การย่อยสลายสารพอลิไซคลิกอะโรมาติกไฮโดรคาร์บอนในดินที่ปนเปื้อนสารปิโตรเลียม โดยการเติมใบมะขามที่หมักกับดินที่ปนเปื้อน

นายวรพงษ์ เลิศธำรงศักดิ์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN PETROLEUM –CONTAMINATED SOIL BY THE ADDITION OF TAMARIND LEAVES COMPOSTED CONTAMINATED SOIL

Mr. Waurapong Lertthamrongsak

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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By	Mister Waurapong Lertthamrongsak
Field of Study	Environmental Management
Thesis Advisor	Associate Professor Kanchana Juntongjin, Ph.D.
Thesis Co-advisor	Ekawan Luepromchai, Ph.D.

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

.....Dean of the Graduate School
(Professor Suchada Kiranandana, Ph.D.)

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.....Thesis Co-advisor
(Ekawan Luepromchai, Ph.D.)

.....Member
(Associate Professor Pairoh Pinphanichakarn, Ph.D.)

.....Member
(Assistant Professor Suthep Thaniyavarn, Ph.D.)

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พอลิไซคลิกอะโรมาติกไฮโดรคาร์บอนจัดว่าเป็นสารมลพิษที่ปนเปื้อนในสิ่งแวดล้อม เนื่องจากสมบัติการแพร่กระจายและความเป็นพิษต่อสุขภาพร่างกายมนุษย์ จุดประสงค์ของการวิจัยเพื่อพัฒนาเทคนิคการบำบัดสารพอลิไซคลิกอะโรมาติกไฮโดรคาร์บอนในดินที่มีการปนเปื้อนน้ำมันปิโตรเลียม โดยการใช้ใบมะขามที่ทราบแน่ชัดแล้วว่า เป็นแหล่งของจุลินทรีย์ที่สามารถย่อยสลายสารพิษนี้ได้อย่างมีประสิทธิภาพ มาใช้ในการเตรียมหัวเชื้อ และนำไปใช้ในระบบบำบัด นำตัวอย่างดินปนเปื้อนน้ำมันปิโตรเลียมบริเวณ ป้อมพระจุลจอมเกล้า ผสมกับ ใบมะขามในอัตราส่วน ดิน : ใบมะขาม เท่ากับ 9: 1 โดยปริมาณดินและใบมะขามรวมกันได้ 2 กรัม ในวันที่ 49 ของการทดลองพบว่ามีอัตราการย่อยสลายดีที่สุด คือมีปริมาณ ฟีแนนทรีน, ฟลูออแรนธีน, และ ไพรีน ถูกย่อยสลาย 13 %, 20 %, และ 8 % ตามลำดับ จนในวันที่ 56 มีการย่อยสลายเพิ่มขึ้น 5 %, 4 %, และ 1 % ตามลำดับ ในขณะที่ ในชุดควบคุมไม่พบการลดลงของสารเหล่านี้อย่างมีนัยสำคัญ ดังนั้นจึงนำดินที่ผสมที่ย่อยสลายในวันที่ 49 ไปใช้เป็นหัวเชื้อ โดยผสมระหว่างดินกับหัวเชื้อ ในอัตราส่วน ดิน : หัวเชื้อ เท่ากับ 9 : 1 และมีการเติมหญ้าจากสนามกอล์ฟเพื่อเพิ่มประสิทธิภาพการย่อยสลาย โดยปริมาณดินรวมกับใบมะขาม และ ดินรวมกับ ใบมะขามกับหญ้า เท่ากับ 20 กรัม จากผลการวิเคราะห์พบว่า ดินปนเปื้อนน้ำมันปิโตรเลียมบริเวณ สถานีรถไฟบางกอกน้อยที่ผสมกับหัวเชื้อ มีประสิทธิภาพในการย่อยสลายสารปนเปื้อนได้ดีที่สุด และเมื่อตรวจแบคทีเรียที่สามารถย่อยสลายฟีแนนทรีนที่เกิดขึ้นในชุดการทดลอง โดยนับจำนวนโคโลนีที่มีวงใสล้อมรอบบนอาหารเลี้ยงเชื้อ CFMM ที่พ่นทับด้วยสารละลายฟีแนนทรีน พบว่าในชุดการทดลองนี้สามารถตรวจพบปริมาณแบคทีเรียได้มากที่สุด โดยแบคทีเรียเหล่านี้สามารถจะตรวจพบเฉพาะในดินที่เติมหัวเชื้อเท่านั้น กล่าวโดยสรุปหัวเชื้อที่เตรียมจากการหมักใบกับดินที่ปนเปื้อน ให้ผลการย่อยสลายสารปนเปื้อนได้อย่างมีประสิทธิภาพสูงในดินที่ปนเปื้อนที่มาจากแหล่งต่างกัน

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จุฬาลงกรณ์มหาวิทยาลัย

สาขาวิชาการจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต.....วรพงษ์ เลิศธำรงศักดิ์.....

ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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WAURAPONG LERTTHAMRONGSAK: BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS IN PETROLEUM – CONTAMINATED SOIL BY THE ADDITION OF TAMARIND LEAVES COMPOSTED CONTAMINATED SOIL.
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Polycyclic Aromatic Hydrocarbons (PAHs) are considered as environmental pollutant due to its widespread distribution and potential health threat. This research aimed to develop PAHs bioremediation in petroleum-contaminated soil by using tamarind leaves which have been proven to be a source of effective degrading microorganisms in preparation of a seed culture for the treatment system. A 2 g soil reactor was set up by using contaminated soil collected from Phrachulachomklao Royal Navy Dockyard mixed with tamarind leaves at the ratio of 9:1. Moisture content, temperature and aeration in the reactor were adjusted to the optimal conditions. At 49 days of incubation, degradation rate seemed to be the highest in which 18 % phenanthrene, 24 % fluoranthene and 9 % pyrene were degraded. Biodegradative efficiency was increased 5%, 4%, and 1% respectively after 56 days while only 1-3 % of these PAHs were eliminated in the control. The scale-up experiment was further achieved in 20 g. reactor. The tamarind leaves-soil mixture incubated for 49 days was used as a seed culture by using the ratio of 9:1 (contaminated soil: seed culture). The experimental results were observed in the soil collected from two contaminated sites: Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station. Phenanthrene degrading bacteria were determined as representative of PAHs degraders by detecting colonies surrounded with clear zones on CFMM agar plates after spraying with phenanthrene solution. The highest numbers of phenanthrene degrading bacteria were observed in Bangkoknoi Railway Station's soil mixed with seed culture. However, numerous phenanthrene degraders were found in the soil mixed with seed culture. Thus the seed culture prepared from leaves composting in contaminated soil provided highly effective PAHs degradation in both of the soils collected from different contaminated sites.

Inter-department Environmental Management	Student's signature Waurapong Lertthamrongsak
Field of study Environmental Management	Advisor's signature Kanchana Juntongjin
Academic year 2003	Co-advisor's signature Ekawan

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NOMENCLATURES

B	=	Bangkoknoi Railway Station's soil
BS	=	Bangkoknoi Railway Station's soil mixed with seed culture
BSY	=	Bangkoknoi Railway Station's soil mixed with seed culture and yard waste
CFMM	=	Carbon Free Mineral Medium
EPA	=	Environmental Protection Agency
P	=	Phrachulachomklao Royal Navy Dockyard's soil
PS	=	Phrachulachomklao Royal Navy Dockyard's soil mixed with seed culture
PSY	=	Phrachulachomklao Royal Navy Dockyard's soil mixed with seed culture and yard waste
PAHs	=	Polycyclic Aromatic Hydrocarbons
ppm	=	part per million
RT	=	Retention time

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CHAPTER 1

INTRODUCTION

1.1 Statement of problem

During the past 200 years, there is a rapid increase of populations world-wide, resulting in the need for even greater amounts of fuel and development of industrial chemicals, fertilizers, pesticides and pharmaceuticals to sustain and improve quality of life (Chakrabarty *et al.*, 1988). Although many of these chemicals are utilized or destroyed, a high percentage is released into the air, water and soil, representing a potential environmental hazard (Alexander, 1995).

Polycyclic aromatic hydrocarbons (PAHs) are considered environmental pollutants resulting from oil spills, automobile exhaust, coal refining process, and petrochemical industries. PAHs are priority pollutants due to their potential toxicity, mutagenicity and carcinogenicity. Low-molecular weight PAHs (containing less than four benzene rings) are acutely toxic, which effect the reproduction and mortality of aquatic animals and most high-molecular weight PAHs (containing four or more benzene rings) are mutagenic and carcinogenic. Various soil remediation techniques including in-situ treatment, agitated tank, soil washing and biological soil treatment have been applied to degrade toxic compounds (Kastner *et al.*, 1995).

Bioremediation is the use of biological treatments, for the clean-up of hazardous chemicals in the environment. At the present, employing the biochemical abilities of microorganisms is the most popular strategy for the biological treatment of contaminated soils and waters (Head, 1998). Microorganisms, more than any other class of organisms, have a unique ability to interact both chemically and physically with a huge range of both man-made and naturally occurring compounds leading to a structural change to, or the complete degradation of, the target molecule (Head, 1998).

Microbial degradation represents the major route responsible for the ecological recovery of PAHs-contaminated sites (Cerniglia, 1992). For example,

many species of bacteria have been shown in recent years to be susceptible to using PAHs extending to four-ring compounds as sole sources of carbon and energy (Bouchez *et al.*, 1996). The application of bioremediation techniques to PAHs is based on the finding that adding fertilizer or degrading bacteria decreased the amount of soil PAHs (Manilal and Alexander 1991; Wang *et al.*, 1990).

In Thailand, PAH biodegradation and bioremediation study have been carried out in the Biodegradation Research Unit, Microbiology Department, Science Faculty, Chulalongkorn University. They found that plant waste materials such as peanut shells and leguminous leaves were found to effectively stimulate PAHs biodegradation rate in 2 g soil microcosm (Charoenchang 2001: Siriwarasin 2002). To improve the technique, this research is aim to develop a scale-up PAH bioremediation treatment while using smaller amount of plant waste material. It will focus on the degradation of three or four ring PAHs that contaminated in petroleum-contaminated soil.

1.2 Objectives

This study aimed to apply microorganisms inherent on tamarind leaves for biodegradation of PAHs and unknown hydrocarbons in petroleum-contaminated soil as well as to find the appropriate incubation time to prepare a seed culture for using in a scale-up treatment.

The sub-objectives of this study were:

(1) To select an appropriate incubation time from the highest decreasing rate of PAHs in petroleum-contaminated soil incubated with tamarind leaves.

(2) To prepare a seed culture by incubating contaminated soil with tamarind leaves using the condition obtained from (1).

(3) To construct a scale-up bioremediation treatment by applying the seed culture to petroleum-contaminated soil and then supplementing with yard waste material from a golf field to increase organic matter content in the system.

(4) To monitor the decrease in the number of PAHs degrading bacteria in the bioremediation system.

1.3 Outline of the study

Bioaugmentation is generally performed by adding pure bacterial culture into the contaminated soil. The efficiency of this treatment would depend on the activity of added bacteria as well as their persistence and survival in soil. In the study, we focused on a potent microbial source from natural waste material which could be inoculated into the contaminated site in the form of microbial consortia and later as seed culture. This microbial addition technique would be more advantages than adding the pure culture, because diverse groups of bacteria would be added to the contaminated soil. These bacteria would simultaneously degrade the mixture of soil contaminants i.e. PAHs and other hydrocarbons. In this study, we used agricultural waste, tamarind leaves, to enhance PAHs biodegradation rate as well as to prepare seed cultures. The incubation time suitable for degradation of phenanthrene, fluoranthene and pyrene in the presence of leaves composting in contaminated soil was also investigated. The time selected was then applied for preparation of the seed culture which could be used in the degradation of PAHs and other hydrocarbon in the other problem areas. The results would prove that tamarind leaves were a useful source of PAH degrading microorganisms and the preparation of seed culture would minimize the amount of leaves added for the bioremediation of petroleum-contaminated soil.

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CHAPTER 2

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

Theoretical Background

2.1. Source of PAHs

PAHs consist of two or more fused benzene rings in linear, angular, or cluster arrangements. By definition, they contain only C and H atoms, although N, S, and O atoms may readily substitute in the benzene ring to form heterocyclic aromatic compounds, commonly grouped with the PAHs. Some of the more common PAHs are shown in Fig. 1. They are formed whenever organic materials are burnt, with temperature influencing the specific mixture of PAHs formed. PAHs are formed naturally during thermal geologic reactions associated with fossil-fuel and mineral production, during the burning of vegetation in forest and bush fires, and also by some plant and bacterial reaction (Blumer, 1976).

However, anthropogenic sources, particularly the burning of fossil fuels (Freeman and Cattell, 1990; Benner *et al.*, 1990) are significant sources of PAHs to the environment (Sims and Overcash, 1983). Anthropogenic combustion activities are a principal source of PAHs to soils in industrialized countries via atmospheric deposition. As a result, soil concentrations of PAHs are likely to increase over the last 100-150 years, particularly in urban areas (Jones *et al.*, 1989a, 1989b).

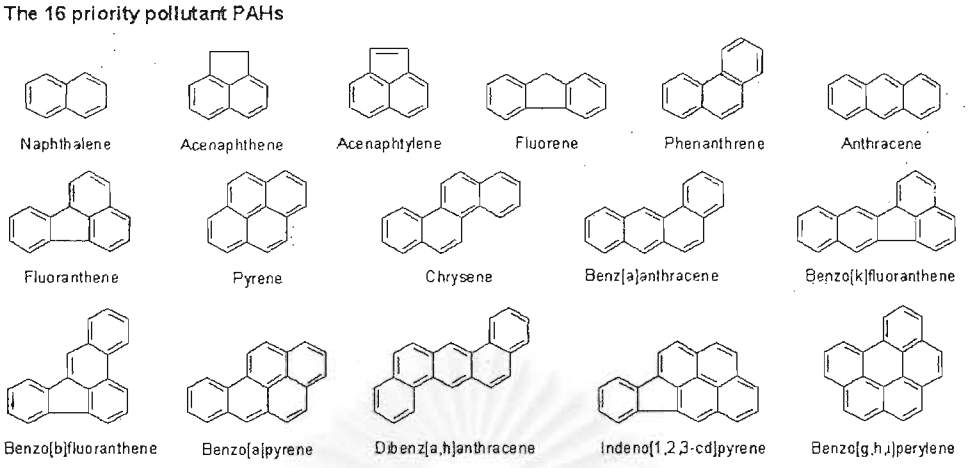


Figure 1. Chemical structures of some common polycyclic aromatic hydrocarbons. (Source: Jinno Laboratory, School of Material Science, Toyohashi University of technology)

The presence of PAHs in contaminated sites is associated with industrial activities, usually involving the processing combustion, and disposal of fossil fuels, or fossil-fuel-derived products such as coal tar and carbon black (Nishioka *et al.*, 1986). Fractionation products derived from crude oil such as diesel, petroleum, fuel oil, lubricating oil, etc., contain PAHs. The crude-oil source (Grimmer *et al.*, 1983) and fractionation process used have an effect on the PAHs content of the final fuel products, and higher concentrations of PAHs are associated with the higher-boiling-point distillation products (Nyer amd Skladany, 1989).

Sites where refining and distillation have occurred are frequently contaminated with PAHs and other aromatic and aliphatic hydrocarbons. PAHs contamination on industrial sites is commonly associated with spills and leaks from storage tanks (under or above ground) and with the conveyance, processing, use, and disposal of these fuel/oil products. PAHs contamination is also commonly associated with wood-treatment activities since PAHs are major constituents of creosote (approximately 85% PAHs by weight) and anthracene oil, which are commonly used wood-treatment pesticide (Bos *et al.*, 1984; Bumpus, 1989; Mueller *et al.*, 1989; Walter *et al.*, 1990). Source of PAHs can conclude and summarized in table 1.

Table 1. Activities associated with the production, processing, use, and disposal of PAHs-containing material (Wilson and Jones, 1993).

