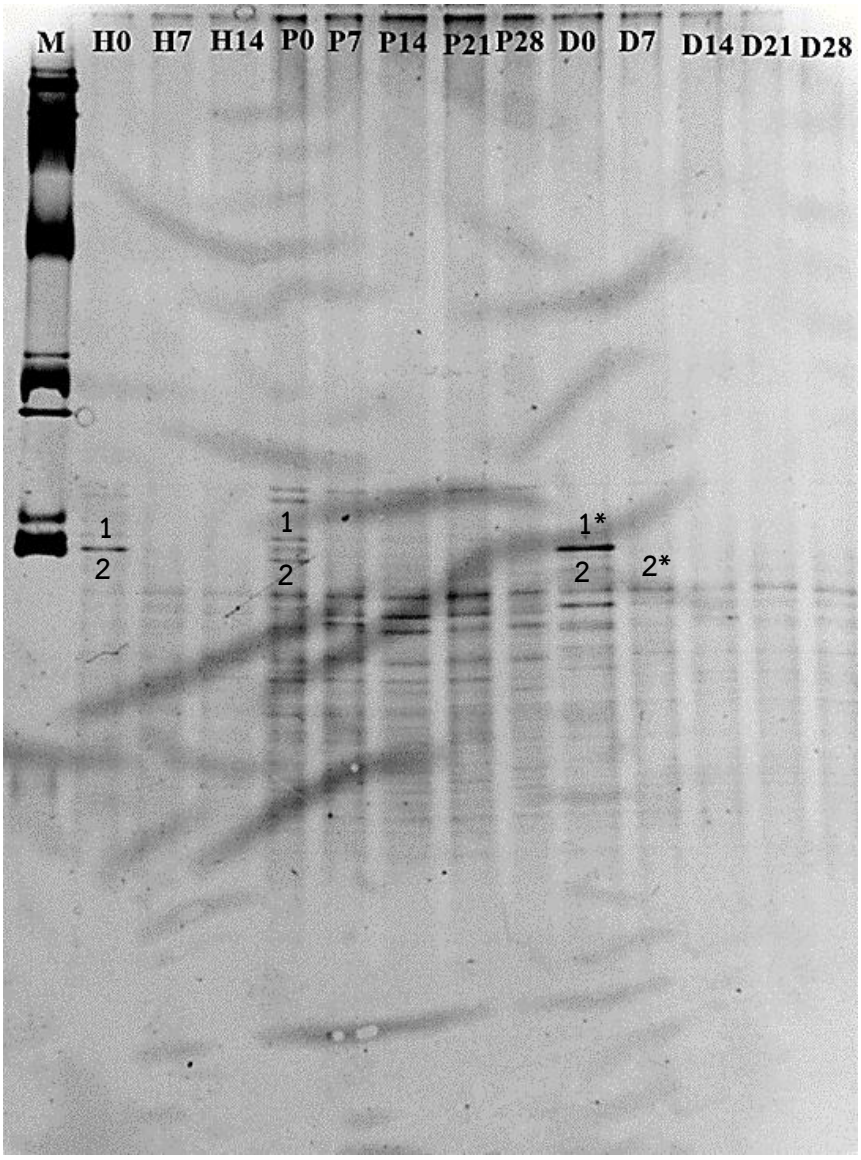

Figure 17 DGGE profile of microcosms from second time point samples band 1 = the dominant indigenous strain at the beginning of the microcosm, band 2 = the dominant indigenous strain after the incubation time (* represented the band that was sent for sequencing)

Table 10 Summary of the sequencing result of the analyzed DGGE bands\ in Figure 17

Band	Sample name	Closest strains (Accession number)	Identities	Source
1*	Original sample 1st Dominant band	*Thiomicrospira sp.* (AJ011073.1)	150/153 (98%)	Coastal sediment of the Jadebusen Bay, Germany
		Thiomicrospira thyasirae strain DSM 5322 (NR_024854.1)	150/153 (98%)	Deutsche SammLung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)
		Thiomicrospira sp. JB-A1F (AF013976.1)	150/153 (98%)	Deep-sea hydrothermal vent
		Thiomicrospira pelophila strain 4B (NR_025926.1)	150/153 (98%)	Deep-sea hydrothermal vent

Band	Sample name	Closest strains (Accession number)	Identities	Source
2*	DNA from diesel Microcosm at 7-day incubation	Uncultured *Nitrosomonas* sp. (LN878313.1)	91/94 (97%)	Sediment at 50m from Thondi Coastal region, Palk Bay, India

4.2.4 Enrichment and isolation of diesel-, hexadecane- and phenanthrene-degrading bacteria from marine sediment

There were many types of colonies grew on BHMS agar such as actinomyces, fungi and other bacteria. In BHMS with hexadecane plates, there were the most variety of microbial colonies. It might be because the hexadecane structure is easier to degrade compare to phenanthrene and diesel. However, only the colony that found more than one repetitive on the plate were selected for purification by streak plate technique. The criteria was used because the study focused on isolation of dominant populations in the sediment. The colony morphology of isolates retrieved from diesel, hexadecane, and phenanthrene were shown in tables 11, 12 and 13, respectively. Examples of isolated bacterial strains growth on BHMS were shown in Figure 18

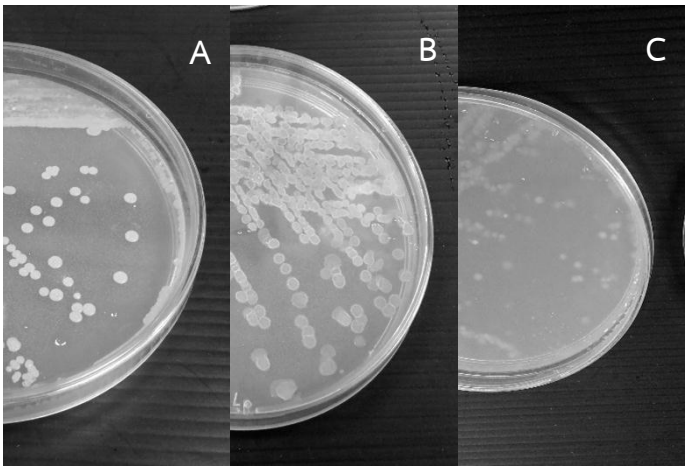

Figure 18 Examples of colony morphologies of the isolated strains
A) Isolate ERH-002, B) ERH-005 and C) ERH-007

Table 11 Colony morphology of the isolated diesel-degrading bacteria

Time point	Colony No.	Morphology					
		Size	Color	Form	Elevation	Margin	Surface
2	ERD-001	Small	Light yellow	Circular	Convex	Entire	Moist
	ERD-002	Small	Creamy	Circular	Convex	Entire	Dry
	ERD-003	Moderate	White	Circular	Convex	Entire	Dry
	ERD-004	Small	Light yellow	Circular	Convex	Entire	Dry
	ERD-005	Small	White	Circular	Convex	Entire	Mucoid

Table 12 Colony morphology of the isolated hexadecane-degrading bacteria

Time point	Colony No.	Morphology					
		Size	Color	Form	Elevation	Margin	Surface
1	ERH-001	Large	yellow	Circular	Convex	Entire	Dry
	ERH-002	Small	Orange	Circular	Convex	Entire	Dry
	ERH-003	Large	Orange	Circular	Convex	Entire	Mucoid
	ERH-004	Small	Light yellow	Circular	Convex	Entire	Mucoid
	ERH-005	Small	Yellow	Circular	Flat	Entire	Moist
2	ERH-006	Moderate	Orange	Circular	Flat	Entire	Moist
	ERH-007	Moderate	Yellow	Circular	Flat	Entire	Moist
	ERH-008	Small	White	Circular	Convex	Entire	Muciod
	ERH-009	Pin point	Yellow	Circular	Flat	Entire	Moist

Table 13 Colony morphology of the isolated phenanthrene-degrading bacteria

Time point	Colony No.	Morphology					
		Size	Color	Form	Elevation	Margin	Surface
1	ERP-001	Moderate	Yellow	Circular	Convex	Entire	Moist
	ERP-002	Moderate	White	Irregular	Flat	Undulate	Moist
	ERP-003	pinpoint	White	Circular	Convex	Entire	Moist
	ERP-004	Moderate	White	Irregular	Flat	Undulate	Mucoid
	ERP-005	Moderate	Yellow	Circular	Pulvinate	Undulate	Dry
	ERP-006	Pin Point	Golden yellow	Circular	Flat	Entire	Moist
2	ERP-007	Moderate	White	Circular	Flat	Entire	Moist
	ERP-008	Moderate	Orange	Circular	Flat	Entire	Moist

Only ten isolates were tested for hydrocarbon-degrading activity (Table 14) and the raw data were showed in appendix C. Those isolates were cultured in LB broth, mixed with 30% glycerol and sent to Macrogen Company for sequencing. The

sequencing results were modified by Bioedit program before blast by nucleotide database. The nucleotide blasting results of each isolates were shown in Table 15 and raw data of the sequencing result was shown in Appendix D.

Table 14 Hexadecane-, phenanthrene- and diesel- degrading activity test of each isolate.

Hydrocarbon	Isolates	Activity test		
		Initial concentration (mg L media^{-1})	Final concentration (mg L media^{-1})	% degradation After 7-day incubation
Hexadecane	ERH-002	629.39	397.81	36.79
	ERH-003	629.39	405.09	35.64
	ERH-006	629.39	396.54	37.00
	ERH-007	629.39	259.46	58.78
	Control*	629.39	629.39	0
Phenanthrene	ERP-002	439.10	273.56	42.56
	ERP-003	439.10	252.22	44.78
	ERP-007	439.10	242.47	37.70
	Control*	439.10	439.10	0
Diesel	ERD-002	384.49	175.48	42.97
	ERD-004	384.49	219.27	35.47
	ERD-005	384.49	248.12	54.36
	Control*	384.49	384.49	0

*control = BHMS media with no bacterial inoculum which was used as a negative control

Table 15 Classification of diesel-, hexadecane, phenanthrene- degrading bacteria

Isolates	Hydrocarbons	Closest strains	Identities	Source
ERH-002	Hexadecane	*Acinetobacter venetianus* strain MTCC 11369 (AB859738.1)	1459/1478 (99%)	Seawater
		Acinetobacter sp. LS-1 (JQ359016.1)	1459/1478 (99%)	Oil-water mixture in Dagang oilfield, China
		Acinetobacter beijerinckii strain 302-PWB-OH1 (HQ425646.2)	1459/1478 (99%)	Sea water from South China Sea
ERH-003	Hexadecane	*Acinetobacter venetianus* strain MTCC 11369	1459/1478 (99%)	Seawater
		Acinetobacter sp. LS-1 (JQ359016.1)	1459/1478 (99%)	Oil-water mixture in Dagang oilfield, China
		Acinetobacter beijerinckii strain 302-PWB-OH1	1459/1478 (99%)	Sea water from South China Sea

Isolates	Hydrocarbons	Closest strains	Identities	Source
ERH-006	Hexadecane	*Bacillus sonorensis* strain IHB B 12523 (KJ767389.1)	1484/1487 (99%)	Plant
		Bacillus sp. JS (CP003492.1)	1484/1487 (99%)	Plant roots
ERH-007	Hexadecane	*Staphylococcus warneri* strain: SH13 (LC035464.1)	1435/1501 (97%)	Fermented small shell
		Staphylococcus warneri strain 07P (KR809427.1)	1433/1499 (97%)	Neonate blood
		Staphylococcus warneri strain 82W (KR809419.1)	1433/1499 (97%)	Neonate blood
ERP-002	Phenanthrene	*Pseudomonas* sp. 40M1 FL01 (KM357382.1)	1475/1482 (99%)	Marine sediment
		Pseudomonas stutzeri clone M14 (HE646774.1)	1475/1482 (99%)	Maize rhizospheric soil
		Pseudomonas stutzeri strain Gr45 (FR667889.1)	1475/1482 (99%)	Roots of cereal crops

Isolates	Hydrocarbons	Closest strains	Identities	Source
ERP-003	Phenanthrene	*Pseudomonas* sp. 40M1 FL01 (KM357382.1)	1470/1481 (99%)	Marine sediment
		Pseudomonas stutzeri strain Gr45 (FR667889.1)	1470/1481 (99%)	Roots of cereal crops
		Pseudomonas stutzeri isolate Gr17 (FN813477.1)	1470/1481 (99%)	*Triticum turgidum* var. durum rhizosphere
		Pseudomonas sp. E1-4 (DQ227347.1)	1470/1481 (99%)	Deep sea
ERP-007	Phenanthrene	*Pseudomonas mendocina* (HM231168.1)	1471/1491 (99%)	Contaminated water
		Pseudomonas sp. R2(2013) (KF544922.1)	1470/1490 (99%)	Rhizosphere Trifolium pratense
		Pseudomonas sihuiensis strain KCTC 32246 (LT629797.1)	1469/1490 (99%)	N/A
		Pseudomonas sp. S6(2013) (KF544920.1)	1469/1490 (99%)	PAH-contaminated soil

Isolates	Hydrocarbons	Closest strains	Identities	Source
ERD-004	Diesel	*Bacillus subtilis* subsp. inaquosorum strain EVI16 (KY002648.1)	1506/1524 (99%)	Insect gut
		Bacillus subtilis strain VV2 (CP017676.1)	1506/1524 (99%)	Fermented rice
		Bacillus subtilis subsp. subtilis strain QB5413 (CP017313.1)	1506/1524 (99%)	Korean soybean paste
		Bacillus subtilis subsp. Subtilis strain QB5412 (CP017312.1)	1506/1524(99%)	
		Bacillus subtilis strain BS16045 (CP017112.1)	1506/1524(99%)	

Isolates	Hydrocarbons	Closest strains	Identities	Source
ERD-005	Diesel	*Pseudomonas* sp. P6 (KX426317.1)	664/722 (92%)	Marine sediment
		Pseudomonas putida strain Oh2-1.3 (LC125152.1)	664/722 (92%)	Seawater
		Pseudomonas pseudoalcaligenes strain RMR17 (KT731539.1)	664/722 (92%)	Oil polluted sea water

The classified hexadecane-degrading bacteria were in *Acinobacter* sp. with 99% identity (isolates ERH-002 and ERH 003), *Bacillus* sp. with 99% identity (isolate ERH-006) and *Staphylococcus* genera with 96% identity (isolate ERH-007). *Acinetobaccter* sp. are generally available in different environment in both marine and fresh water habitat. It is also found in soil and sediment. Several metabolic pathways had been investigated in *Acinetobacter* sp. such as antibiotic resistance, biosurfactant production, hydrocarbon degradation, etc. (Das and Chandran, 2011). *Acinetobacter* sp. is a part of crude oil degrading-community. Many studies (e.g. Maeng et al., 1996, Beilen et al, 2006 and Throne-Holst et al., 2007) supported the hydrocarbon-degradability in *Acinetobaccter* sp.

Bacillus sp. especially *Bacillus subtilis* is a group of bacteria that had been study about the hydrocarbon degradation. A study of Nwaogu (2008) reported the *Bacillus subtilis* had a high potential to degrade diesel oil. Guermouche et al (2015) found that *Staphylococcus* sp. was able to grow in crude oil especially, *Staphylococcus warneri*. Tanase et al. (2013), using culture –dependent techniques, isolated several hydrocarbon-degrading strains from soil around the area of oil pump. They investigated *Rhodococcus erythropolis*, *Acinetobacter baumanii*, *Burkholderia cepacia, Achromobacter xylosoxidans* and *Achromobacter xylosoxidans*.