Gasification / liquefaction of fossil fuels
Heat and power generation by using fossil fuels
Coke production, catalytic cracking, Asphalt production and use
Carbon-black production and use
Coal-tar / coal-tar-pitch production and use
Refining / distillation of crude oil and crude-oil-derived products
Wood-treatment process
Wood-preservative (e.g. creosote / anthracene-oil) production
Fuel / oil storage, transportation, processing, use, and disposal
Landfill / waste dumps, open and burning (types / refuse / coal etc.), incineration

2.2 Bioremediation

Bioremediation involves the use of microorganisms to degrade hazardous organic constituents to harmless substances such as carbon dioxide and water. The degradation process may be enhanced by changing chemical or physical conditions in the soil such as soil pH, moisture, and aeration and also by nutrient addition. The addition of specifically adapted microorganisms may also enhance the process. Bioremediation methods used to treat soil contaminated with organic compounds including PAHs consist of one or a combination of three systems described below.

2.2.1 In-situ treatment

The contaminated soil is treated in place and essentially remains undisturbed during in-situ treatment (Hilberts *et al.*, 1985; Wilson and Brown, 1989). The most common form of in-situ treatment is the biodegradation of contaminants within the saturated zone of the soil. This involves the addition of nutrients, an oxygen source (usually hydrogen peroxide) and sometimes specifically adapted microorganisms to enhance degradation and may be achieved by drilling a series of wells through out the contaminated area and directly injecting the appropriate solutions. Groundwater is often recovered (usually down-gradient) and recirculated

after some form of surface treatment. The soil amendments may be added to the treated groundwater prior to recirculation.

In-situ treatment can be used for unsaturated soils but tends to be less easy to control. Treatment generally relies on the application of nutrient-rich water by percolation or through pressure–injection with multiple injection point. Forced-air/vacuum extraction systems may be used to enhance the air exchange with in the soil.

2.2.2 Bioreactors

The contaminated soil is excavated and removed to a specific reactor for treatment. The bioreactor may have been established on-site or elsewhere in a dedicated treatment area. Usually, the soil is slurred with water and then treated in the reactor where conditions for bioremediation are enhanced. There is considerable control the operating conditions, which often results in relatively quick and effective treatment. The bioreactors are usually of the horizontal-drum and airlift type and may be batch or continuous but are usually batch-mode. An acclimatized microbial population from a previously treated soil batch is usually introduced to each new batch to enhance the degradation rate. After treatment, the material is passed through a water-separation system and the water recycled. Contaminated groundwater / effluent may also be treated in bioreactors that are either the fixed-film or the stirred-tank type.

2.2.3 Prepared bed/On-site treatment

Landfarming of contaminated material was one of the first forms of on-site treatment and has been widely used in the oil industry for the disposal of oily sludge (Morgan and Watkinson, 1989). The waste material is applied to the soil as slurry and the area managed by fertilization, irrigation and lime addition to maintain optimum conditions of nutrient content, moisture content and pH. The area is also tilled to improve aeration and to ensure that degradation and immobilization of contaminants within the upper and underlying soil layer occur. Microorganisms used in the degradation process are most often the indigenous soil population. However, specifically adapted microorganisms may be added to the soil to enhance the process.

The major disadvantage with landfarming is the possibility of contaminant movement from the treatment area.

More recently, prepared beds, which involve a greater degree of engineering and containment, have been used. The contaminated soil is removed to a specifically prepared area, which is usually lined with low permeability material such as high-density polyethylene or clay. The bed is managed to optimize degradation with fertilization, irrigation, pH control and sometimes microbial and surfactant additions. The beds are design to enhance treatment and minimize contaminant movement off-site. They often encompass leachate-collection and sometimes emission-control systems and are usually situated elsewhere on-site or on the area from which the contaminated material is removed.

Composting is another form of prepared-bed type of treatment that has been used to treat highly contaminated material. It is a pool of considerable microbial diversity (Beffa *et al.*, 1996a, b; Nakasaki *et al.*, 1985) that can potentially serve as a source inoculum for the biological treatment of hazardous waste, including PAHs, pesticide and other contaminants (Semple *et al.*, 2001). Although many surface soils contain native bacteria and fungi capable of degrading PAHs and other hydrocarbons (Berry, 1999; Prince and Drake, 1998; Van Agteren *et al.*, 1998), compost material has been blended with PAH-contaminated soils to aid in the degradation of PAHs as an ex-situ remediation process (Crawford *et al.*, 1993).

Composting of PAH-contaminated soils, using admixtures with animal manure/or carbon sources, has also been practiced as a means of bioremediation (Crawford *et al.*, 1993; Haderlein *et al.*, 2001). The biodegradation of PAHs in compost matrix has been studied by measuring residual PAH concentrations and using ^{14}C-radiotracer techniques for $^{14}CO_2$ evolution (Kastner and Mahro, 1996; Reid *et al.*, 1999). While both techniques have confirmed the biodegradation potential of mushroom and yard waste/kitchen waste composts, it seems that native, PAH-degrading microbial communities may vary with the type and source of compost.

Yard waste compost is composed of plant materials such as grass clippings, leaves, trimmings and woody parts (Glenn, 1989). Diverse microbial

decomposition of plant polymers leads to numerous ring structures which are further biodegraded through ring fission to central metabolites. The content of yard waste varies with the season, contributing to the physical and chemical heterogeneity of the compost material. According to McGowin *et al.* (2001) PAHs levels mostly are at low mg kg^{-1} levels in yard waste compost, but variation is to be expected depending on the feed material.

In the present work, the biodegradation potential of yardwaste compost are assessed using phenanthrene, a three-ring PAH, as a model substrate. Fluoranthene, a four-ring entity, was also used in some mineralization experiments.

Advantages of compost (Olds College Composting Technology Centre, 2001).

- No order when spreading
- No weed seeds
- No pathogens- Fecal coliform and Salmonella sp. are indicator organisms that are destroyed during composting
- Add organic matter to the soil
- Improves soil structure (porosity)
- Low moisture content
- High water holding capacity
- Diverse microbial population
- Neutral pH
- Slow release fertilizer –Nutrients are inorganic from less chance of leaching
- Can seed immediately after application

2.3.Factors affecting bioremediation of PAH (Olds College Composting Technology Centre, 2001; Wilson and Jones, 1993).

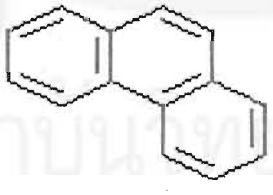
A number of different factors affect the likelihood of successful bioremediation. In the first instance, this is dependent on inherent properties of the PAHs

compounds and the soil. Soil properties that alter the rate of degradation such as organic matter, structure, and particle size have been reviewed previously environment factors, including temperature, pH and soil aeration also affect the rate of degradation and may be modified to enhance degradation. The presence of contaminants that are toxic to microorganisms such as metals and cyanides may hinder degradation.

2.3.1 Properties of PAHs

Athough PAHs behave in certain general ways in the environment each PAHs compound obviously has a unique set of physical and chemical properties. The stability of PAHs is indicated by the ring arrangement, linear being the most unstable and angular (rings in step) are the most stable (Blumer, 1976). PAHs are relatively insoluble in water and are therefore associated primarily with the particulate phase, in particular the organic matter (Means *et al.*, 1980). A wide range of solubility is displayed with solubility generally decreasing with an increasing number of fused rings (Sims and Overcash, 1983). Volatility also decreases with an increasing number of fused rings. Properties of PAHs used in this research (Jinno Laboratory, 1996; Physical and Theoretical Chemistry Laboratory, 2004; Spectrum Laboratory Inc, 2003) are:

Phenanthrene



Physical data

Appearance: white crystals

Melting point: 99 - 101 °C

Boiling point: 336 °C

Water solubility : 7.2×10^{-3} mmol / l

Stability

Stable, combustible, incompatible with strong oxidising agents.

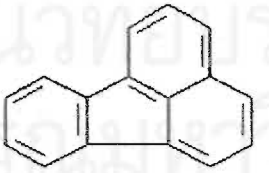
Toxicology

Harmful if swallowed. May be harmful if inhaled or absorbed through the skin. Skin, eye and respiratory irritant. Causes photosensitivity.

Environmental Impact

Release of phenanthrene (Phe) most likely results from the incomplete combustion of a variety of organic compounds including wood and fossil fuels. Release to the soil will likely result in biodegradation. Volatilization is not expected to be significant. Phenanthrene is expected to bind strongly to soil and not leach extensively to groundwater. When released to water, adsorption of Phe to suspended sediments is expected to remove most of the compound from solution. Photolysis is expected to occur near the water surface and biodegradation of phenanthrene in the water column is expected. Oxidation, volatilization and bioconcentration are not expected to be significant. Phenanthrene released to the atmosphere is expected to rapidly adsorb to particulate matter. Phenanthrene adsorbed on fly ash has been shown to photolyze rapidly (half-life 49 hr) and Phenanthrene adsorbed on particulate matter will be subject to wet and dry deposition. Vapor phase phenanthrene will react with photochemically generated, atmospheric hydroxyl radicals with an estimated half-life of 1.67 days. Phe is a contaminant in air, water, sediment, soil, fish and other aquatic organisms and food. Human exposure results primarily from ingestion of food contaminated with Phe.

Fluoranthene



Physical

Appearance: solid

Melting point: 105 - 110 C

Boiling point: 375 C

Water solubility: 1.3 x 10^{-3} mmol / l

Stability

Stable. Incompatible with strong oxidizing agents.

Toxicology

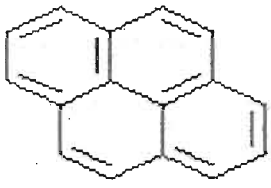
Harmful if swallowed. Limited evidence that this may act as a carcinogen. Skin, eye and respiratory irritant.

Environmental Impact

Fluoranthene's release into air and water is quite general since it is a universal product of combustion of organic matter and is present in fossil fuel products. Its release is greatest in areas of high anthropogenic activity. Both in air and water it is largely associated with particulate matter. When released into water, it will rapidly become adsorbed to sediment and particulate matter in the water column, and bioconcentrate into aquatic organisms. In fact, concentrations in shellfish such as clams and mussels are an excellent indicator of pollution in a localized area. In the unadsorbed state it will degrade by photolysis (half-life days to wk). It appears to be stable in sediment for decades or more. Because it is strongly adsorbed to soil, it should remain in the upper few centimeters of the soil.

However, its detection in groundwater demonstrates that it can be transported there by some processes. It should biodegrade in a few years in the presence of acclimated microorganisms. The fluoranthene released in the atmosphere will photodegrade in the free state (half-life 4-5 days). Aerosols and particulate matter containing sorbed fluoranthene is sufficiently stable to be transported long distances while being subject to gravitational settling and rainout. Photochemical smog situations enhance the degradation of both the sorbed molecule and the free vapor. Human exposure is from ambient air and ingesting food contaminated with products of combustion or prepared in such a manner (smoking, charcoal broiling) as to generate polynuclear aromatic hydrocarbons. Exposure from drinking water is less common since water treatment such as filtration and chlorination removes fluoranthene. Distribution systems lined with coal tar or asphalt can sometimes contribute measurable amounts of fluoranthene to the drinking water.

Pyrene



Physical data

Appearance: yellow or white crystals and powder

Melting point: 149 - 151 °C

Boiling point: 404 °C

Water solubility: 7.2×10^{-4} mmol / l

Stability

Stable. Incompatible with strong oxidizing agents. Flammable.

Toxicology

Harmful if swallowed. May act as a carcinogen. May be harmful by inhalation or through skin contact - readily absorbed through skin. Irritant. Toxicology not fully investigated.

Environmental Impact

Pyrene's release to the environment is ubiquitous since it is a ubiquitous product of incomplete combustion. It is largely associated with particulate matter, soils and sediments. Although environmental concentrations are highest near sources, its presence in places distant from primary sources indicates that it is reasonably stable in the atmosphere and capable of long distance transport. When released to air it may be subject to direct photolysis, although adsorption to particulates apparently can retard this process. If released to water, it will adsorb very strongly to sediments and particulate matter, bioconcentrate in aquatic organisms slightly to moderately, but will not hydrolyze.

It may be subject to significant biodegradation, and direct photolysis may be important near the surface of waters. If released to soil it will be expected to adsorb very strongly to the soil and will not be expected to appreciably leach to the

groundwater, although its presence in groundwater illustrates that it can be transported there. It will not be expected to hydrolyze or significantly evaporate from soils and surfaces. It may be subject to appreciable biodegradation in soils. Human exposure will be from inhalation of contaminated air and consumption of contaminated food and water. Especially high exposure will occur through the smoking of cigarettes and the ingestion of certain foods (eg, smoked and charcoal-broiled meats and fish).

2.3.2 Fate of PAHs in soil

The soil-removal and decomposition process that determine the fate of PAHs are volatilization, abiotic losses (i.e. leaching, hydrolysis) and biodegradation.

Biodegradation by natural surface and subsurface soil microorganisms appears to be the process primarily responsible for the removal of PAHs in a multiphase soil system (Sims and Overcash, 1983). Song and Bartha (1990) illustrated the importance of biodegradation in removing jet fuel from soil. In a study of fourteen PAHs in two soils under unsaturated conditions, Park *et al.* (1990) found that volatilization was negligible, except for naphthalene and substituted naphthalenes. Mechanisms of abiotic loss appeared to be potentially importance for two and three- rings PAHs compounds, although neither volatilization nor abiotic losses played a significant role in determining the fate of PAHs with more than three rings.

2.3.3 Microbial degradation in the field

The ultimate usefulness of any microorganism to degrade PAHs in a clean-up of contaminated soils depends on its survival and performance in diverse, contaminated ecosystems. Limitations to growth will include competition from other microorganisms, microorganisms' die-off, the presence of substrates that are degraded in preference to PAHs and site condition. Heitkamp and Cerniglia (1989) demonstrated that *Mycobacterium sp.* survived in microcosms for six weeks and mineralized multiple doses of pyrene. Competition with indigenous microorganisms did not adversely affect survival of the bacteria or pyrene degradation. However, few studies have demonstrated the survival in the natural environment of laboratory cultured microbes isolated from contaminated soil that are known to have the

potential to degrade PAHs, particularly those with more than three rings. Demonstration of effectiveness in diverse environments is essential for successful application in the field.

2.3.4 Microorganisms

PAHs are microbially degraded in one of two ways, either as the sole source of carbon and energy to the microorganisms or by co-metabolism (or co-oxidation if oxidation occurs). In the latter, an enzyme produced for degradation of a certain growth-supporting compound also degrades a PAH that is neither used nor essential for growth. Little is known about the mechanism and factors controlling co-metabolism. Co-oxidation has been observed to enhance the degradation of high-molecular-weight (HMW) PAHs in particular, i.e. those with four or more aromatic rings (Sims and Overcash, 1983). Sims *et al.* (1988) observed that HMW PAHs were more resistant to degradation when present as pure compounds in soil than in the same soil in complex waste mixtures.

During soil bioremediation, use of the PAHs as the sole carbon and energy sources is considered favorable because complete oxidation to carbon dioxide and water can occur. Complete mineralization of low-molecular-weight (LMW) PAHs (two- and three-rings) in contaminated soils by a variety of naturally occurring microorganisms that use these PAHs as sole carbon and energy sources has been demonstrated by a number of researchers (Cerniglia, 1984b: Mueller *et al.*, 1989). Bacteria genera are the most commonly reported and include: *Aeromonas*, *Alicaligenes*, *Bacillus*, *Beijerinckia*, *Corynebacterium*, *Cyanobacter*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Flavobacterium* and *Vibrio*.

PAHs with four or more condensed rings are less soluble, more stable and therefore more recalcitrant. Only recently, in laboratory studies, have a number of researchers isolated microorganisms from contaminated soil that have demonstrated the ability to mineralise four-rings PAHs as their sole carbon source and energy total source. Bacterial mineralization of PAHs with four or more rings has generally been reported to occur via co-metabolism (Heitkamp and Cernilglia, 1989; Mueller *et al.*, 1990).

Table 2.1. Bacterial species that have been reported to use PAHs as their carbon and energy source.