In contrast, there were only *Pseudomonas* sp. (99% identity) that had been isolated by BHMS broth enriched by phenanthrene as a sole carbon source. *Pseudomonas* sp. are famous in petroleum degradation. There were a lot of study that had been successfully investigated *Pseudomonas*'s ability to degrade different hydrocarbons types (i.e. PAH) (Das and Chandran, 2010).

The isolated strains enriched by diesel were not different from the other two hydrocarbons (hexadecane and phenanthrene). The result was similar to Panda et al. (2013), they were successfully isolated *Pseudomonas aeruginosa* from marine sediment via BHMS broth supplemented with 0.5% diesel. They also claimed that the *Pseudomonas aeruginosa* has the highest hydrocarbon-degrading potential in nature. From the results above, it could imply that there were more bacterial strains with alkane (hexadecane) degradability than aromatics (phenanthrene) or a mixture of alkanes and aromatics (diesel).

Chapter V
Conclusions, recommendations and future study

5.1 Conclusions

The preliminary results of hexadecane and phenanthrene degradations confirmed that the methodology and time frame used for setting microcosms were appropriated for determination of hydrocarbon biodegradation in marine sediment. An artificially hydrocarbon contaminated microcosm was successfully conducted with marine sediment in order to estimate the hydrocarbon biodegradation rate. The biodegradation rate of diesel, hexadecane and phenanthrene after incubation could be estimated as 81.96 mg kg sediment^{-1} day^{-1} (R^2=0.89), 86.43 mg kg sediment^{-1} day^{-1} (R^2=0.97) and 9.03 mg kg sediment^{-1} day^{-1} (R^2=0.83), respectively. The data could be used as the reference to estimate the degradation time when the actual oil spill occurred. This information could be useful for planning a bioremediation process for oil spill cleanup.

The investigation of hydrocarbon- degrading community was achieved via PCR-DGGE techniques. The dominant bacterial species obtained from DGGE were reported as bacterial hydrocarbon-degrading community. The dominant DGGE bands detected in original sediment were closely related to *Thiomicrospira* sp. and *Ferrimonas* sp. The results indicated that the bacterial populations in the study area had high potential to degrade hydrocarbons.

Through hydrocarbon-enrichment method, 10 indigenous bacteria were isolated from the marine sediment sample. The isolated strains were belonged to *Pseudomonas* sp., *Bacillus* sp., *Acinetobacter* sp. and *Staphylococcus* sp. The isolated bacterial results confirmed that this area contained a powerful hydrocarbon-degrading strains. When compared the results from DGGE analysis and enrichment step, the dominant populations were totally different. From the sequencing results, most of DGGE bands were represented of uncultured bacteria. The reason why some significant strains could not be isolated in this experiment might be due to the

enrichment condition (i.e. pH, temperature and nutrient) that promoted fast growing bacteria. The uncultured bacteria might not compete with fast growing bacteria in the enrichment experiment. If we need to enrich the specific stain, we may try to find its specific condition or try to adjust some condition to favor the uncultured strains.

The results from this study could be used as a database for microbial community that may play an important role in case of petroleum hydrocarbon contamination and their degradation potential. For further study, the activity of the isolated strains at higher hydrocarbon concentrations should be investigated. The isolates with high potential degradation could be incorporated in bioaugmentation process.

5.2 Recommendations and future study

5.2.1 Incorporation of SIP (Stable Isotope Probing) techniques in the experiment to improvement of hydrocarbon community study.

The isotopic labeling of element (i.e. carbon and nitrogen) can represent substrate utilization of target bacterial community. Due to low existence of isotopic element in nature, we can separate substrate usage of community base on their cell structures, especially nucleic acid, because the bacteria that uptake isotopic labeling element will contain heavier nucleic acid (Sheppard, 2013). Therefore, ^{13}C labeled hexadecane and phenanthrene can be used. So, the application of this technique can facilitate a study of hydrocarbon-degrading community.

5.5.2 Investigation of physiological properties (i.e., optimum condition) of each isolated bacterial strain for further development of effective hydrocarbon bioremediation process

For example, Moghadam et al (2013) varied optical density, pH, temperature, NH_4Cl and salinity during biodegradation. After optimization, the biodegradation rate of PAHs was 28.4% increased. For further study, pH, temperature, and hydrocarbon concentration should be varied to observe an optimum condition for each isolated strain.

5.5.3 Application of the isolated strain for efficient removal of hydrocarbons from contaminated marine environments

The isolated bacteria can be used as a consortium to remediate hydrocarbon compounds in a closed system. For example, Yu et al. (2005) successfully incorporated a consortium consisted of *Rhodococcus* sp., *Acinetobacter* sp. and *Pseudomonas* sp. to remediate PAHs mixture and found that up to 100% of the interesting PAHs was degraded by this consortium.

5.5.4. Metagenomics analysis using next generation sequencing for understanding microbial community in depth

Oulas et al. (2015) reviewed an advantage of next-generation sequencing (NGS) in the environmental community analysis. The sequencing allowed us to access to the community existing in the environment, without using cultured-dependent techniques in one time. Daghio et al. (2015) incorporated the MiSeq Illumina for studying the biodiversity of hydrocarbon degrading microbial communities. They found *Acidovorax, Bdellovibrio, Hydrogenophaga, Pseudoxanthomonas* and *Serpens* were dominated in groundwater treatment without inoculum while *Rhodococcus* sp. was dominated the groundwater treatment with inoculum.

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APPENDIX

Appendix A: Media preparation

Appendix A-1 BHMS broth (Panda et al., 2013) for 1 Liter

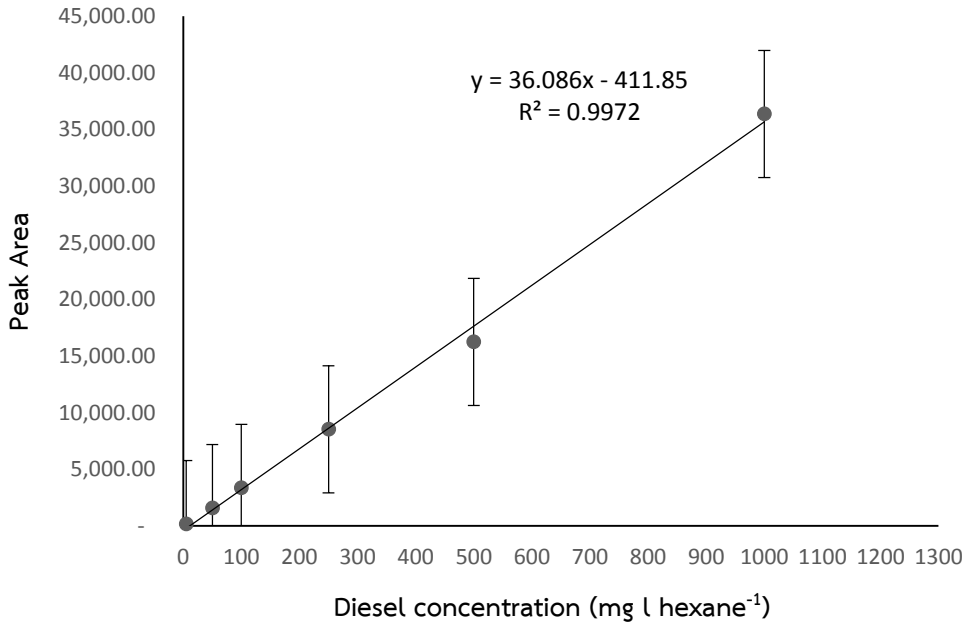
$MgSO_4.7H_2O$	0.20 g
$CaCl_2$	0.02 g
KH_2PO_4	1.00 g
K_2HPO_4	1.00 g
NH_4NO_3	1.00 g
60% $FeCl_3$	2 drops