PAHs	PAHs-degrading bacteria	Reference
Fluoranthrene	*Pseudomonas paucimobilis* *Alicalienes denitrificans*	Mueller *et al.*, 1990 Weissenfels *et al.*,1990b
Chysene	*Rhodococcus sp.*	Walter *et al.*, 1991
Pyrene	*Rhodococcus sp.* strain UW1 *Mycobacterium sp.* strain VF1	Walter *et al.*, 1991 Kastner *et al.*, 1994
Phenanthrene	*Aeromonas sp.* *Pseudomonas paucimobilis*	Kiyohara *et al.*, 1976 Mueller *et al.*, 1990
Fluorene	Pseudomonas sp. F274	Grifoll *et al.*, 1994

Fungal metabolism of PAHs with more than three aromatic rings is less well studied but has been reviewed by a number of researchers (Cerniglia, 1984a, 1984b; Cerniglia & Heitkamp, 1989). Most mechanisms reported are co-metabolic. However, at least 22 two-, three- and four-rings PAHs including phenanthrene, fluorene, fluoranthene, anthracene and pyrene, underwent 70-100% removal in the laboratory, some to CO_2, by the white-rot fungus, *Phanerochaete chysossporium*, during 27 days of incubation with nutrient-limited cultures (Bumpus, 1989). Degradation of benzo[a]pyrene to carbon dioxide and water has also been reported. Although the potential of this fungus has yet to be demonstrated effectively in the field, it may used in the future to degrade HMW substrates, with bacteria aiding degradation of the resulting lower-molecular-weight (LMW) metabolites.

2.3.5 Soil Hydrophobicity

Soil hydrophobicity can be a severe problem in the remediation of petroleum contaminated soils even after petroleum hydrocarbons have been significantly reduced and toxicity is no longer observable. Some research reported that bioremediation of a petroleum contaminated soil had little effect on hydrophobicity. Hydrophobicity significantly decreases soil water holding capacity. After remediation, hydrophobic soils make the establishment of plant growth

difficult, because the first time the field soil dries out vegetation dies from lack of water.

Addition of compost to remediated soils might be a worthwhile means of overcoming hydrophobicity and establishing plant cover. Compost, by increasing soil organic matter, can increase soil aggregation and greatly increase water holding capacity, thereby mitigating hydrophobicity.

2.3.6 Bioavailability

Bioavailability is an important technical and regulatory issue. It is the major reason that biological decomposition of petroleum in soils can take years. Bioavailability is determined by various chemical, physical, physiological, and ecological factors. Generally, the *n*-alkanes and aromatics are the most biodegradable, followed by the branched alkanes and cycloalkanes. Of the various petroleum fractions, polycyclic aromatic hydrocarbons (PAH's) appear to be the most studied because of potential hazard and environmental persistence. Therefore, biodegradation studies of PAHs tend to pre-dominate the literature over other petroleum.

Bioavailability can be limited by sequestration when petroleum fractions are added to a soil or compost (Means *et al.*, 1980). Clays can oligomerise PAHs into unextractable forms with low bioavailability; this can happen as quickly as a 100% loss of recovery within a few weeks (Karimi-Lotfabad *et al.*, 1996). PAHs can also form complexes with organic matter, which is unextractable and poorly available (Hogan, 1995). Alternately, a great number of microorganisms can produce biosurfactants, which can greatly increase bioavailability of petroleum components (Zajic and Mahemedy 1984, Deziel *et al.*, 1996), and such organisms would grow during composting.

2.3.7 Moisture

Generally, optimum microbial activity is achieved by the maximum water content that does not restrict oxygen diffusion. Water is crucial to transport of many microbial cells and therefore substrate colonization (Griffin 1981; Harris 1981,

Miller 1989). In land farming of petroleum wastes Dibble and Bartha (1979) found 30 - 90% of soil moisture capacity was optimal. Composting petroleum contaminated soil (1.7% oil and grease) with added leaves and alfalfa in a laboratory system; Beaudin *et al.* (1996) maintained moisture within a range of 50-60%. McMillen and Gray (1994) composted an oily sludge with added tree waste, horse manure and soil, with a total mix extractable hydrocarbon content of 5.94% and found 39% moisture good.

2.3.8 Temperature

Temperature control is a critical management issue in composting. Biological systems in general exhibit the 'Q10 effect', in that for every 10°C increase in temperature activity doubles. Of course, microorganisms have lethal temperature limits at which activity stops. Much available information related to optimal temperatures for biodegradation of petroleum fractions comes from land farming situations. In the composting of plant or animal derived materials, the rate of composting increases with temperature until about 60°C. Above 60°C, activity rates drop rapidly with additional increases in temperature (MacGregor *et al.*, 1981, Finstein *et al.*, 1983).

With the usage of organic amendments in appreciable amounts to carry out soil composting, formal temperature control would be necessary to avoid exceeding the upper lethal limit for the petroleum degrading populations. The tendency for composting systems to overheat until biological activity comes to a halt is intrinsic to such systems (Miller, 1993a).

2.3.9 Oxygen

Oxygen is important to the degradation of petroleum factions as they tend to be highly reduced. Bossert and Bartha (1984) reviewed a number of studies of oil decomposition in soils, and oil degradation rates were always highest when aeration was maximized. In research with Diesel invert mud and cuttings, Aasen *et al.* (1996) reckon from their results that oxygen concentrations lower than 10 - 16% could slow the rate of hydrocarbon biodegradation.

2.3.10 Inorganic Nutrients

While petroleum can supply a good deal of metabolic carbon, petroleum is deficient in other nutrients, such as nitrogen and phosphorus. Suitable ratios of C:N:P are not readily apparent for petroleum degradation, however, in that much of the carbon in petroleum is of limited availability. McMillen and Gray (1994) found a standard C:N:P ratio of 100:5:1.7 worked well for degrading petroleum wastes. However, best ratios for C:N:P are likely to be contaminant specific and related to carbon availability. A slowly available carbon source can be degraded with wider C:N:P ratios because N and P will be recycled during decomposition.

2.3.11 Organic Nutrients Agents

Addition of significant amounts of plant materials and manures to petroleum-contaminated sub-soils can be used to initiate a 'soil composting' process. Advantages of soil composting include;

a. significantly increasing the populations of microorganisms by providing a readily available carbon source and provision of the required macro and micro-nutrients in organic form.

b. improvement of physical structure for decomposition by reducing bulk density and increasing porosity.

c. provide sufficient nutrition to increase temperatures into favorable ranges for sustained periods. One issue observed by Hogan (1995) was that for some otherwise readily degradable PAHs, nutrient addition can, on the short term, spare these compounds from degradation.

2.3.12 Effects of Soil Texture in Remediation

Soil type, including texture and classification, is an important consideration in bioremediation. Soil texture, i.e. the particle size distribution of the soil, has a great effect on structure and activity. Amount of clay in a soil is very important in bioremediation because of the effect of clays on soil porosity and more complex chemical aspects.

2.3.13 Use of Special Inoculant Cultures

In reference to the role of specific populations in petroleum remediation, seeding with specific cultures for bioremediation of petroleum products has not been of value to date in natural environments (Atlas and Bartha, 1992). On the other hand, use of adapted seed cultures is a very useful method with a long history of application in biological waste treatment. Danielson (1994) did find that the use of an adapted seed culture significantly increased petroleum decomposition, and especially when treatment temperatures (35°C) were higher than the normal soil ambient temperatures.

Table 2.2. Environmental conditions affecting degradation of organic contaminants in soil.

Parameter	Conditions required for microbial activity	Optimum values for PAH degradation
Soil moisture	25-85% for water-holding capacity	30-90% (1)
Soil pH	5.5-8.5	7.5-7.8(1), 7.0
Temperature (°C)	15-45	30 (2,4), 35 (5), 24-30 (3), 27
Nutrient content	N and P for microbial growth C:N:P 120:10:1 (optimum value approxi-mately)	C:N 60:1 (1) C:P 800:1 (1) Salt concentration less than 4%
Oxygen content	Aerobic, minimum air-filled pore space of 10%, Anaerobic < 1% by volume	10-40 % O_2 (2)

References:

(1) Dibble and Bartha (1979)

(2) Bauer and Capone (1985)

(3) Heitkamp *et al.*, (1988)

(4) Walter *et al.*, (1991)

(5) Siriwarasin (2002)

Literature review

Bioremediation of soil contaminated by organic compounds may remove the contaminants to a large extent. Numerous studies have, however, shown that a residue will remain in the soil, reflecting that certain compounds are not or only very slowly biodegraded (Alexander, 1994). The residual contamination levels may exceed the clean-up standards set by environmental control authorities and thereby limit the use of bioremediation for site clean-up.

PAHs are major components of wastes from wood preservatives, oil spilled and manufactured gas (MFG) plant. Fate of PAHS in the environment is of great concern due to their cytotoxic, carcinogenic and mutagenic effects. Bioremediation of PAH-contaminated soils is desired compared with other abiotic processes: chemical oxidation, photooxidation and volatilization that are often more costly and less effective. PAH components with 2-5 condensed rings (naphthalene to benzo[a] pyrene) are found to be biodegradable in laboratory cultures of microorganisms isolated from contaminated sites (Cerniglia, 1992; Kastner *et al.*, 1994).

Walter *et al.* (1990) used bacterial mixed cultures to degrade PAHs including phenanthrene, anthracene and pyrene in liquid media. They indicated that the maximum PAHs oxidation rates and optimum specific bacterial growth were obtained at pH 7.0 and 30°C. Nevertheless, there are other factors that affected PAHs biodegradation process in soil as Carmichale and Pfaender (1997) reported that the addition of inorganic and organic compounds could enhance phenanthrene and pyrene degradation. In addition, they found that organic or inorganic substances, characteristics of soil, history and the amount of PAHs in soil affected the efficiency of biodegradation process.

Trzesicka-Mlynarz and Ward (1995) isolated a mixed culture from soil contaminated with PAHs. They found that, Better degradation of a defined PAHs mixture was observed with the mixed culture than with individual isolates. The mixed culture was capable of degrading a range of other PAHs, including benzo[a]pyrene, anthracene, phenanthrene, acenaphtene and fluorene. The mixed culture contained four predominant isolates, all of which were Gram-negative rods,

three of which were identified as *Pseudomonas putida*, *Flavobacterium* sp. and *Pseudomonas aeruginosa*. Increased complex nitrogen levels in the medium levels in the medium promoted bacterial growth and a greater extent of fluoranthene degradation.

Elizabeth *et al.* (1998) divided their experiments in three set: soil alone, soil augmented with pyrene-degrading community, and soil inhibited with sodium azide (NaN_3). After 9 month, they found that pyrene mineralization was occurred only in the degrader-amended soil set.

Because of the biodegradation rate in the controlled process is still less effective therefore biostimulation and bioaugmentation in form of addition the nutrients and microorganisms inherent to agricultural wastes or plant, respectively to improve biodegradation process of PAH in contaminated soil was considered. As Cho *et al.* (1997) added basic nutrients for microorganism and twelve kinds of materials (baked diatomite, microporous glass, coconut charcoal, an oil decomposing bacteria and eight kinds of surfactants) to accelerate the biodegradation of oil–hydrocarbons. The result showed that 15% to 30% of contaminated oil was decomposed during 43 weeks incubation.

Robert *et al.* (2000) used microbial consortium to covert [7-^{14}C benzo[a]pyrene to $^{14}CO_2$ when it was grown on diesel fuel. This consortium was recovered from soil. They concluded that addition of diesel fuel at concentration ranging from 0.007% to 0.2% (wt/vol) stimulated the mineralization of 10 mg /l benzo[a]pyrene from 33 to 65% during a 2-week incubation period. When the benzo[a]pyrene concentration was 10 to 100 mg/l and the diesel concentration was 0.1 % (wt/vol), an inoculumn containing 1 mg of cell protein per liter resulted in mineralization of up to 17.2 mg of benzo[a]pyrene per liter in 16 days.

Charoenchang (2001) used agricultural wastes (peanut shell, rice straw, and rain tree leaves) to enhance microbial degradation of polycyclic aromatic hydrocarbons in soil. She found that addition of peanut shells and rain tree leaves could facilitate the undetectable concentration of phenanthrene within 28 days, and 42 days for fluoranthrene and pyrene in the soil mixture. Biotic factors from the

leaves were found to be a major role in enhancing degradation rate. Moreover, the leaves addition was found to increase bioavailability of the PAHs contaminated in the soil as well.

Miya and Firestone (2001) analyzed the impacted of root exudates and root debris on phenanthrene biodegradation and studied microbial degrader community dynamics. Root exudates significantly enhanced phenanthrene biodegradation in rhizosphere soils, either by increasing contaminant bioavailability or increasing microbial population size and activities. They reported that both root debris-amended and exudates-amended soil maintained larger phenanthrene degrader populations than in control soils even after much of phenanthrene had been utilized.

Siriwarasin (2002) added leguminous leaves (from rain tree, tamarind tree and yellow flame tree) for enhancing phenanthrene and pyrene degradation in artificially PAHs contaminated soil. By using 2 grams of soil microcosm, she found the optimal conditions for high concentration of pyrene and phenanthrene (2 mg/g soil) degradation in the soil. The major parameters preferable to this small scale treatment were 70% water holding capacity, 35°C incubation temperatures, one time opening of the vial cap in a week to maintain aerobic condition. Phenanthrene and pyrene degrading bacteria were detected in the system, all of them were found to come from the additive used.

However, there are many researches reported biodegradation process may enhanced or inhibited by abiotic factors for examples: Molina *et al.* (1993) reported that pyrene mineralization could be inhibited by addition of other PAHs, although this inhibition could have been related to toxicity as well as substrate utilization patterns. Weissenfels *et al.* (1995) concluded that the kind of PAH binding in soil appear were completely prevent biodegradation so sorption of organic pollutants onto soil organic matter significantly affects biodegradability as well as biotoxicity.

Jose-Julio *et al.* (1998) studied the efficiency of phenanhtrene mineraliza-tion in pure cultures of *Pseudomonas fluorescence* strain, isolated from soil, in the presence of soil humic fraction. A higher mineralization rate was

measured in treatment with humic acid at 100 µg/ml. Humic acid at 10 µg/ml stimulated the transformation only in the presence of 10 g of clay per litter.

Although composting of yard wastes, municipal wastewater sludge, and municipal solid waste have been performed for decades, composting of solid contaminated with hazardous materials is still an emerging ex-situ biological technology. Composting has been demonstrated to be effective in biodegrading explosives and PAHs in soils (USEPA, 1996). Composting of soil contaminated with petroleum hydrocarbons, especially diesel fuel, has been demonstrated by only a few researchers (Stegmann *et al.*, 1991; Van Den Munckhof and Veul, 1993).

Beaudin *et al.* (1996) reported that maple leaves and alfalfa cocomposted with hydrocarbon-contaminated soil can degrade contaminants in a laboratory-scale reactor. From soxhlet extraction, 50% of the mineral oil and grease (MOG) of soil origin was degraded in the first 105 days comparing favorably with landfarming studies with the same soil where only 20% of the MOG of soil origin had been degraded after 180 days. MOG fractionation demonstrated that 60% of the aliphatics, 54% of the aromatics and 83% of the polars were degraded during the first 180 days of co-composting. After 287 days, at least 73 % of MOG of soil origin had been degraded.

Potter *et al.* (1999) applied the composting technique and amendment condition to degraded PAHs in soil. All amendment conditions resulted in decreased concentrations of PAHs with two to four rings in their molecular structure. No reduction in concentrations of five or six-ring PAHs occurred during the 12 week study. Composting of PAH contaminated soil decreased toxicity to earthworms and oat roots but had no significant effect on lettuce root toxicity.

Namkoong *et al.* (2002) added compost as an amendment for supplementing organic matter for composting of contaminated soil and they varied the ratio between contaminated soil and compost. The ratio of contaminated soil to organic amendments were 1:0.1, 1:0.3, 1:0.5, 1:1 as wet weight basis. The result showed that degradation rates of total petroleum hydrocarbons (TPH) and *n*-alkanes were the

greatest at the ratio of 1:0.5 of contaminated soil to organic amendments on wet weight basis.

Carlstrom and Tuovinen (2003) evaluated the potential of phenanthrene and fluoranthene biodegradation in yardwaste compost materials. Composted samples were incubated in biometers with ^{14}C-labeled phenanthrene and the evolution of $^{14}CO_2$ was assessed as a measure of mineralization. They found that active mineralization occurred at mesophilic and thermophilic temperatures. Mineralization of phenanthrene reached about 40% extent of $^{14}CO_2$ evolution at best before leveling off, but the maximum varied from sample to sample and could be as low as 1 % after three months.

From the literature review, there is little information about the composting quantity in contaminated soil that gives the most effective PAHs biodegradation. However, we can anticipate that the more microorganisms contain in the compost, the more biodegradation efficiency. It would be very useful for economic and technical reasons if the amount of compost, which should be added to the contaminated soil to remove PAHs and the other contaminants in a certain amount of time effectively, is known.

To improve the composting technique, this research is aim to develop a PAH bioremediation treatment by using smaller amount of microbial source due to the amount of tamarind leaves with effective degrading microorganisms are limited for treating in the big size of contaminated area. If using the seed culture is successful, it may be a trial to develop a competent inoculum to remediate PAHs contaminated soil in the problem areas in the future. This thesis will focus on the degradation of three- or four-rings PAHs that involve in petroleum-contaminated soil by using fallen tamarind leaves as a source of PAHs degrading microorganisms in the preparation of seed culture that would be able to minimize the amount of leaves added whn performed in the scale-up treatment.

CHAPTER 3
METHODOLOGY

3.1 Materials and apparatus

3.1.1 Preparation of soil and leaf materials

Soil samples were collected from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station. All debris was removed and soil samples were then air dry for over night. Dried soil was ground and sieved through sieve No. 8 to be powder (2.36 mm) before used (Figure 3.1). Dried ground and sieved soil was separated into 3 parts. First part was used for GC analysis to determine PAH and four unidentified hydrocarbons (i.e. unknown 1-4) background concentration The study focused on three PAHs (i.e. phenanthrene, fluoranthene, and pyrene) and four unidentified hydrocarbons. The four hydrocarbons were selected because they had highest concentrations on chromatogram from GC analysis of the soil samples as well as determine total bacteria by spread plate technique on Luria Bertani medium. The second part was sent to the Department of Soilscience, Faculty of Agriculture, Kasetsart University for analyzing their physical and chemical properties. Another one was kept for the experimental treatment at 4°C until use.

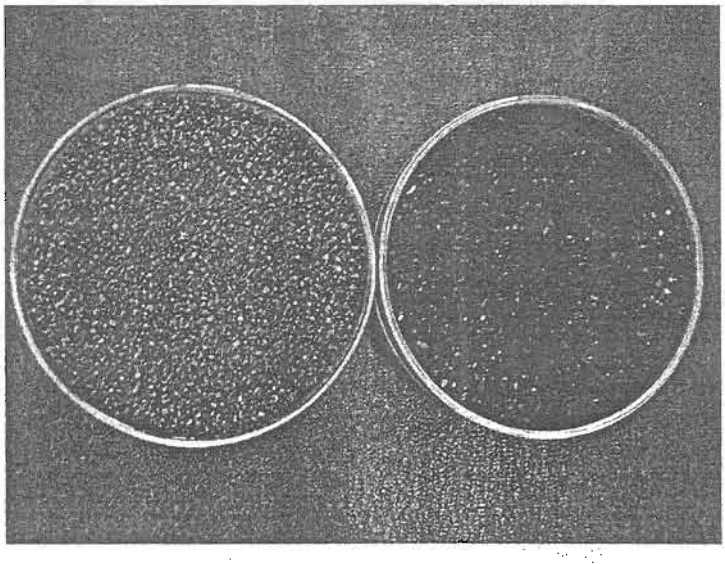


Figure 3.1 Dried-ground and sieved soil samples from Phrachulachomklao Royal Navy Dockyard (left) and Bangkoknoi Railway Station (right).

Tamarind leaves collected from a garden in Chonburi Province were dried at room temperature prior to grinding and sieving to a particle size of 2.36 mm. or less and the yard waste collected from golf-field were dried at room temperature. Dried ground and sieved tamarind leaves and the yard waste were separated into 2 parts: first part was used for GC analysis to determine PAHs and four unidentified hydrocarbons. The second part was kept at 4°C until used.

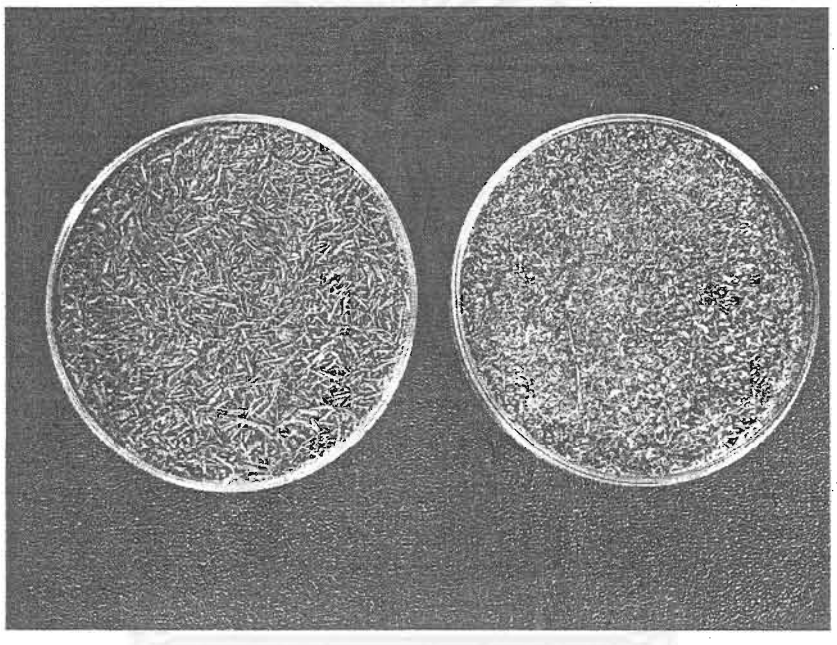


Figure 3.2 Dried-ground and sieved tamarind leaves (left) and yard waste materials (right).

3.1.2 Soil reactors

Two sizes of soil reactors were constructed from 24 ml vials and 225 ml glass bottles and used as batch experiment and larger scale bioremediation treatment, respectively.

Figure 3.3 Two sizes of soil reactors.

3.1.3 Chemicals

1. Phenanthrene, Cyclohexamide were purchased from Sigma Chemical, USA.

2. Fluoranthene, Pyrene were purchased from Kanto Chemical, Japan.

3. Yeast extract, Tryptone, Bacto agar were purchased from Difco Laboratories, USA.

4. Sodium chloride (NaCl), Sodium hydroxide (NaOH), Methanol (CH_3OH), Diethyl ether ($(C_2H_5)O$), Acetone (CH_3COCH_3), Sodiumsulfate anhydrous (anhydrous Na_2SO_4) were purchased from E.Merck, Germany.

5. Ammonium nitrate (NH_4NO_3) was purchased from BDH Chemicals, Australia.

6. Disodiumhydrogen phosphate ($Na_2HPO_4.12H_2O$), Magnesium sulfate ($MgSO_4.7H_2O$) were purchased from Carlo ERBA, France.

7. Calcium chloride ($CaCl_2.2H_2O$), Potassiumdihydrogen phosphate (KH_2PO_4) were purchased from AJEX Chemicals, Australia.

8. Ferrous chloride ($FeCl_3.6H_2O$) was purchased from May & Baker, England.

9. Dichloromethane (CH_2Cl_2) was purchased from Mallinckrodt, France.

10. Triton-x 100 was purchased from Amersham Biosciences Co.,Ltd.

11. 96 % Multisolvent N-Hexane was purchased from Becthai Bangkok Equipment & Chemical Co.,Ltd.

3.1.4 Equipments

1. Flame ionizing detector (FID) Gas liquid chromatography (Agilent 6890N) equipped with a HP-5 capillary column (inner diameter, 0.2 mm and length 25 m) was used for PAHs analysis.

2. pH meter Spectronic 21 from Bausch & Lomb, USA.

3. Pasteur pipette was purchased from Becthai Bangkok Equipment & Chemical Co.,Ltd.

4. Ultrasonicator (bath model) FS4000 from Decan Ultrasonics, USA.

5. 20, 100, 200, 1000 and 5000 µl of Micropipette from Drummond Sciencetific, USA.

6. 1, 5, 10 ml of Pipette from Gilson, France.

7. Incubator Hereaus type B 5050 E from Hereaus, Germany.

8. "ISSCO" laminar flow BVT-124 from International Sciencetific Supply, USA.

9. Autoclave from Kakusan, Japan.

10. Blender MX-T31GH from Matsushita Electric, Taiwan.

11. Standard Sieve O.S.K. 119 with 2.36 mm of pore size from Okawa Seiki, Japan.

12. Weighing L2200P and A200S from Sartorius, USA.

13. Vortex mixer G-560 E from Scientific Industries, USA.

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3.2 Biodegradation of PAHs in the contaminated soil

3.2.1 Batch experiment for selection of an appropriate incubation time in the preparation of seed culture

Two sets of experiments (15 vials of each set) were performed in order to verify the degradative efficiency of tamarind leaves collected as described by Siriwarasin, 2002. The incubation time provided the highest rate of degradation was selected for further preparation of leaves composted contaminated soil and then used as seed culture for the scale-up bioremediation treatment. Tamarind leaf powder was mixed with petroleum-contaminated soil collected from Phrachulachomklao Royal Navy Dockyard. The decreasing rates of PAHs were compared among this group and control group (no tamarind leaves addition) as shown in figure 3.4.

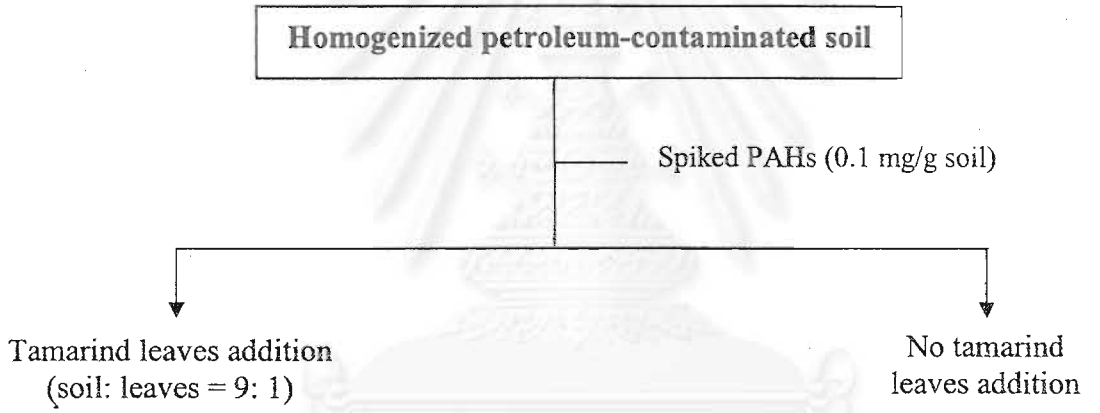

Figure 3.4 Diagram of the experiment to verify the efficiency of microorganisms inherent to the tamarind leaves on biodegrdation of PAHs in contaminated soil.

One set of triplicate experiment was performed on bioaugmentation treatment. Briefly, our reactors consisted of 1.8 grams of non-sterile soils spiked with 200 µl of 100 ppm PAHs in acetone solution (phenanthrene, fluoranthene and pyrene) after standing overnight for acetone removal then 0.2 grams of tamarind leaves was mixed into the soil. Non-sterile control was done by using non-sterilized soil, spiked with 200 µl of 100 ppm PAHs solution but no addition of tamarind leaves. Water content of 70% of water holding capacity was adjusted by dropping sterilized distilled water, and the two sets were incubated at 35°C in the dark. At every 14-day interval of 56 days incubation, one reactor from experimental set and

another from the control were collected in triplicate to quantify the remaining of phenanthrene, fluoranthene, pyrene and 4 high-concentration unknown hydrocarbons by Gas Chromatography. Soil reactor with an incubation time that provides the highest decreasing rate of PAHs was selected to apply as a seed culture on the next experiment.

3.2.2 Preparation of seed culture in batch experiment

This experiment was set up to prepare tamarind leaves composting in the contaminated soil as a seed culture based on the appropriate incubation time selected from 3.2.1.

Sixty reactors (24 ml vials) containing 1.8 g contaminated soil spiked with 200 µl of 1000 ppm phenanthrene, fluoranthene and pyrene in acetone solution were prepared. After acetone evaporation overnight, 0.2 grams tamarind leaf powder was filled into each of the reactor. Water content of 70% of water holding capacity was adjusted by dropping sterilized distilled water. All reactors were incubated at 35°C in the dark. After 49 days of incubation time, all of reactors were collected to use as seed culture in a larger scale experiment.

3.2.3 Scale-up bioremediation treatment using the prepared seed culture

Two soil samples from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station were used as a model for petroleum-contaminated soil. Contaminated soils were further spiked with 1 ml of 1000 ppm phenanthrene, fluoranthene and pyrene in acetone solution and put into each reactor. After acetone evaporation overnight, the experiment was divided into four groups as follows:

Control 1: 20 g of each contaminated soil were sterilized by autoclaving once a day and repeated 3 times then added with cyclohexamide to prevent fungal contamination.

Control 2: 20 g of each non-sterile contaminated soil.

Treatment 1: 18 g of each non-sterile contaminated soil mixed with 2 g. of seed culture obtained from 3.2.2.

Treatment 2: 16.2 g of each non-sterile contaminated soil mixed with the 2 g. of seed culture from 3.2.2 and supplemented with 1.8 g. golf-yard waste material that was ground and sieved to a particle size similar as the leaves.

Water content of the soil and the soil mixtures were adjusted to 70 % of the water holding capacity by sterile distilled water and incubated at 35°C in the dark. Twenty grams of each sample were collected every 14 days for analyzing the remaining PAHs and 4 unknown compounds by Gas Chromatography.

Figure 3.5 showed a diagram of four sets of contaminated soils from Phrachulachomklao Royal Navy Dockyard (P) and Bangkoknoi Railway Station (B) treated with seed culture prepared from the method above.

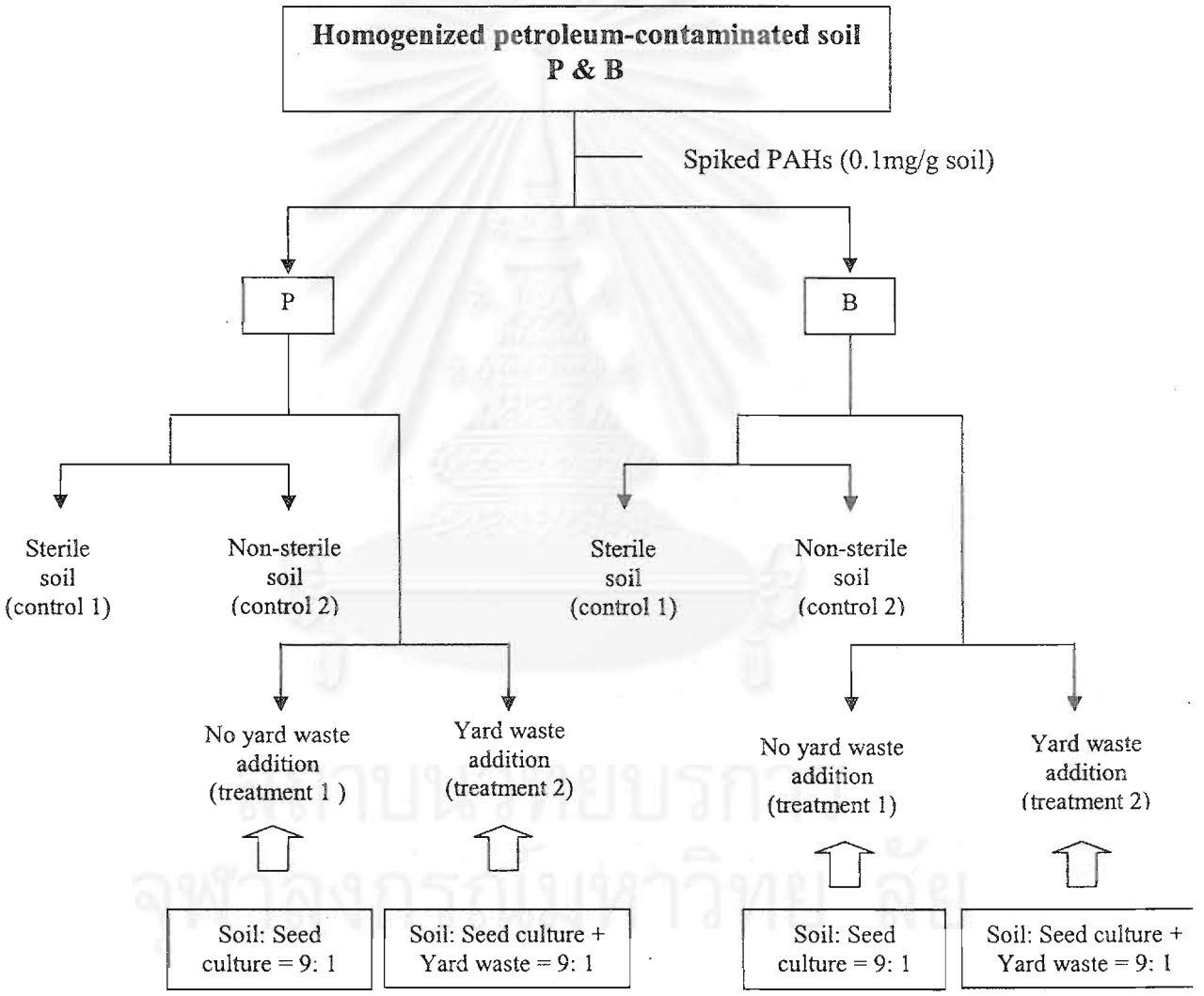

Figure 3.5 Diagram for scale-up bioremediation treatment of contaminated soils from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station.