pH was adjust to 7.0-7.5 before sterile by autoclave with 121°C, 15 min

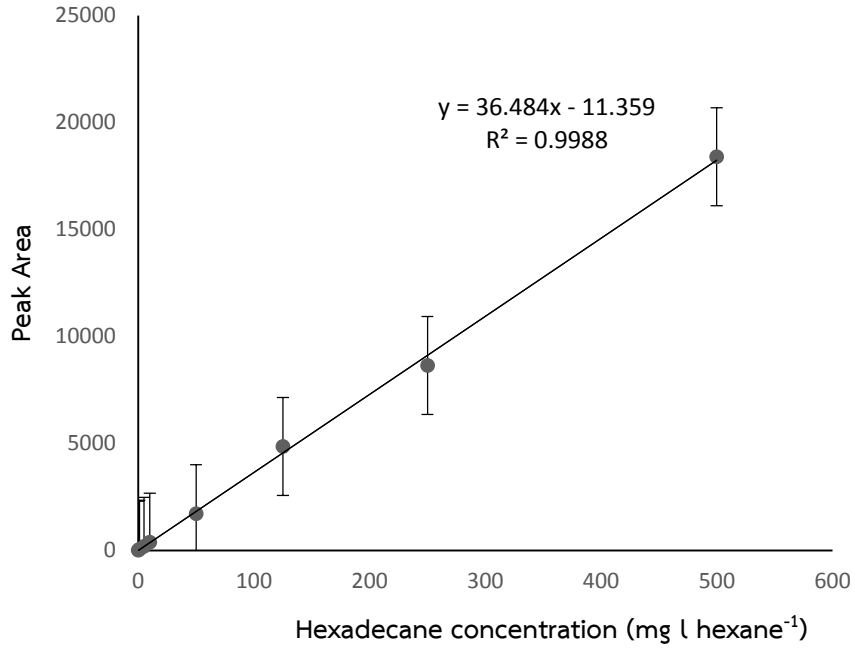
Appendix A-2 BHMS agar

The solution in Appendix A-1 with 2g agar added

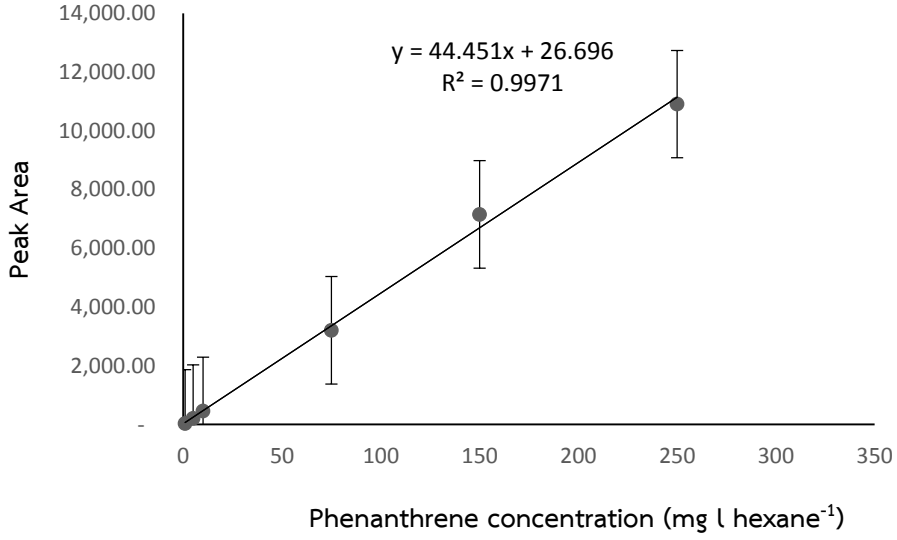
Appendix B: Diesel, hexadecane and phenanthrene Standard curve



Appendix B-1 Diesel standard curve



Appendix B-2 Hexadecane standard curve



Appendix B-3 Phenanthrene standard curve

Appendix C Raw data of hydrocarbon degradation graph and hydrocarbon degrading activity test

Appendix C-1 Raw data of hexadecane degradation in seawater control set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	9,339.92	256.31	254.52	7.72
	8,966.04	246.06		
	9,517.97	261.19		
1	9,696.57	266.09	254.94	18.19
	9,648.58	264.77		
	8,524.15	233.95		
3	9,277.13	254.59	254.12	3.31
	9,131.44	250.60		
	9,371.27	257.17		
5	9,572.80	262.69	259.88	2.46
	9,428.34	258.74		
	9,408.72	258.20		
7	8,738.89	239.84	249.10	8.87
	9,384.26	257.53		
	9,106.89	249.92		
14	9,065.2	248.78	236.17	11.01
	8,325.65	228.51		
	8,424.00	231.21		

Appendix C-2 Raw data of hexadecane degradation in seawater experimental set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	9,728.14	266.95	252.95	12.27
	8,893.64	244.08		
	9,030.08	247.82		
1	9,348.43	256.54	252.05	6.08
	8,931.89	245.13		
	9,272.93	254.48		
3	9,034.13	247.93	239.88	12.19
	8,958.51	245.86		
	8,229.10	225.86		
5	6,875.77	188.77	200.17	20.74
	6,834.14	187.63		
	8,165.13	224.11		
7	8,884.13	243.82	233.01	13.27
	8,635.85	237.01		
	7,949.17	218.19		
14	8,957.85	245.84	244.93	12.52
	9,363.95	256.97		
	8,452.45	231.99		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-3 Raw data of hexadecane degradation in sediment control set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	5,025.64	276.12	265.20	10.08
	4,790.47	263.23		
	4,663.37	256.26		
1	4,816.57	264.66	224.13	51.40
	3,022.64	166.32		
	4,392.67	241.42		
3	4,667.74	256.50	251.21	14.25
	4,276.91	235.08		
	4,769.20	262.06		
5	3,742.93	205.80	214.99	26.59
	4,457.08	244.95		
	3,531.40	194.21		
7	3,687.44	202.76	216.00	16.09
	4,255.55	233.91		
	3,843.56	211.32		
14	N/A	0.00	226.98	3.99
	4,180.64	229.80		
	4,077.69	224.16		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-4 Raw data of hexadecane degradation in sediment experimental set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	4,814.72	264.56	263.50	9.43
	4,956.98	272.36		
	4,614.59	253.59		
1	4,351.17	239.15	230.23	17.91
	4,401.85	241.93		
	3,812.46	209.62		
3	3,889.49	213.84	220.83	15.28
	3,824.66	210.29		
	4,336.66	238.35		
5	355.04	20.09	33.12	18.44
	830.69	46.16		
	N/A	N/A		
7	1,542.87	85.20	66.65	21.05
	1,283.24	70.97		
	787.19	43.78		
14	78.43	4.92	11.52	13.42
	480.40	26.96		
	37.49	2.68		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-5 Raw data of Phenanthrene degradation in seawater control set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	5,731.47	128.34	132.49	1.73
	5,727.27	128.24		
	5,596.50	125.30		
5	5,738.91	128.51	136.75	1.30
	5,712.38	127.91		
	5,823.29	130.40		
7	5,781.81	129.47	134.49	1.89
	5,900.08	132.13		
	5,944.20	133.12		
14	5,672.62	127.01	125.93	10.19
	4,802.00	107.43		
	5,452.94	122.07		
21	5,958.05	133.44	130.11	3.00
	6,141.18	137.56		
	5,882.20	131.73		
28	5,752.58	128.81	124.88	0.29
	5,773.07	129.27		
	5,776.79	129.36		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-6 Raw data of Phenanthrene degradation in seawater experimental set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	6,131.31	137.33	132.49	5.96
	5,996.22	134.29		
	5,620.01	125.83		
5	6,115.17	136.97	136.75	1.07
	6,147.07	137.69		
	6,053.40	135.58		
7	5,871.62	131.49	134.49	3.16
	5,991.82	134.20		
	6,151.91	137.80		
14	5,772.12	129.25	125.93	11.75
	6,056.93	135.66		
	5,044.41	112.88		
21	5,553.23	124.33	130.11	6.61
	6,130.87	137.32		
	5,746.49	128.68		
28	5,703.49	127.71	124.88	3.28
	5,417.99	121.29		
	5,611.09	125.63		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-7 Raw data of Phenanthrene degradation in sediment control set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	2,689.55	119.81	116.15	7.31
	2,713.92	120.91		
	2,421.14	107.73		
5	2,348.15	104.45	111.28	6.81
	2,650.70	118.06		
	2,500.74	111.32		
7	3,064.45	136.68	116.61	18.18
	2,514.09	111.92		
	2,276.58	101.23		
14	2,397.82	106.68	101.87	6.59
	2,123.87	94.36		
	2,350.95	104.58		
21	2,250.17	100.04	97.85	2.32
	2,206.46	98.07		
	2,147.52	95.42		
28	1,964.13	87.17	88.09	2.64
	1,938.94	86.04		
	2,050.68	91.07		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-8 Raw data of Phenanthrene degradation in sediment experimental set (Wet season)