3.2.3.1 Determining of phenanthrene degrading bacteria

Phenanthrene degrading bacteria, as a representative of PAHs degraders were determined by collecting 1 g of each sample in the reactor every 2 weeks, dissolving in 0.85% sodium chloride solution and spreading on Carbon Free Mineral Medium (CFMM) agar plate after making ten-fold dilution. Then they were sprayed with phenanthrene solution in diethyl ether. After incubation at 30° C up to 14 days, the numbers of colonies surrounded with clear zone on the plate were counted.

3.2.4 Determining of total bacteria

One gram of soil, soil mixed with seed culture, and soil mixed with seed culture and yard waste were collected to dissolve in 0.85% sodium chloride solution, after making ten-fold dilution, 0.1 ml of solution was spreaded on Luria Bertani (LB) agar plate then incubate at 30 °C for 24-48 hours in order to study the number of total bacteria in the system.

3.3 Extraction method

As for the PAHs extraction from contaminated soils and soil mixture, a reactor contained 2 grams of contaminated soil was filled with 4 ml of n-hexane and 1.5 ml of 15 % triton-x 100 solution while 40 ml of n-hexane, 15 ml of 15 % triton-x 100 solution (surfactant) were put directly to each of the reactor contained 20 grams of the soil samples tested, whereas the optimum ratio of hexane: Triton x-100 solution have been tested before direct adding to contaminated soil in 24-ml glass bottles. The samples were shaken by orbital rotary shaker at 250 rpm for 6 hours. The reactors were then frozen at -4 ^{0}C to solidify the lower aqueous layer, and then transferred the solvent fraction with Pasteur pipette to second glass-bottle where a few grams of anhydrous sodium sulfate was added to eliminate water from the sample. PAHs dissolved in solvent fraction were transferred to gas chromatography (GC) auto sampler vials for analysis using gas chromatography with flame ionization detector (GC-FID).

3.3.1 Recovery

Recoveries of compounds were determined for soild phase in triplicate experiment by the following procedures. In solid phase, 2 and 20 grams of non-sterilized, dry soil was added to 225 ml glass bottle with nothing addition and spiked with PAHs (phenanthrene, fluoranthene and pyrene) to give final concentration of 0.1 to 100 ppm then tested glass bottle were hexane extracted and injected to GC-FID and the detail of PAHs recovery efficiency was presented in Appendix B. Percent recovery was determined as the following;

$$\% \text{ Recovery} = \frac{\text{Amount recovered- background concentration}}{\text{Original amount spiked}} \times 100$$

3.4 Gas Chromatography analysis

All sample quantifications were performed with external standard using Gas Chromatography model 6890 N equipped with flame ionization detector. A volume of 2 μl of each sample extract was injected under the following conditions; Temperature injector: 280°C, Temperature detector: 250°C, initial column temperature: 80°C hold for 1 minute then programmed at 80°C to 160°C at a rate of 25°C/min hold for 3 minutes and 160°C to 240°C at a rate of 3°C/min and hold for 3 minutes and 240°C to 300°C at a rate of 40°C/min and hold for 8 minutes The carrier gas was helium (average linear volume of 13.3 ml/min) and the make up gas was nitrogen at 60 ml/min. Split ratio was kept at 5:1 The retention time of phenanthrene, fluoranthene and pyrene are 13.991± 0.5_ min, 21.745 ± 0.5 min, and 23.269 ± 0.5 min, respectively under these conditions. Each sample was analyzed for concentration of PAHs by comparing PAHs recovered from soil to a standard curve of PAHs.

3.4.1 Calibration curve

A calibration curve was developed for contaminated soil by dissolving PAHs in hexane, then the stock standard were diluted to obtain the desired concentration (triplicate per each). The calibration standards were analyzed similar to sample procedures. Calibration curves were shown in Appendix B.

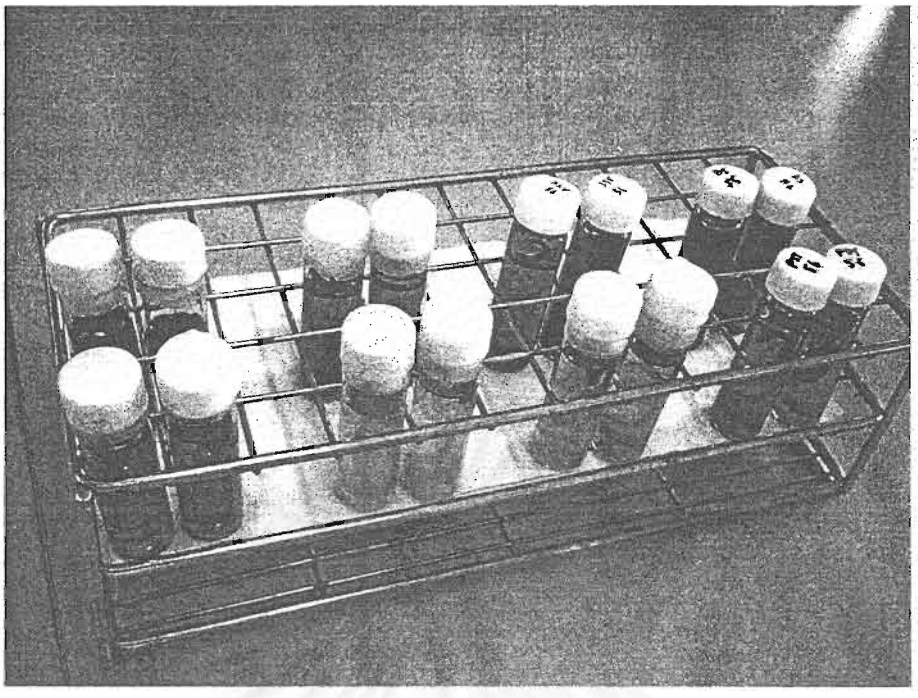

Figure 3.6 Extract (n-hexane layer) from reactors.

Figure 3.7 Gas chromatography.

CHAPTER 4
RESULTS AND DISCUSSIONS

4.1 Characteristics of soil and leaf materials

Petroleum contaminated soil samples were collected from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station. The soil sample was contaminated with diesel oil from the ship engine that the officers dumped into the soil once a week or twice. The chemical and physical properties of the soil samples were showed in the Tables 4.1. Phrachulachomklao Royal Navy Dockyard (P) soil was sandy soil with very low amount of nutrients (C:N:P ratio = 31,200:150:1) while Bangkoknoi Railway Station (B) soil was loamy sand soil with higher amount of nutrients (C:N:P ratio = 221:22:1).

The soil samples were extracted and analyzed by GC-FID to identify the contaminated hydrocarbons. GC chromatograms showed various peaks of hydrocarbon compounds as well as PAHs (Appendix C).The present of PAHs was confirmed by spiking standard compounds into the samples before analysis. In the study, we focused on three PAHs (i.e. phenanthrene, fluoranthene, and pyrene) and four unidentified hydrocarbons (Table 4.2). The hydrocarbons were selected from GC chromatogram based on their high concentrations and the retention time (RT) closed to other studied PAHs. Phenanthrene is a three-ring PAH, whereas fluoranthrene and pyrene are four-ring PAHs. PAHs with more than three rings are often referred to high-molecular-weight (HMW) PAHs and found to be stable and persistent in the environment (Kanaly and Harayama, 2000).

Background concentrations of PAHs and the unknown hydrocarbons in the soil samples collected were ranged from 6-10 ppm and 15-54 ppm from B and P soil, respectively (Table 4.2). Soil from Phrachulachomklao Royal Navy Dockyard was heavily contaminated. It was suggested that petroleum oil wastes were probably dumped into the sampling area at the Phrachulachomklao Royal Navy Dockyard more frequent than at Bangkoknoi Railway Station. Tamarind leaves and yard waste contained low amount of PAHs but no other hydrocarbons. There were 5.0 ppm phenanthrene and 8.6 ppm pyrene in leaves, while yard waste contained only 3.9 ppm phenanthrene (Table 4.2) and chromatograms were shown in Appendix C.

Table 4.1 Properties of petroleum-contaminated soil collected from Phrachulachomklao Royal Navy Dockyard (P) and Bangkoknoi Railway Station (B).

Parameters*	P soil	B soil
Soil texture	Sandy soil	Loamy sand soil
Sand (%)	95	82
Silt (%)	4	16
Clay (%)	1	2
Organic matter (%)	10.80	20.60
Organic carbon (%)	6.24	11.98
Total – nitrogen (%)	0.03	1.21
Phosphorus (ppm)	2	541
C:N:P ratio	31,200:150:1	221:22:1
Potassium (ppm)	20	165
Calcium (ppm)	92	4,848
Magnesium (ppm)	7	14
Moisture (%)	89.36	22.63
EC (electrical conductivity) (mS/cm)	1.48	0.16
pH	4.1	7.8
Water holding capacity (%)	10.31	35.30

*Soil properties were analyzed by Soil and Water group, Agriculture Chemistry Division, except the water holding capacity which was analyzed by Department of Soil Science, Faculty of Agriculture, Kasetsart University.

Table 4.2 Background concentrations of phenanthrene, fluoranthene, pyrene, and unknown hydrocarbons (1 to 4) in tamarind leaves, yard waste and soil samples.

Compounds	RT (min)	Concentrations (ppm)			
		P soil	B soil	Leaves	Yard waste
Phenanthrene	13.86	14.8	6.0	5.0	3.9
Unknown 1	16.68	43	7	-	-
Unknown 2	19.57	54	7	-	-
Fluoranthene	21.53	17.5	7.5	-	-
Pyrene	23.01	19.2	10.0	8.6	-
Unknown 3	25.41	42	8	-	-
Unknown 4	31.06	34	4	-	-

Table 4.3 presented the efficiency of extraction technique used in the study. The tested soil samples were spiked with 100 ppm phenanthrene, fluoranthene and pyrene as the control PAHs. The soil was extracted by hexane and triton-X 100 (surfactant) and then PAHs were analyzed by GC-FID detector. Different in percent PAH recovery may be due to the adsorption of PAHs to soil organic matter.

Table 4.3 Recovery of phenanthrene, fluoranthene and pyrene from different types of soil.

Sources	Recovery (%)	
	P soil	B soil
Phenanthrene	102 ± 2	99 ± 1
Fluoranthene	99 ± 2	98 ± 1
Pyrene	96 ± 2	97 ± 1

4.2 Batch experiment for seed culture preparation

According to Siriwarasin (2002), the microorganisms inherent to tamarind leaves increased phenanthrene and pyrene biodegradation rate in 2 g artificial PAHs contaminated soil microcosms. To scale-up the experiment, this bioremediation approach would require large amount of tamarind leaves to provide sufficient number of important microorganisms. However, it may not possible to obtain similar microorganisms from tamarind leaves collected from difference areas or difference time periods. Therefore, this study aimed to minimize the amount of leaves by preparing seed culture before added to 20 g petroleum contaminated soil microcosms.

The seed culture was made from tamarind leaves composted with petroleum contaminated soil. To identify the appropriate incubation time (composting period) for the seed culture, we studied the decreasing of PAHs and the selected hydrocarbons in 2 g soil batch experiment with and without tamarind leave additions (or treated and control soil, respectively). Soil from Phrachulachomklao Royal Navy Dockyard was used in the study since the amount of collected sample was much more than the sample from Bangkoknoi Railway Station. Although, the soil contained some background PAHs as well as hydrocarbons (Table 4.2), it was further spiked with 100 ppm each of phenanthrene, fluoranthene, and pyrene to accelerate the growth of effective PAHs degrading bacteria.

Percentages of fluoranthene remaining in the treated soil was significantly lower than the control soil at day 42, while the amount of phenanthrene and pyrene was significantly lower than the control soil at day 56 (Figure 4.1). Of all PAHs, phenanthrene was decreased the most. There was about 80% of the initial phenanthrene concentration left in the soil after treatment; whereas almost 100% of all PAHs were remained in the control soil. For the unknown hydrocarbons, around 35 to 50% of the initial unknown hydrocarbon was remained in the treated soil after 56 days, while almost 100% was remained in the control soil at the same period (Figure 4.2). The unknown hydrocarbons in treated soil were decreased more rapid than PAHs. At day 28, the amount of unknown 1 and 4 were significantly lower than the control soil, while the unknown 2 and 3 required longer incubation period and their concentrations in treated soil were lower than control after day 42.

The results suggested that the decreasing of PAHs and other hydrocarbon compounds in this experiment depended on the addition of tamarind leaves. These also confirmed the results from Siriwarasin (2002). Since, PAHs biodegradation reached the highest efficiency between 42 and 56 days of incubation, we then selected 49 days of incubation to prepare the seed culture for further scale-up biodegradation experiment. At this time, we assumed that microorganisms had the highest activity for the biodegradation and the number of PAHs-degraders was probably highest at this point.

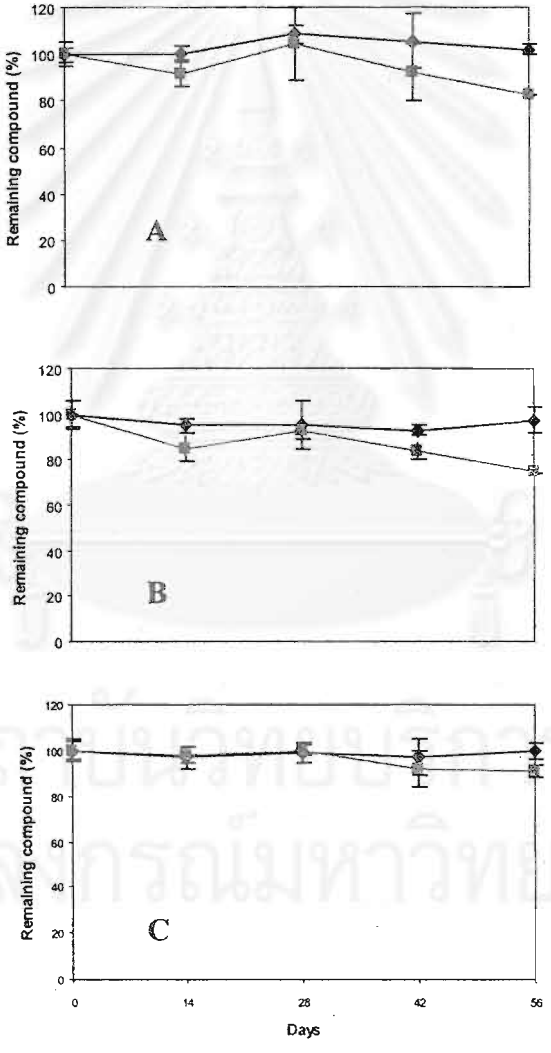

Figure 4.1 Percentage of remaining phenanthrene (A), fluoranthene (B) and pyrene (C) in the treated soil (with tamarind leaves; ■) and the control sets (without tamarind leaves; ♦).

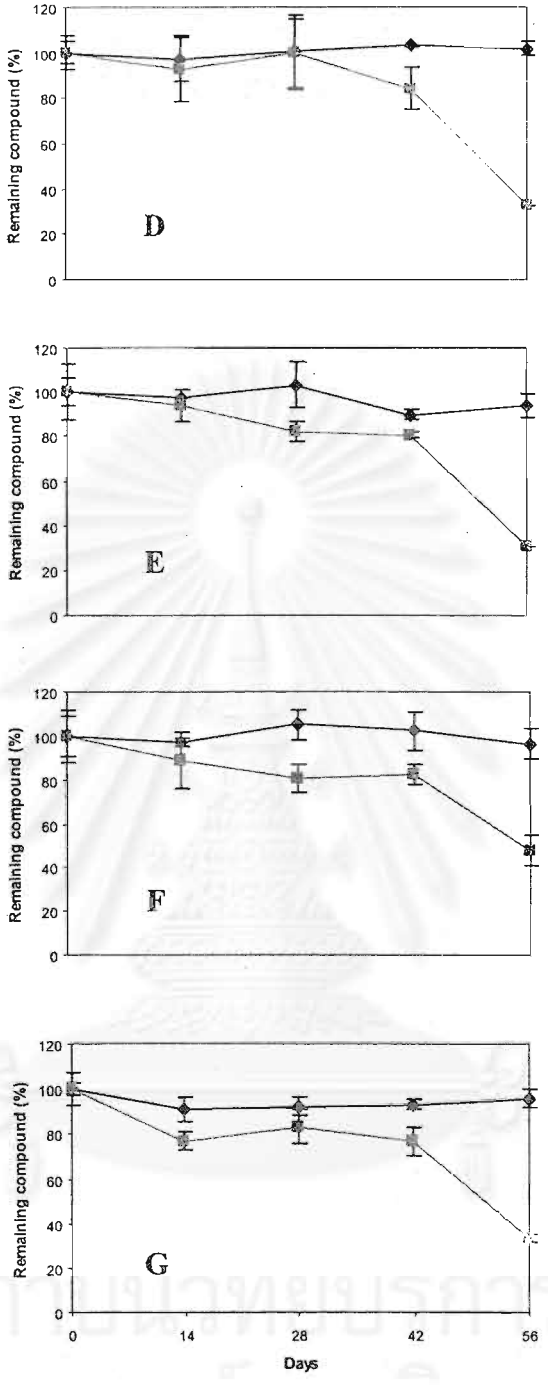

Figure 4.2 Percentage of remaining unknown 1 (D), unknown 2 (E), unknown 3 (F), and unknown 4 (G) in the treated soil (with tamarind leaves; ■) and the control sets (without tamarind leaves; ♦).

4.3 Scale-up bioremediation treatment

Biodegradation of PAHs and the unidentified hydrocarbon compounds in the contaminated soil were studied in a scale-up experiment using 20 g soil microcosm. There were two types of soil; Phrachulachomklao Royal Navy Dockyard (P) soil and Bangkoknoi Railway Station (B) soil. Both soil samples were further spiked with 100 ppm each of phenanthrene, fluoranthene, and pyrene. The treatments were consisted of a) soil mixed with seed culture and b) soil mixed with seed culture and yard waste materials. Seed culture was prepared as in batch experiment (2 g microcosm). At day 49, the compost was harvested and added to each soil microcosm. In the study, yard waste material had been used as nutrients supplement to compare to the addition of seed culture only. All treatments were performed in the same manner, and then compared with the control treatments, which consisted of sterilized and non-sterilized soil without the addition of seed culture or yard waste.

4.3.1 Initial PAHs concentrations and the amount of bacteria in mixed samples

At the beginning of study, concentrations of PAHs and the unknown hydrocarbons in the mixed soil samples were ranged from 67-156 and 3-54 ppm, respectively (Table 4.4). The concentration of each compound from different treatments was not identical; this was probably due to the different amount of contaminated soil in the microcosms, which were 20, 18, and 16.2 g in control soil, soil mixed with seed culture, and soil mixed with seed culture and yard waste treatments, respectively. PAHs remained in the seed culture might also contribute to the increased concentrations of the contaminants. Meanwhile, percentages of initial contaminants at time zero were set up to be 100% and their remaining amount in percent were observed at designated time to investigate the effect of amendments on PAHs degradation.

Phenanthrene degraders, representative of PAHs degrading bacteria was detected by sampling 1 g from each microcosms, diluted and spreaded on Carbon Free Mineral Medium (CFMM) agar sprayed with PAHs solution in diethyl ether. Only colonies surrounded with clear zone were counted as phenanthrene degraders. In both soil types, the results showed that the addition of seed culture increased the

number of phenanthrene-degrading bacteria almost 10 times (Table 4.5). Number of phenanthrene-degrading bacteria was ranged from 1.4 x 10^2 to 10.6 x 10^2 CFU/g in soil mixed with seed culture, while there were less than 10^2 CFU/g of the bacteria found in the control soil.

Number of total bacteria in the soil and soil mixtures at the beginning of study were used to determine the population of bacteria in the contaminated soil and analyzed the biological effect from indigenous microorganisms. The initial population density of bacteria detected in non-sterile P soil and non-sterile B soil were 4.5 x 10^6 and 2.8 x 10^9 CFU/g soil, respectively (Table 4.5). The results indicated that the soil from Bangkoknoi Railway Station was more fertile than Phrachulachomklao Royal Navy Dockyard's soil. It was also found that total numbers of bacteria in all experiments were significantly increased when added the seed culture and/or the yard waste into the soils. The results suggested that the seed culture was a rich source of bacteria.

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Table 4.4 Initial concentrations of phenanthrene, fluoranthene, pyrene, and the unknown hydrocarbons in mixed soil.

Parameters	Non-sterile control soil		Soil mixed with seed culture		Soil mixed with seed culture and the yard waste	
	P	B	P:S	B:S	P:S:Y	B:S:Y
Ratio of soil to tamarind leaves	100:0	100:0	99: 1	99: 1	90:1:9*	90:1:9*
PAHs concentration (ppm)						
Phenanthrene	148	89	156	96	144	96
Fluoranthene	83	67	90	72	92	74
Pyrene	102	73	108	80	102	92
Hydrocarbon concentration (ppm)						
Unknown 1	42	5	43	7	38	8
Unknown 2	52	4	54	7	47	10
Unknown 3	42	5	42	8	38	10
Unknown 4	33	3	34	4	26	6

P: Soil from Phrachulachomklao Royal Navy Dockyard.

B: Soil from Bangkoknoi Railway Station.

S: Seed culture.

Y: Yard waste materials.

* : Ratio of soil and tamarind leaves to yard waste.

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Table 4.5 Initial number of phenanthrene degrading bacteria and total bacteria found in the mixed soil.

Experimental sets	Phenanthrene-degrading bacteria (CFU/g soil x 10^2)	Total bacteria (CFU/g soil x 10^6)*
Sterile-P	0^A	0^A
Non-sterile P	0^A	4.5^B
PS	2.2^B	13^B
PSY	1.4^B	$1,900^C$
Sterile-B	0^A	0^A
Non-sterile B	0.12^C	$2,800^D$
BS	10.6^D	$24,000^E$
BSY	9.2^D	$27,100^F$

P: Soil from Phrachulachomklao Royal Navy Dockyard.

B: Soil from Bangkoknoi Railway Station.

PS: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture.

BS: Non-sterile soil from Bangkoknoi Railway Station mixed with seed culture.

PSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

BSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

*Comparisons between treatments within each column are significantly different (LSD, $P < 0.05$) if marked with different capital letters.

4.3.2 PAHs concentrations in the mixed samples after treatment

A) Soil from Phrachulachomklao Royal Navy Dockyard

As clearly shown in Figure 4.3 and 4.4, the percentages of contaminant degradation were considerably low in the untreated soil and sterile soil comparing to the soil mixed with seed culture. The results suggested that physical and biological

factors in the contaminated soil had no effects on the contaminants since the concentration of these compounds was decreased less than 1% after treatment. Comparison on percentages of the remaining PAHs in the soil collected from Phrachulachomklao Royal Navy Dockyard when treated with seed culture, seed culture supplemented with the yard waste and original tamarind leaves were shown in Figure 4.3.

The seed culture prepared prior to the onset of larger-scale bioremediation was showed to enhance the efficiency of biodegradation process. In soil mixed with seed culture, the concentration of phenanthrene was decreased gradually and reached the undetectable concentration within 56 days; meanwhile around 70-80% of fluoranthene and pyrene were still remained in the soil at the end of treatment. When yard waste materials were added to the soil, higher amounts of PAHs (around 90%) were remained in the soil but still lower than in control soil (100%) at day 56. When compared between scale-up experiment (Figure 4.3) and batch experiment (Figure 4.1), it was found that PAHs were decreased at higher extent and more rapid than in batch experiment.

Percentages of unknown hydrocarbon remaining in the soil mixed with seed culture were significantly lower than the control soil after day 42 (Figure 4.4). At day 56, the amounts of all unknown hydrocarbons were less than 50% of the initial concentration in the mixed soil. Yard waste materials also effected hydrocarbon degradation. The results showed lower amount of unknown hydrocarbons remained in the soil mixed with yard waste and seed culture than the soil mixed with seed culture alone. This was different from the results of PAHs degradation, which showed lower degradation when yard waste was present. Meanwhile, the unknown hydrocarbons were decreased at higher extent than PAHs when compared between similar treatments.

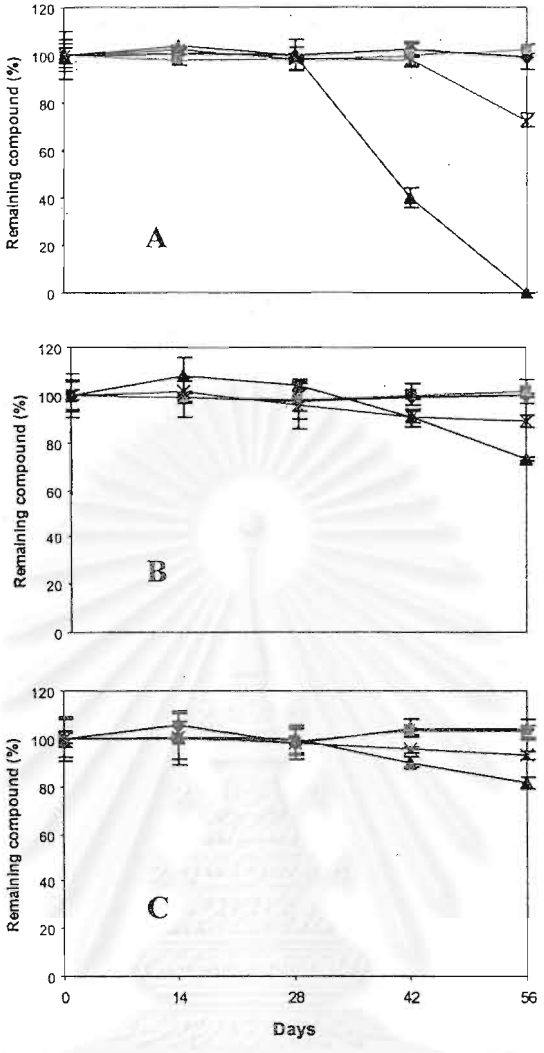

Figure 4.3 Percentage of remaining phenanthrene (A), fluoranthene (B) and pyrene (C) in soil from Phrachulachomklao Royal Navy Dockyard. The treatments were soil mixed with seed culture (▲), soil mixed with seed culture and yard waste (x) compared with the sterile soil (♦) and non-sterile soil (■).

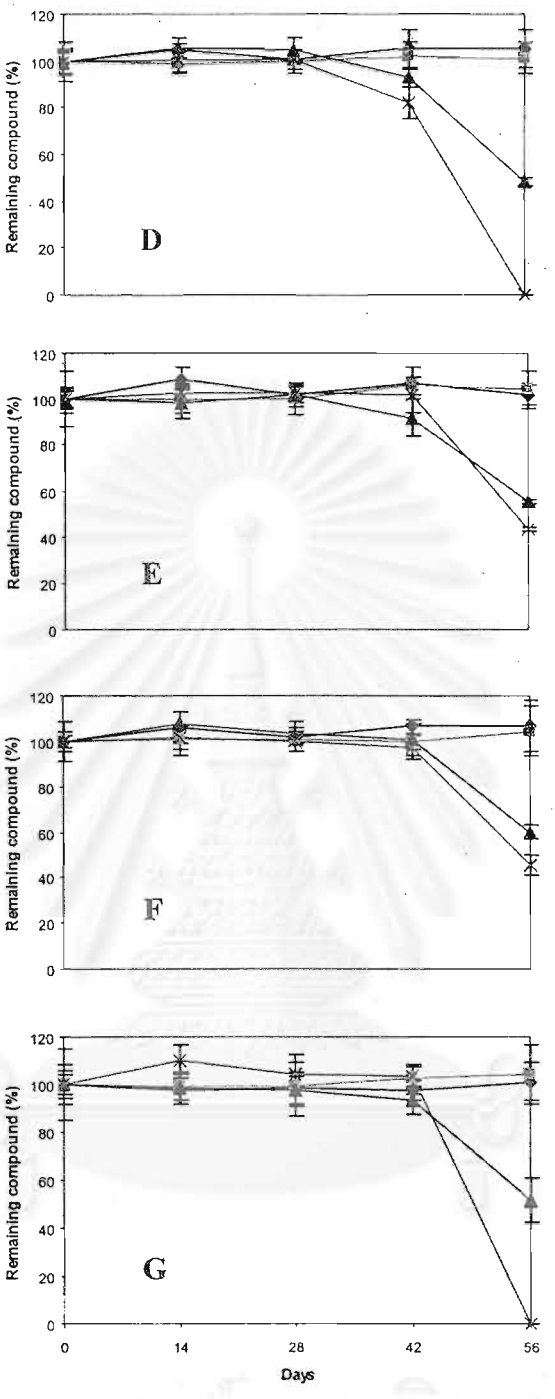

Figure 4.4 Percentage of remaining unknown 1 (D), unknown 2 (E), unknown 3 (F), and unknown 4 (G) in from Phrachulachomklao Royal Navy Dockyard. The treatments were soil mixed with seed culture (▲), soil mixed with seed culture and yard waste (x) compared with the sterile soil (♦) and non-sterile soil (■).

B) Soil from Bangkoknoi Railway Station.

The seed culture prepared was tested for its efficiency to degrade PAHs and the 4 unidentified hydrocarbons that contaminated in the soil collected from Bangkoknoi Railway Station. From the untreated soil and sterile soil experiment, physical factor (volatilization) and biological factor (indigenous biodegradation) had little effects to the amount of the contaminants as showed by the slight decreasing of the contaminants in all reactors within the experimental period. Total volatilization (physical factor) and indigenous biodegradation (biological factor) loss was in the range of 0-27% during the experimental period (Figure 4.5-4.6).

Rapid degradation of all contaminants was observed in the early stage (within 14 days) of incubation period in all experiments, especially in the soil samples mixed with seed culture (Figure 4.5-4.6). In soil mixed with seed culture, the concentration of phenanthrene was decreased rapidly and reached the undetectable concentration within 28 days; meanwhile around 60% of fluoranthene and pyrene were still remained in the soil at the same period (Figure 4.5). The results suggested that seed culture accelerated the onset of biodegradation and increased the relative amount of contaminant degradation. Effects of yard waste materials on enhanced PAHs degradation were distinct when compared to soil mixed with seed culture only at day 14 and only for phenanthrene and pyrene.

Percentages of unknown hydrocarbon remaining in the soil mixed with seed culture only were significantly lower than the control soil after day 14 (Figure 4.6). At day 56, the amounts of all unknown hydrocarbons were less than 10% of the initial concentration in the mixed soil. Yard waste materials also effected hydrocarbon degradation but at lower extent than seed culture. The results showed the amounts of unknown 2-4 in the soil mixed with seed culture alone were below detection limit at day 28; however similar unknown hydrocarbons remained in the soil mixed with yard waste and seed culture at the same period. The result was similar to the degradation of PAHs, which showed slower degradation when yard waste was present.