Incubation time (day)	Peak area	Concentration (mg L seawater^{-1})	Average concentration (mg L seawater^{-1})	SD
0	2735.44	121.88	119.96	1.89
	2691.52	119.90		
	2651.33	118.09		
5	2616.45	116.52	112.45	4.11
	2433.89	108.31		
	2527.59	112.52		
7	2301.53	102.35	106.40	6.38
	2554.93	113.75		
	2317.84	103.09		
14	1424.66	62.90	55.78	19.58
	774.17	33.63		
	1600.21	70.80		
21	0.00	0.00	20.76	35.97
	0.00	0.00		
	1411.21	62.29		
28	175.24	6.68	2.23	3.86
	0.00	0.00		
	0.00	0.00		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-9 Raw data of hexadecane degradation in sediment control set (Dry season)

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
0	4915.97	268.86	269.92	1.89
	4987.55	272.79		
	5108.19	279.40		
	4707.33	257.43		
	4790.27	261.97		
	5102.03	279.06		
1	4582.01	250.56	242.54	1.89
	ND	ND		
	4314.09	235.87		
	4698.01	256.92		
	ND	ND		
	4149.02	226.82		
3	4470.34	244.43	238.97	1.89
	4041.59	220.93		
	4675.84	255.70		
	4329.09	236.69		
	4064.35	222.18		
	4642.42	253.87		
5	2965.63	161.95	211.40	1.89
	4723.36	258.31		
	3963.23	216.64		
	2928.56	159.92		
	4687.69	256.35		
	3938.19	215.26		
7	4142.95	226.49	217.74	1.89
	3640.21	198.93		
	3945.44	215.66		
	4324.09	236.42		
	3764.61	205.75		

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
	4082.91	223.20		
14	2683.14	146.46	204.49	1.89
	3634.74	198.63		
	4906.87	268.37		
	5035.74	275.43		
	3642.76	199.07		
	2750.08	150.13		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-10 Raw data of hexadecane degradation in sediment experimental set (Dry season)

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
0	5,372.72	293.90	274.72	23.79
	4,505.49	246.36		
	5,388.06	294.74		
	5,316.49	290.82		
	4,451.89	243.42		
	5,102.35	279.08		
1	2,598.99	141.85	142.99	5.26
	2,678.74	146.22		
	2,702.17	147.51		
	2,437.13	132.98		
	2,657.55	145.06		
	2,644.16	144.33		
3	145.60	7.36	6.37	1.31
	139.39	7.02		
	97.64	4.73		
	149.15	7.55		
	136.74	6.87		
	97.05	4.70		
5	35.67	1.33	3.29	1.59
	91.16	4.37		
	91.12	4.37		
	32.59	1.16		
	86.12	4.10		
	91.65	4.40		
7	49.17	2.07	1.43	0.68
	40.42	1.59		
	21.75	0.57		
	49.34	2.08		
	41.43	1.65		

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
	22.42	0.61		
14	24.69	0.73	0.97	0.99
	13.56	0.12		
	48.80	2.05		
	15.23	0.21		
	52.07	2.23		
	12.43	0.06		

*ND= non detected therefore the concentration was set as 0.0

Appendix C-11 Raw data of Phenanthrene degradation in sediment control set (Dry season)

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
0	2,907.31	129.61	119.07	10.38
	2,813.63	125.39		
	2,794.90	124.55		
	2,417.46	107.57		
	2,431.64	108.21		
	ND	0.00		
5	2,417.23	107.56	113.65	4.33
	2,458.25	109.40		
	2,614.59	116.44		
	2,548.29	113.45		
	2,654.11	118.22		
	2,622.75	116.81		
7	2,565.31	114.22	107.45	5.78
	2,336.05	103.91		
	2,258.27	100.41		
	2,555.02	113.76		
	2,450.00	109.03		
	2,323.66	103.35		
14	2,233.69	99.30	98.20	4.77
	2,074.55	92.14		
	2,201.02	97.83		
	2,303.45	102.44		
	2,102.36	93.39		
	2,340.31	104.10		
21	2,009.58	89.22	88.81	0.66
	2,006.88	89.10		
	2,011.23	89.29		
	1,975.73	87.69		
	2,010.81	89.27		

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
	1,988.95	88.29		
28	1,519.82	67.18	96.77	27.54
	2,037.32	90.46		
	2,882.51	128.49		
	1,632.28	72.24		
	2,035.79	90.40		
	2,956.78	131.83		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-12 Raw data of Phenanthrene degradation in sediment experimental set (Dry season)

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
0	3188.11	142.24	11.59	137.32
	3023.28	134.83		
	3174.58	141.63		
	3181.68	141.95		
	3316.34	148.01		
	2588.20	115.25		
5	2272.16	101.03	11.93	112.88
	2509.51	111.71		
	2839.42	126.55		
	2365.09	105.21		
	2341.35	104.14		
	2885.43	128.62		
7	657.95	28.40	34.30	67.17
	2302.89	102.41		
	1534.49	67.84		
	647.70	27.94		
	2406.67	107.08		
	1568.33	69.36		
14	206.21	8.08	16.09	29.25
	898.99	39.25		
	876.80	38.25		
	225.55	8.95		
	941.10	41.14		
	911.86	39.83		
21	80.66	2.43	1.36	1.18
	ND	0.00		
	ND	0.00		
	ND	0.00		
	ND	0.00		

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
	77.42	2.28		
28	ND	0.00	5.62	3.63
	ND	0.00		
	270.85	10.99		
	ND	0.00		
	ND	0.00		
	266.28	10.78		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-13 Raw data of Diesel degradation in sediment control set (Dry season)

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
0	7,273.97	425.97	440.77	106.64
	7,476.51	437.20		
	10,024.20	578.40		
	4,795.41	288.60		
	6,328.74	373.58		
	9,347.15	540.87		
1	2,981.64	188.08	303.05	77.75
	5,978.62	354.18		
	4,886.32	293.64		
	4,001.19	244.58		
	5,644.53	335.66		
	6,844.21	402.15		
3	5,058.51	303.18	257.46	59.84
	4,602.11	277.89		
	3,008.49	189.57		
	ND	0.00		
	5,332.10	318.35		
	3,166.44	198.32		
5	3,796.78	233.26	244.74	10.23
	4,250.17	258.38		
	4,169.64	253.92		
	3,810.82	234.03		
	4,030.46	246.21		
	3,966.22	242.65		
7	5,086.59	304.74	284.89	10.20
	4,591.20	277.28		
	4,669.46	281.62		
	4,755.54	286.39		
	4,642.95	280.15		