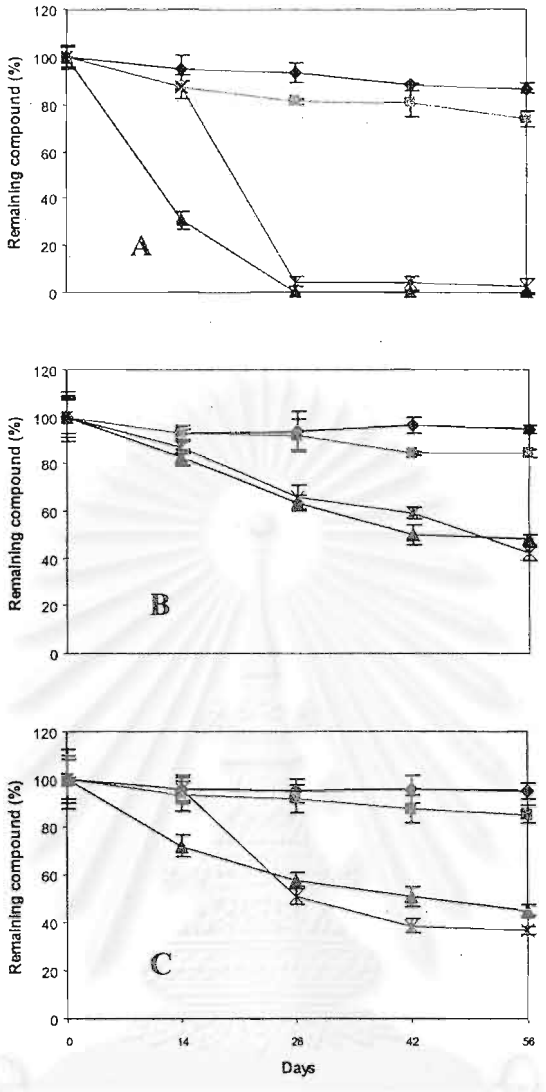

Figure 4.5 Percentage of remaining phenanthrene (A), fluoranthene (B) and pyrene (C) in soil from Bangkoknoi Railway Station. The treatments were soil mixed with seed culture (▲), soil mixed with seed culture and yard waste (x) compared with the sterile soil (♦) and non-sterile soil (■).

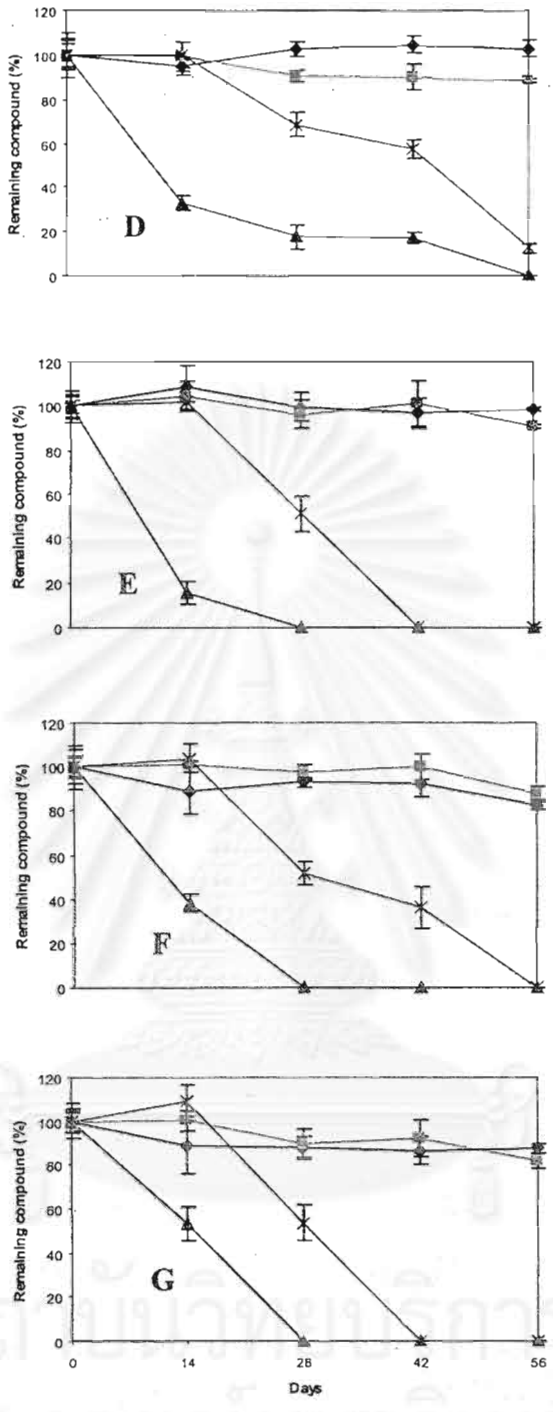

Figure 4.6 Percentage of remaining unknown 1 (D), unknown 2 (E), unknown 3 (F), and unknown 4 (G) in soil from Bangkoknoi Railway Station. The treatments were soil mixed with seed culture (▲), soil mixed with seed culture and yard waste (×) compared with the sterile soil (♦) and non-sterile soil (■).

4.3.3 The amount of phenanthrene-degrading bacteria in mixed samples after treatment

Number of phenanthrene degraders was used to represent PAHs degrading bacteria in soil. Figure 4.7 showed typical agar plate with colonies of phenanthrene degraders. During the first day of incubation, there were a few numbers of phenanthrene-degrading bacteria in all treatment sets. However, the number of phenanthrene-degraders increased rapidly within 14 days (Table 4.6). When these studies were finished, there were a lot of phenanthrene-degrading bacteria in PS, BS and BSY set, while PSY set provided the lowest number of phenanthrene-degrader. This indicated that yard waste material used in this study affected the numbers of phenanthrene-degrading bacteria and the observed difference was likely demonstrated that slowly increasing of the number of bacteria may be due to nutrients limitation, competition between indigenous and exogenous microorganisms, and predators. These factors may affect microbial growth and microbial activities of phenanthrene-degrading bacteria. However, clear zone could not be observed in non-sterile from Phrachulachomklao Royal Navy Dockyard and in sterile soil from two contaminated sites but a few numbers of phenanthrene-degraders could be counted in non-sterile soil collected from Bangkoknoi Railway Station. Since this site was contaminated with petroleum hydrocarbons for a long time, the indigenous microorganisms probably acclimated and were able to use these contaminants as c-source to support growth.

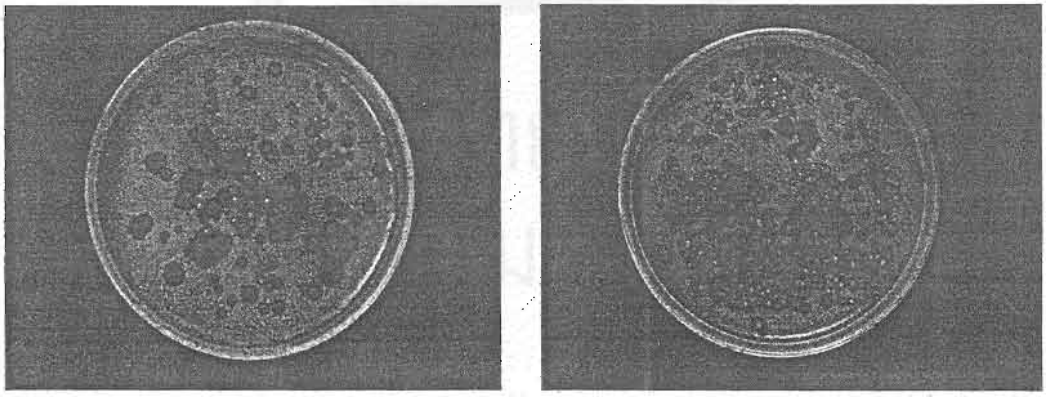

Figure 4.7 Phenanthrene-degrading bacteria in the contaminated soil collected from Phrachulachomklao Royal Navy Dockyard mixed with seed culture (A) and soil collected from Bangkoknoi Railway Station mixed with seed culture (B).

Table 4.6 The number of phenanthrene-degrading bacteria from scale-up bioremediation treatments.

Experimental set	Phenanthrene-degrading bacteria (CFU/ g soil x 10^2)				
	0 day	14 days	28 days	42 days	56 days
Sterile-P	0^A	0^A	0^A	0^A	0^A
Non-sterile P	0^A	0^A	0^A	0^A	0^A
PS	2.2^B	6900^B	8200^B	10000^B	15800^B
PSY	1.4^B	5.5^C	10^C	70^C	92^C
Sterile-B	0^A	0^A	0^A	0^A	0^A
Non-sterile B	0.12^C	0.42^C	0.7^C	0.9^C	1.2^C
BS	10.6^D	11800^D	54700^D	780000^D	950000^D
BSY	9.2^D	120^E	4700^E	91000^E	107300^E

P: Soil from Phrachulachomklao Royal Navy Dockyard.

B: Soil from Bangkoknoi Railway Station.

PS: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture.

BS: Non-sterile soil from Bangkoknoi Railway Station mixed with seed culture.

PSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

BSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

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4.4 Summary of results

The effectiveness of PAHs and hydrocarbon degradation in seed culture and scale-up bioremediation treatment was compared in Table 4.7. The results showed that PAHs and hydrocarbons in soil were degraded well when tamarind leaves were added. Meanwhile, amount of tamarind leaves could be reduced by apply as seed culture to the soil, this way the ratio of soil to tamarind leaves could be as low as 99:1 and 90:1. The degradation of PAHs and hydrocarbon in soil mixed with seed culture were significantly higher than non-mixed soil, however the extent of degradation was also depended on the source of soil samples. We found that PAHs and hydrocarbons were degraded more in soil from Bangkoknoi Railway Station than from Phrachulachomklao Royal Navy Dockyard.

It was found that adding seed culture with and without yard waste supplementation could increase the efficiency of biodegradation process more than 50 times. This research suggests that besides the external nutrients sources (C-source or N-source), seed culture quality was considered as an important factor for the PAHs and unidentified hydrocarbons degradations in the soil since it would be a major source of degrading microorganisms which were able to accelerate the biodegradation capacity in contaminated soil. Korda et al. (1997) reported that the reintroduction of indigenous microorganisms isolated from the contaminated site after culturing seems to be a highly effective bioremediation method, especially when microorganism growth is supplemented with oxygen and fertilizers. It can be concluded that all treatments tested on degradation of petroleum hydrocarbon compounds in the contaminated soil by using the seed culture were able to remove the contaminated compounds much more than control sets during 56 days, thus seed culture was likely suitable for facilitating PAHs and the other contaminants biodegradation in the soil.

Seed culture increased the efficiency of 7 contaminants degradation focus on this studied in the two soils tested and increased population of PAH-degrading bacteria while the different results were observed in the yard waste. From the result above, we concluded that the seed culture did not necessary to be prepared from Phrachulachomklao Royal Navy Dockyard and use in there. We can prepared seed culture from one site and use it in the other site of the related polluted compounds so in the future if this work will be developed, we will save the remediation cost, degrade many contaminants and improve environmental quality in Thailand much

more than the past. However, this research suggests that supplements should be chosen and applied with care if they are to be used as a part of a successful remediation strategy.

Table 4.7 Comparison of seed culture and scale-up bioremediation treatment on degradation of PAHs and hydrocarbon.

Parameters	**Seed culture (Batch experiment)**	**Scale-up bioremediation treatment**					
		P	B	PS	BS	PSY	BSY
Ratio of soil to tamarind leaves	90:10	100:0	100:0	99: 1	99: 1	90:1:9*	90:1:9*
PAHs degradation (%)**							
Phenanthrene	20^{A}	0^{C}	20^{A}	100^{B}	100^{B}	28^{C}	98^{D}
Fluoranthene	17^{A}	0^{D}	15^{A}	9^{B}	53^{C}	9^{D}	57^{E}
Pyrene	8^{A}	0^{F}	15^{G}	11^{B}	55^{C}	8^{A}	63^{E}
Hydrocarbon degradation (%)							
Unknown 1	69^{A}	0^{E}	12^{F}	51^{B}	100^{C}	100^{C}	88^{D}
Unknown 2	68^{A}	0^{E}	10^{F}	44^{B}	100^{C}	57^{D}	100^{C}
Unknown 3	54^{A}	0^{D}	12^{E}	40^{B}	100^{C}	55^{A}	100^{C}
Unknown 4	68^{A}	0^{D}	18^{E}	47^{B}	100^{C}	100^{C}	100^{C}

P: Soil from Phrachulachomklao Royal Navy Dockyard.

B: Soil from Bangkoknoi Railway Station.

PS: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture.

BS: Non-sterile soil from Bangkoknoi Railway Station mixed with seed culture.

PSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

BSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

*Ratio of soil to tamarind leaves to yard waste.

**Comparisons between treatments within each row (compound) are significantly different (LSD, $P < 0.05$) if marked with different capital letters.

Correlation between the number of phenanthrene-degrading bacteria and the decreasing of phenanthrene in scale-up bioremediation treatment of contaminated soil collected from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station were shown in Figure 4.8-4.9. It was found that that the number of phenanthrene-degrading bacteria were increase with time and related with the decreasing of phenanthrene in all treatment These results suggested that increase in the number of phenanthrene-degrading bacteria may be significant enough to affect the concentration of phenanthrene in two soil tested.

Concentration of phenanthrene and unknown 1 to 4 were decreased more rapidly than fluoranthene and pyrene probably due to the structure and arrangement of these compounds. The efficiency of biodegradation process depend on the number of aromatics rings. There are three aromatic rings in the structure of phenanthrene whereas four aromatic rings could be observed in fluoranthene and pyrene thus phenanthrene was easily to degrade by PAH-degrader more than fluoranthene and pyrene. Although, unknown 3 and 4 may have higher molecular weight than fluoranthene and pyrene but the arrangement of unknown 3 and 4 may be in the form of long chain or branch chain hydrocarbons. Consequently, the unknown 3 and 4 were easily to degrade by microorganisms more than fluoranthene and pyrene.

Decreasing in biodegradation rate of PAHs and the other contaminants were usually due to either preferential use of supplement as carbon source and stimulation of non-PAH degrading bacteria. Many kinds of the supplement increased populations of heterotrophic microorganisms (Lisa and Frederick 1997), as measured by plate count technique but not increased population of PAHs-degrading microorganisms, as measures by the spray plate technique method (Kiyohara et al., 1982). These results suggest that the PAH degrading community at each site may be unique in their response to materials added in an attempt to stimulate PAH degradation. The characteristics of the site including exposure history, soil type, and temporal variations may all influence their response.

The results in all control sets (sterile and non-sterile soil) from Bangkoknoi Railway Station showed the difference in PAHs concentrations within a 56 day incubation period. These suggested that some of the indigenous microorganisms in the soil were able to degrade PAHs. The results were also consistent with the present of phenanthrene degrading bacteria in these soil samples. Meanwhile, there was no difference in PAH degradation in Phrachulachomklao Royal Navy Dockyard soil.

The results from determination of phenanthrene degrading bacteria suggested that PAHs degrading bacteria were not present in this soil. However, the slight depletion of PAH concentrations observed might be due to some physical factors such as volatilization or sorption to the soil matrix.

There are many parameters that would result in the different of PAH degradation between the soil samples obtained from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station, and between control and treatment set. These parameters probably are PAHs structure, physicochemical parameters of the site, type of microorganisms, and organic and inorganic content in the contaminated soils (Shuttleworth and Cerniglia, 1995). PAHs could be sorped to organic matter in soils and prevent the degradation. Consequently the rate of desorption strongly influences biodegradation rate. Residual levels of PAHs after bioremediation were found to be strongly dependent on soil type. The presence of both soil organic matter and asphaltic compounds in the soil was found to be associated with higher residuals levels (Breedveld and Karlsen, 2000). In addition, intermediate from the mineralization of other PAH species, for example, product derived from pyrene transformation have the potential to accumulate in PAH-contaminated system and that such products can significantly influence the removal of other PAHs (Kazunga and Aitken, 2000). Further improvement of this bioremediation technique should take these parameters into consideration.