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
	4,624.61	279.14		
14	4,758.76	286.57	294.84	20.90
	4,481.23	271.19		
	5,395.51	321.86		
	5,018.12	300.95		
	4,538.56	274.37		
	5,255.23	314.09		
21	9,108.04	527.62	306.47	177.12
	2,515.35	162.23		
	3,649.54	225.10		
	9,258.30	535.95		
	2,443.47	158.25		
	3,732.31	229.68		
28	5,268.94	314.85	300.01	13.25
	4,914.63	295.21		
	5,070.10	303.83		
	5,245.80	313.56		
	4,654.74	280.81		
	4,853.13	291.80		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-14 Raw data of Diesel degradation in sediment experimental set (Dry season)

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
0	7,384.05	432.07	404.80	106.36
	6,503.07	383.25		
	9,390.16	543.26		
	6,409.51	378.06		
	5,466.76	325.81		
	6,198.09	366.34		
1	3,448.47	213.95	238.38	35.02
	3,845.43	235.95		
	4,796.48	288.66		
	3,466.83	214.97		
	ND	0.00		
	ND	0.00		
3	3,137.05	196.69	142.03	54.26
	1,004.21	78.48		
	2,043.77	136.10		
	1,286.90	94.15		
	3,460.20	214.60		
	1,972.51	132.15		
5	2,423.36	157.14	130.67	24.68
	1,468.31	104.20		
	1,914.79	128.95		
	2,476.96	160.11		
	1,443.70	102.84		
	1,948.30	130.81		
7	3,144.38	197.10	163.27	33.20

Incubation time (day)	Peak area	Concentration (mg L sediment^{-1})	Average concentration (mg L seawater^{-1})	SD
	2,538.92	163.54		
	1,836.28	124.60		
	3,187.17	199.47		
	1,833.48	124.44		
	2,664.37	170.49		
14	1,267.28	93.06	119.19	55.67
	3,267.70	203.93		
	909.15	73.21		
	1,283.79	93.98		
	2,736.68	174.50		
	967.92	76.47		
21	367.70	43.21	56.65	9.94
	730.12	63.29		
	712.27	62.30		
	391.14	44.50		
	719.34	62.69		
	741.61	63.93		
28	203.43	34.10	49.44	12.16
	720.96	62.78		
	521.07	51.71		
	221.94	35.13		
	659.96	59.40		
	553.54	53.51		

*ND= non detected therefore the concentration was set as 0.00

Appendix C-15 Raw data of hexadecane, phenanthrene and diesel degrading isolates activity test

Hydrocarbon	Isolates	Peak area	Concentration (mg L media^{-1})	% degraded
Hexadecane	w/o	22,951.30	629.39	0.00
	ERH-002	14,502.40	397.81	36.79
	ERH-003	14,767.80	405.09	35.64
	ERH-006	14,456.00	396.54	37.00
	ERH-007	9,454.67	259.46	58.78
Phenanthrene	w/o	19,545.20	439.10	0.00
	ERP-002	12,186.73	273.56	42.56
	ERP-003	11,238.13	252.22	44.78
	ERP-007	10,804.82	242.47	37.70
Diesel	w/o	13,462.80	384.49	0.00
	ERD-002	5,920.52	175.48	42.97
	ERD-004	7,500.85	219.27	35.47
	ERD-005	8,541.68	248.12	54.36

*w/o = BHMS media with no bacterial inoculum which was used as a negative control

Appendix D Nucleotide sequence data

Appendix D-1 Nucleotide sequence of band D0.1 (~ 150 bp)

GCCTATCTGACGCACTCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCCTAGGGTTGTAAAGC
ACTTTTAGTGAGGAGGAAAGGTTAGTAGTTAATACCTGCTAGCTGTGACGTTACTCACAGAAAAA
GCCCCGGCTAACTCTGTGCCCGCAGCCGCGGTAATAA

Appendix D-2 Nucleotide sequence of band H0.2 (~ 150 bp)

GCGGGTTAGGGGGCAACCCTGAGCAGCACGCCCGCGTGTGTGATGAAGGCCTTCGGGTTGTAAAG
CTCTTTCGTAGGGAAGAAGTGGTTGGGGTTTATACCCCTGGGTCTTGGCCTGTCCCTTCCAAGGA
GCCCGGGTTAATCCCGGGCCGCCCGCCCGGGTATAA

Appendix D-3 Nucleotide sequence of isolate ERH-002 (~ 1500 bp)

TTTTTTGGTTCCCCCTTTTAGACTTCACCCCAGTCATCGGCCACACCGTGGTAAGCGTCCTCCTT
GCGGTTAGACTACCTACTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGC
CCGGGAACGTATTCACCGCGGCATTCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCG
AGTTGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCATCCTATCGCTAGGTAGCA
ACCCTTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACG
TCGTCCCCGCCTTCTTCCAGTTTGTCACTGGCAGTATCCTTAAAGTTCCCATCCGAAATGCTGGC
AAGTAAGGAAAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAGCCATGCAGCACCTGTATCTAGATTCCCGAAGGCACCAATCCATCTCTGGAAAGTTTTTAGTA
TGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCG
GGCCCCCGTCAATTCATTTGAGTTTTAGCCTTGCGAAACTGAACTCCCCAGGCGGTCTGACTTAT
ACTGCGTTAGCTGCGCCACTAAAGACTCTCAAAGACCCCAACGGCTAGTAGACATCGTTTACGGC
ATCGACTACCAGGGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCAGCGTCAGTATTAG
GCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGG
AATTCTACCATCCTCTCCCATACTCTAGCCATCCAGTATCGAATGCAATTCCCAAGTTAAGCTCG
GGGATTTCACATTTGACTTAAATGGCCGCCTACGCACGCTTTACGCCCAGTAAATCCGATTAACG
CTCGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGCGAGTAAC
GTCCACTATCCAGTAGTATTAATACTAGTAGCCTCCTCCTCGCTTAAAGTGCTTTACAACCATAA
AGCCTTCTTCACACACGCGGCATGGCTGGATCAGGGTTCCCCCCATTGTCCAATATTCCCCACTG
CTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCGGATCATCCTCTCAAACCCG
CTACAGATCGTCGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACTTAGGCTCATCTA
TTAGCGCAAGGCCCGAAAGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCATTCCTTT
CGGAATGTTGTCCCCCACTAATAGGCAGATTCCTAAGCATTACTCACCCGTCCGCCGCTAAGTCC
AGTAGCAAGCTACCCTTTCCCCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCAATCTG
AGCTGAGAAAAAAAATCTAAAAAA

Appendix D-4 Nucleotide sequence of isolate ERH-003 (~ 1500 bp)

TTGGTTCCCCCTTTACGACTTCACCCCAGTCATCGGCCACACCGTGGTAAGCGTCCTCCGTGCGG
TTAGACTACCTACTTCTGGTGCAACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGCGGCATTCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGTT
GCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCATCCTATCGCTAGGTAGCAACCC
TTTGTACCGACCATTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTGGACGTCGT
CCCCGCCTTCTTCCAGTTGGTCACGGCCAGTATCCTTAAAGTTCCCATCCGAAATGCTGGCAAGT
AAGGAAAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGC
CATGCAGCACCTGTATCTAGATTCCCGAAGGCACCAATCCATCTCTGGAAAGTTTTTAGTATGTC
AAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCC
CCCGTCAATTCATTTGAGTTTTAAAATTGCGACCGTACTCCCCAGGCGGTCTGACTTATACTGCG
TTAGCTGCGCCACTAAAGATCTCAAAGATCCCAACGGCTAGTAGACATCGTTTACCGGCATGGAC
TACCAGGGTATCTAATCCTGTTTGCTCCCCATGCTTTCGTACCTCAGCGTCAGTATTAGGCCAGA
TGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCT
ACCATCCTCTCCCATACTCTAGCCATCCAGTATCGAATGCAATTCCCAAGTTAAGCTCGGGGATT
TCACATTTGACTTAAATGGCCGCCTACGCACGCTTTACGCCCAGTAAATCCGATTAACGCTCGCA
CCCTCTGTATTACCGCGGCTGCTGGCACAAAGTTAGCCGGTGCTTATTCTGCGAGTAACGTCCAC
TATCCAGTAGTATTAATACTAGTAGCCTCCTCCTCGCTTAAAGTGCTTTACAACCATAAAGCCTT
CTTCACACACGCGGCATGGCTGGATCAGGGTTCCCCCCATTGTCCAATATTCCCCACTGCTGCCT
CCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCGGATCATCCTCTCAAACCCGCTACAG
ATCGTCGCCTTGGTAGGCCTTTACTCCACCAACTAGCTAATCCAACTTAGGCTCATCTATTAGCG
CAAGGCCCGAAAGTCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCATTCCTTTCGGAAT
GTTGTCCCCCACTAATAGGCAGAATTCCTAAGCATTACTCACCCCGTCCGCCGCCTAAGTCCGGT
AGCAAGCTTCCCTTTCCCCGCTCGACTTTGCATGTGGTTAGCCCTGCCGGCCAGCGTTTCAATCT
GAGCCTGTGTGTAAAACTCTAAAAAAGGCCCCAAGGAAAAAAAAAGGGG

Appendix D-5 Nucleotide sequence of isolate ERH-006 (~ 1500 bp)

TTGGTTCCCCTTGTAGACTTCACCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGG
TTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTT
GCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCC
TTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT
CCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTA
AGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACC
ATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCA
AGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGACTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAG
CTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGATTAGCGGCGTGGACTACC
AGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGT
CGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTC
TCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCAC
ATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTAC
CGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATC
ACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCG
TAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCG
TCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAG
CCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAAGCATCCGGTATTAGCCCCGGTTTC
CCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATC
AGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCT
GAGCCAGAATCAAAACTCTAAA

Appendix D-6 Nucleotide sequence of isolate ERH-007 (~ 1500 bp)

TTGGTTCCCCTTGTAGACTTCACCCCAATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGG
TTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTT
GCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCC
TTTGTTCTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT
CCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTA
AGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACC
ATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCA
AGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGACTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAG
CTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGATTAGCGGCGTGGACTACC
AGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGT
CGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTC
TCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCAC
ATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTAC
CGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATC
ACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCG
TAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCG
TCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAG
CCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAAGCATCCGGTATTAGCCCCGGTTTC
CCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATC
AGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCCT
GAGCCAGAATCAAAACTCTAAA

Appendix D-7 Nucleotide sequence of isolate ERP-002 (~ 1500 bp)

TTTTGGTTCCCCCTTTTAGACTTCACCCCAGTCATGAATCACTCCGTGGTAACCGTCCCCCCGAA
GGTTAGACTAGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCC
GGGAACGTATTCACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGCA
GTGGCAGACTGCGATCCCAGGACGACGAGTCGCTTATATGCGATTAGCTCCAGCACTAAGCGGCT
TGGAAACCCCCTATACCGACCATTAGCACTCATCGTGTGTAGCCCAGGCCGTAAGGACCATGACC
ACTGGACGTCATCCACACCTTCCTCCGGTTTGATCACCGGCAGTCGCCTTAGAGTGCCCACCTCA
ACGTGCAGGTAACTAAGGACAAGAAAGTCGCGCTTCGCCACTGGTGACTTAACCTCAACATCTCA
GCGCATTTCACCAGCTGACGACATGCAATGCAGCACCTGTGTCAGAGCTCCTCGAAGGCACTCAA
GTTTTCCAGTCTCCGGAAAGATCCCTCCACGGTCAAGGCCTGGTAAGGCTCTTCACATCAGACTT
AGAATTAAACCACATGCTACACGCGCTTGTACGGGCCCAAGTCAATTCATTTGAGTTATAACGCT
TGCCACCTACGTACTACCGCAGGCGGCTCGCACGTAATGCGTTAGCCGTGCGCCATCTAAGATCT
CAAGGATACCGTCAACGACTAGTGCGACAGTCTACGGACACATTTGGTCTTCCCTGATAACAGAG
TTTTACGAACCGAAGACCTTCATCACTCACGCGGCGTTGCTCCGTCCGGATTTCGCCCATTGGGG
AAGAATCCCTACTGGTGGCTCCCGTAAGAATCTGGACCGTGGTCTCAGTTCCAGTGTGGCCCAAC
ACCCTCTCAGGGCCGCTACGTATCGATGCCCTGGTAAAACGTTACCTTACCAACTAGCTAATACG
GCGTGGATCCATCTATAAATGACAGCAAAACCGCCTTTCACTAGTGAAACATGCGGGTACAATAT
GTATATCCGGTATTATCTCCCGGTTTCTCGAAGATATCCCCAGTCTTATACGCTAGATTATCCAC
GTGTTTACTCACGCCGTCCGCGCTAATGTCAAAGGAGAAAGCTTCCTTAACTGGTTCGCTCGAAC
TTGCATGTATTAAGGCACGCCGGCCAGCGTTCATCCTGAGCAGGAGAAAAACTCTAAAAAA

Appendix D-8 Nucleotide sequence of isolate ERP-003 (~ 1500 bp)

TTGGTTACCCCTTTACGACTTCACCCCAGTCATGAATCACTCCGTGGTAACCGTCCCCCCGAAGG
TTAGACTAGCTACTTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGG
GAACGTATTCACCGTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTT

GCAGACTGCGATCCGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCC
TTTGTACCGACCATTGTAGCACGTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCAT
CCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCTTAACGTGCTGGTAAC
TAAGGACAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAG
CCATGCAGCACCTGTGTCAGAGCTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGCATGT
CAAGGCCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCATTTGAGTTTTAACCTTGCAAAGTCGCGTACTCCCCACGCGGTCGACTTAATG
CGTTAGCTGCGCCACTAAGATCTCAAGGAACCCAACGGCTAGTCGACATCCATTTACTGGCGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATTAGCCCA
GGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATT
CCACCACCCTCTGCCATACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGC
TTTCACATCCAACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTG
CACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTCGGTAACGTCA
AAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCT
TCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCC
TCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACG
GATCGTCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAGC
GTGAGGTCCGAAGATCCCCCACTTTCTCCCGTAGGACGTATGCGGTATTAGCGTTCCTTTCGAAA
CGTTGTCCCCCACTACCAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTGAATCATGGAG
CAAGCTCCACTCATCCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCTG
ATTCAAAACTCTAAAACGGGCTGGTAGAAATATAC

Appendix D-9 Nucleotide sequence of isolate ERP-007 (~ 1500 bp)

GTAGACTTCACCCCAGTCATGAATCACTCCGTGGTAACCGTCCCCCCGAAGGTTAGACTAGCTAC
TTCTGGAGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC
GTGACATTCTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATC
CGGACTACGATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCA
TTGTAGCACGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTC
CGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGACAAGGGT
TGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCT
GTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAA
GGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCA
TTTGAGTTTTAACAAGTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCAC
TAAGTATCTCAAGGAATCCAACGGCTAGTTGACATCGTTTACGGCCCGTGGACTACCAGGGTATC
TAATCCTGTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCG
CCACTGGTGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTAC
CGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACATCCAACT
TAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTA
CCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAACGTCAAAACAGTAGGATAT
TAGGCTACTGCCCTTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCG
GCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCT
GGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGG
TGAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGG
TCCCCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCGCTCGTTTCCGAACGTTATCCCCCACT
ACCAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTGAATCCAGGAGCAAGCTCCTCTCAT
CCGCTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGACCATGATTCAAACTCTA

Appendix D-10 Nucleotide sequence of isolate ERD-002 (~ 1500 bp)

CCCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACTGGTGTCTGAGTTCCCGAAGGC
ACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTAGA
ATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCAG
CCGTACTCCCCTAGACGGTCAACTTAATGCGTTAGCTGCGCCACTATTAGTATCTCATAGGATCC

TTCAACGGATAGTTGACATCGTTTTACAGCGTGGACTTACTTTCAGGGTATTACGTATATCTTCT
GTTTGCTCCCCACGCTTTCGCACCTCAGTGTCAAAGTGTCAGTCCAGGTAGTCGCCTTCGCCACT
GGTGTTCCTTCCAATGATCTACGCATTTTCACCGCTACACTGGAAATTCCACTACCCTCTACCGC
AAGAAGGCCTCAGACAGTTTGGATTGCAGTTCCCATGGTTGAGCCAGGGGATTTCACATCCAACT
TATCAAACCAACCTACACGCGCTTTTTACGCACCAGAAATCCGAGCCATTAACGCTAGCGCCGCT
TCGCATTATCGCGGCTGCGGTTACAAATTTAGCCGGCGCTCATTCTGTGGGAGATGATCAAATTA
TAAAACATTCACTATGATAAAAATCATTCCATTCAAAGGAGGATAAGTTGATGAGTAGGTTTTAC
AAACACAGCAGGTGTGTGCACGATCTTCCTTAGTAGGAGGCAAGAATGACTCCGGGCGTTGCTTG
GATATATGTGAGCATGGTAGGGATCTTGCTGGGAGATAGATCCATTCCTTTCAGTTTTAAGATTG
GCGACGGGCAGCGGGGTTTACCGTATAAATAGAGAGTAGAAAAGAATAAATCTACAAGATCCTGG
GGAATAAGAGTAACTAATCTGCGGCTAAGAGTAGCGTGAAAGAGATTCGGTATAGTGAGAGAATA
CCTACGCTGAGATGACGACGACGAGCAGAGGGAAGACTCACCGGGGGCAGGGTTGAGATAAAACT
CGGGACCTTTCAGGGTTCCGGTACGGGCTGGGGAAGAACGGCGAAGAAGCCTAAATGCACGATAA
ACTAAAAAGGGACGAGGAGAAGGCGTGGAGATTTGAAGTTAACATTTAAGAGCGAACATAAGAGT
TTTATGGGGTTGAAAAGAAAAGAAAAATATTAGAAGACTTTAGAGAAGTGAGGAAGCTGGAAACG
AAGATGGCGCAAGAGTGTTGGTGCGGGAGGGAGGAGGCATAACGAGGTTAAGAACGGGAAGGAGG
GCCACAACTCAAAATACTTAAAAAGAGGGTGAGACTGCGCCAGAGAGGGACTGGGGGCAAAGCAA
ACACCCAAAAAATTTTTTATTTTTTTTTGGTCCCCCCGACCCCCAGGCAA

Appendix D-11 Nucleotide sequence of isolate ERD-004 (~ 1500 bp)

TGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAG
AACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGTCCATTGTAGCAC
GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTC
ACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCC
CCCGAAGGGGACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCG
TTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCA
GTCTTGCGACCGGTACTCCCCAGGGCGGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGG
AAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCG
CTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTC
CTCCACATCTATACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTT
CCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCC
TGCGAGCCCTTTACGCCCAATAATTCCCGGTTACAACGCTTGCCACCTACGTATTACCGCGGCTG
CTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGT
ACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCT
CCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTG
TCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGT
TACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTAT
GTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTC
TTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGAGCAAGCTCCCAT
CTGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTTCGTCTGACG

Appendix D-12 Nucleotide sequence of isolate ERD-005 (~ 1500 bp)

TTTTAGCTAAGCGGGGTTCGATGGGGGGGGTGGGGGGTGGCGAAGTGCTGAGTGTATGGGGGAAG
GGCGTCATTAAAATTTTAACGATGGGAGGGGTGAGGTGGCACGGTGGTGTTGCTGGCGGATAAGT
GTATATAATTTTGGAAATTGAGATTCTTACCCATGGCTGTCCAGGCCTATATCCAGTACATGGAT
GCACGATAGACCTCCAGACGTAGATGGCCTCATGACGCTACGACATCCCCACCTTCGTCCGTTTG
TCACCGGCAGTGTCTTAAAAGCATCCACCATAACGTGCTGGTAATTAGGGACAAGGGTTGCGCTC
TTTACGGGACTTAACCCAACATCTCACGACACGAGACTGTCGACAGCCATGCAGCACCTGTGTCT
GAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCT
TCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAG
TTTTAACCTTGCGGCGATCGTGTACCTCCCGTCAGGCAAGTCAACTTAATGTTCGTTAGCTGCGC

TCACTAAATATCTCAAGGATTCCAACGGCTAGTTGACATCGTTTTACGGCGTGGACTACCAGGGT
ATTCTAATCCTGTTTACTCCCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGATAGTCGC
CTTCTGCCACTGGTGTTCCTTCCTATATATACGCATTTCACCGCTACACAGGAAATTCCACTACC
CTCTACCGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACAT
CCAACTTAACGAACCACCTACGCGCGCTTTACGCACCAGTAATTCCGATTAATACTTGCGCCCTT
CGTATTAGCGCGGCTGCTGGCACCAGGTTGGACGTGGGTTATTTGGTTGTAGGGTCCGAGCTTTA
ATACATGCGTCTTGGGGCTATCACCCCAACTTAGGGAGGTTTACATTGGAAAGCGCTTCGTCGGC
GCCCCGAGGGGGGTGGGTAACGCTCTCACTACTTTATGATAATTTAACTCTCCTGATCTCCTTCT
CACATGAGGACCGCGGATCAGCTTCGGGGTGGGAGGTGAGTAGGTCGGTCCGATTTTTTCGCTTC
GGGGAGGGATTCTGGGGTTTTGCGCCGCGGGAGGCTATGGCGGAGGCTAGGGTCGGAGGGGGTAG
GGGGGGGGGGTAGTGGGTGAACTGAGCGAGGGGGTGGGGGGGGGCATAGGCGGGGTTTGGGGCGG
GGTTCTTTCGCAGTGGCAGGGGCGAACGGGGGCGGATGGGAGCATACCGGGCGCGGGCGATTGAC
CACTTCCGGGCACAGGCATCAAGGCATTCGGATGGGGGGTGGGGGGGGGCGGCGGGCGGAGGGCT
AAG

VITA

Miss Parichaya Tiralerdpanich was born in February, 20 1992. Her academic background are written below;

- Bachelor of Science (Microbiology), Faculty of Science, Chulalongkorn University, Thailand (2010-2014)

- Master of Science (Environmental Management) Inter-Department of Hazardous Substance and Environmental Management, Graduate school Chulalongkorn University, Thailand (2014-2016)

- Participated oral presentation in the 5th International Conference on Environmental Engineering, Science and Management, May 11-13 2016 at The Twin Towers Hotel Bangkok, Thailand, topic "Biodegradation of Hexadecane and Phenanthrene in Sediment ans Seawater Samples from a Canal in Bangkhuntien, Bangkok, Thailand"