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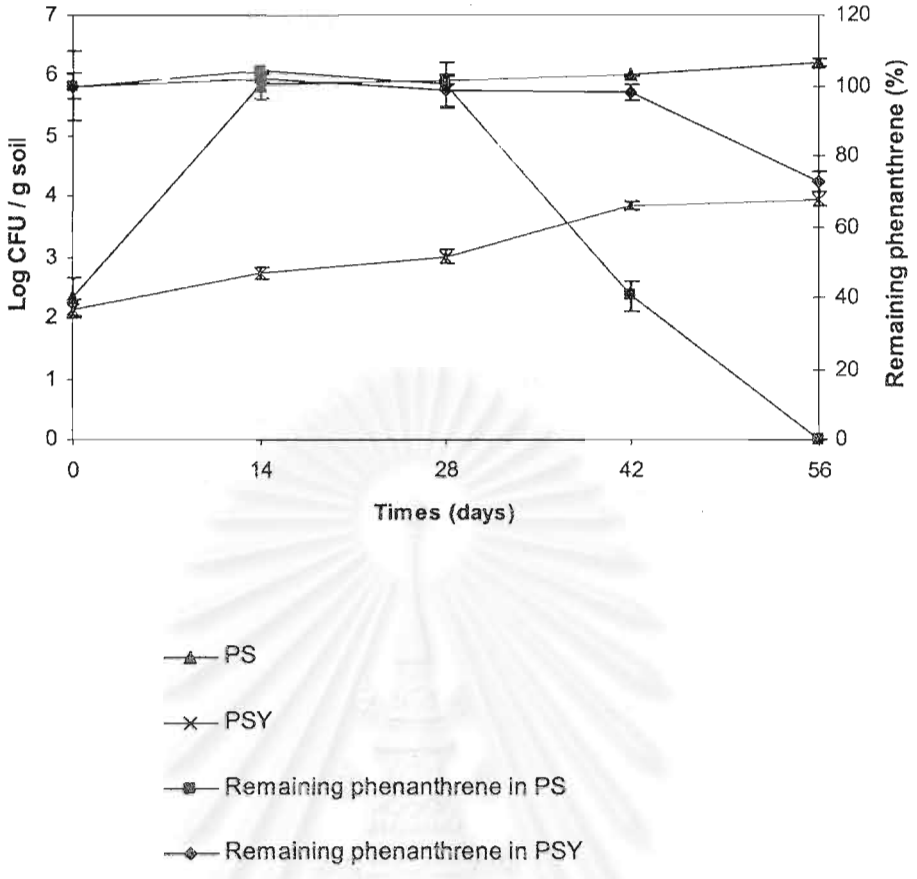

Figure 4.8 Correlation between the number of phenanthrene-degrading bacteria and the decreasing of phenanthere in scale-up bioremediation treatment of Phrachulachomklao Royal Navy Dockyard soil.

PS: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture.

PSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

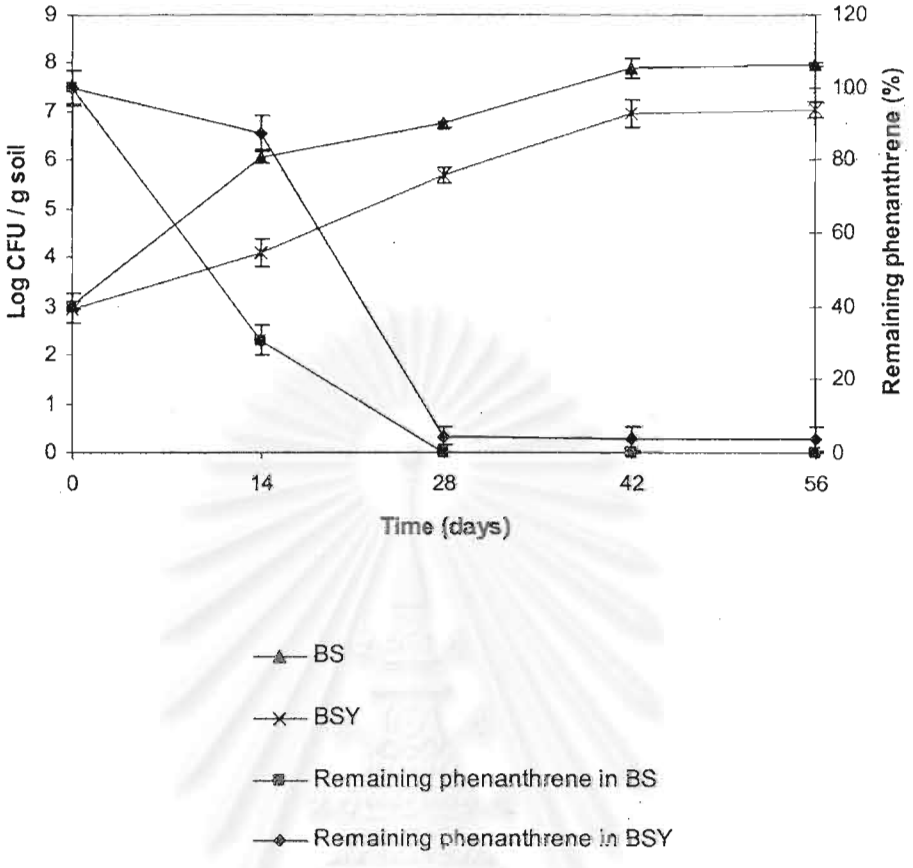

Figure 4.9 Correlation between the number of phenanthrene-degrading bacteria and the decreasing of phenanthere in scale-up bioremediation treatment of Bangkoknoi Railway Station.

BS: Non-sterile soil from Bangkoknoi Railway Station mixed with seed culture.
BSY: Non-sterile soil from Phrachulachomklao Royal Navy Dockyard mixed with seed culture supplemented with the yard waste.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 Conclusions

The main purpose of this study was to apply certain microorganisms that had been inherent to the leaves fallen from tamarind trees on biodegradation of polycyclic aromatic hydrocarbons (PAHs). To minimize the amount of leaves added, seed cultures were prepared by composting the leaves with contaminated soil and applied to 20 g petroleum-contaminated soil reactors. The results showed that:

1) The preparation of seed culture improved the efficiency of PAH bio-degradation in contaminated soil under optimal condition.

2) Seed culture supplementation in contaminated soil from Bangkoknoi Railway Station provided the highest efficiency to degrade PAHs, which was almost 100 times more than the degradation in reactors without seed culture.

3) Supplementation of contaminated soil with yard waste material provided the lower efficiency of PAHs and hydrocarbons degradation than adding seed culture without the yard waste supplementation, however the efficiency was still higher than the control reactors.

4) Phenanthrene degrading bacteria were the most abundant bacteria in contaminated soil from Bangkoknoi Railway Station supplemented with seed culture. These bacteria may be responsible for the decreasing of PAHs and unknown hydrocarbons in treated soil.

5) Seed culture could be prepared from a specific soil sample and then applied to enhance PAH degradation in another soil sample (site). For example, seed culture from Phrachulachomklao Royal Navy Dockyard soil could be used to enhance PAHs biodegradation in the soil from Phrachulachomklao Royal Navy Dockyard and Bangkoknoi Railway Station contaminated sites.

5.2 Suggestions for future work

The results from this research can be used as baseline data for developing other biodegradation treatment as well as for the demonstration of bioremediation approach for clean-up PAHs and hydrocarbon pollutants. Further study is needed to optimize the most suitable condition, improve the supplementation and then apply to the larger contaminated samples. However, PAH structure, physicochemical parameters of the site, type and concentration of contaminants, nutrient source, exposure history, type and number of microorganisms in the soil should be considered since these parameters may affect the efficiency of biodegradation process and microbial activity. The degradation products of PAHs and the degrading bacteria found in this system should be analyzed to confirm the biodegradation process. Although, it was found that using less amount of seed culture led to lower biodegradation efficiency, but for remediation respect, using such a high quantity of seed culture would be costly. If time is not limited in the remediation program, using lower amount of seed culture and longer incubation period would be more cost effective. From our study, the ratio of seed culture to contaminated soil at 1:9 still provided the decreasing of contaminants, therefore lower amount of seed culture nay be applied to achieve the best cost effectiveness if higher potential of seed culture would be further developed in the future.

Surveying of petroleum-contaminated site in Thailand should be done in order to conduct laboratory-scale treatability study as well as field trial. Other type of plant waste materials and yard wastes should be examined for the biodegradation of PAH with lower or higher molecular weight comparing to the result from this study. Moreover, seed culture prepared from other sites that have history of PAHs exposure and contain acclimated microorganisms, for example oil refining plant, garage, and other sites where petroleum wastes have been discharged, was anticipated to provide high activity for biodegradation of PAHs.

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APPENDICES

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APPENDIX A
CHEMICAL SOILUTIONS

1. Phenanthrene, Fluoranthene and Pyrene in acetone solution

Dissolve 0.03 g of each PAHs in acetone 30 ml mixed vigorously by vortex mixer then filled though filter with 0.20 μm of pore size. This solution should be fresh prepared and keep in 0 °C until use and spiking of PAHs should be hurry performed because acetone solution volatile very fast so the concentration of PAHs may be changed.

2. Phenanthrene in diethyl ether solutio

Dissolve 2 g of phenanthrene in 100 ml of diethyl ether then mix completely and filled though filter with 0.20 μm of pore size. This solution should be fresh prepared before use.

3. 0.85 % Sodium Chloride

Dissolve 8.5 g of sodium chloride in 1000 ml of distilled water and sterile by autoclave with pressure 15 pond/inch^2 temperature 121 °C 15 minutes.

4. Standard PAHs for Gas chromatography

Dissolve PAHs 1 mg in methanol 1 ml mixed vigorously by vortex mixer then filled though filter with 0.20 μm of pore size and sealed with parafilm after that cover with Floyd paper for prevent this solution from photo oxidation. Keep it in -20 °C until use.

5. 15 % Triton –x 100

Mixed 15 ml of triton x-100 with 85 ml of distilled water keep in room temperature until use.

6. Nutrient media

Nutrient media used in all experiments were Carbon free minimum mineral medium (CFMM medium) and Luria Bertani (LB agar) as shown in table A.1 and A.2, respectively.

Table A.1 Composition of Carbon free minimum mineral medium (CFMM medium).

Constituent	Concentration (g/l)
NH_4NO_3	3
KH_2PO_4	0.8
$Na_2HPO_4.12H_2O$	5.5
$MgSO_4.7H_2O$	0.1
$FeCl_3.6H_2O$	0.05
$CaCl_2.2H_2O$	0.05
Distilled water	1000 ml

Adjust pH to 7.5 autoclaved at pressure 15 pond/inch^2 temperature 121°C 15 minutes.

Table A.2 Composition of Luria Bertani (LB agar).

Constituent	Concentration (g/l)
Tryptone	10
Yeast extract	5
NaCl	5
Agar	15
Distilled water	1000 ml

Adjust pH to 7.0 autoclaved at pressure 15 pond/inch^2 temperature 121°C 15 minutes.

APPENDIX B

1. Standard curve of phenanthrene

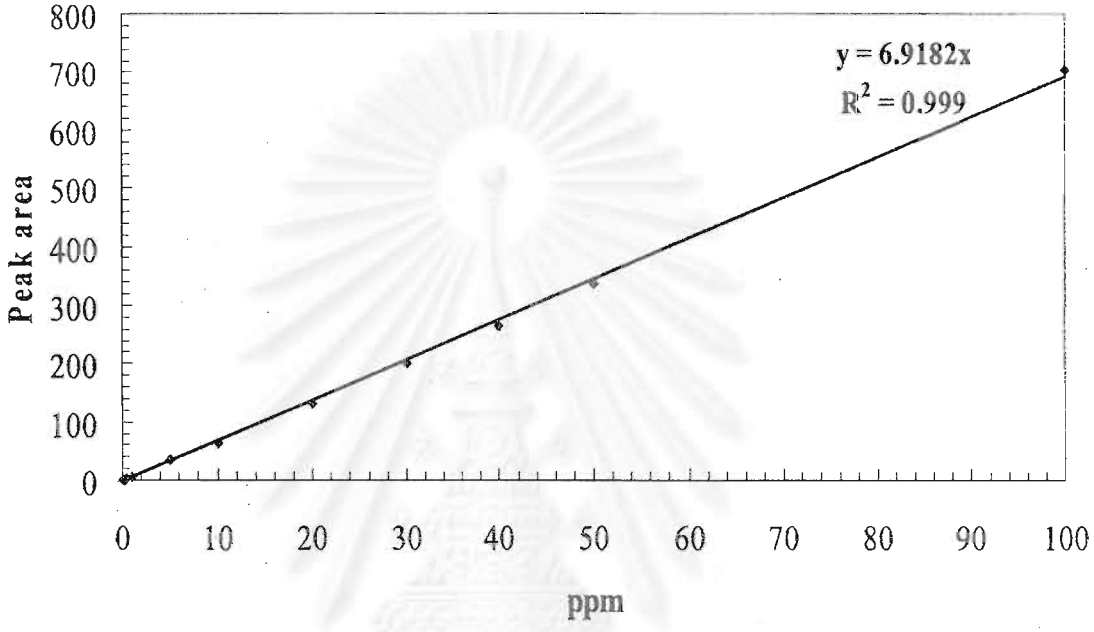

Figure B.1 Standard curve between phenanthrene and peak area analyzed by Gas Chromatography.

The concentration of phenanthrene can calculate by substitute peak area values in linear equation:

Peak area = (slope of standard curve x phenanthrene) – y intercept

From this graph slope = 0.999

y intercept = 6.9182

2. Standard curve of fluoranthene

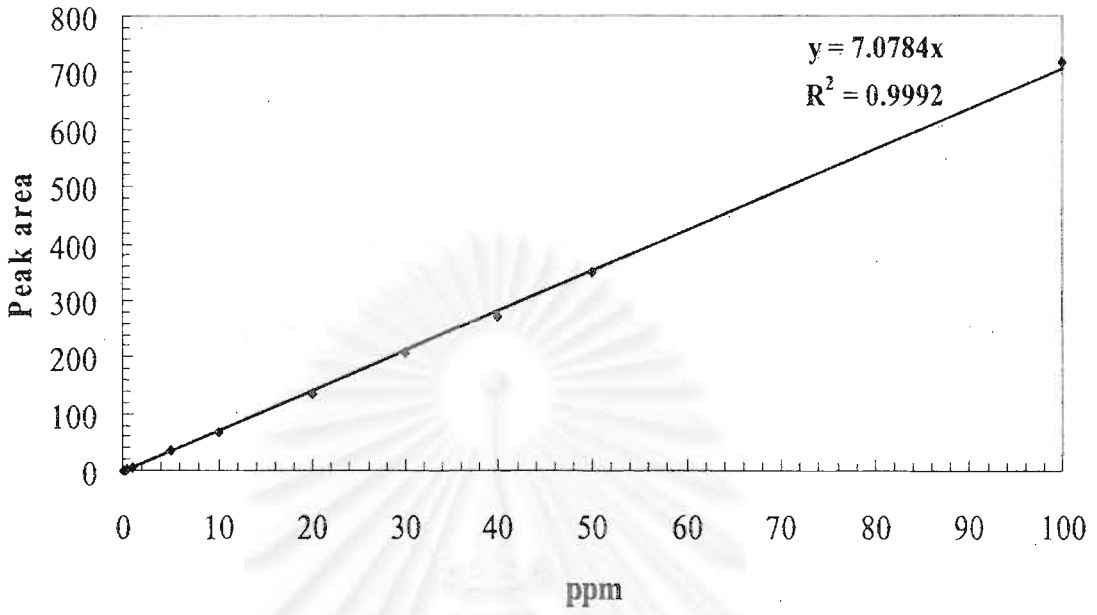

Figure B.2 Standard curve between fluoranthene and peak area analyzed by Gas Chromatography.

The concentration of fluoranthene can calculate by substitute peak area values in linear equation:

Peak area = (slope of standard curve x fluoranthene) – y intercept

From this graph slope = 0.9992

y intercept = 7.0784

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3. Standard curve of pyrene

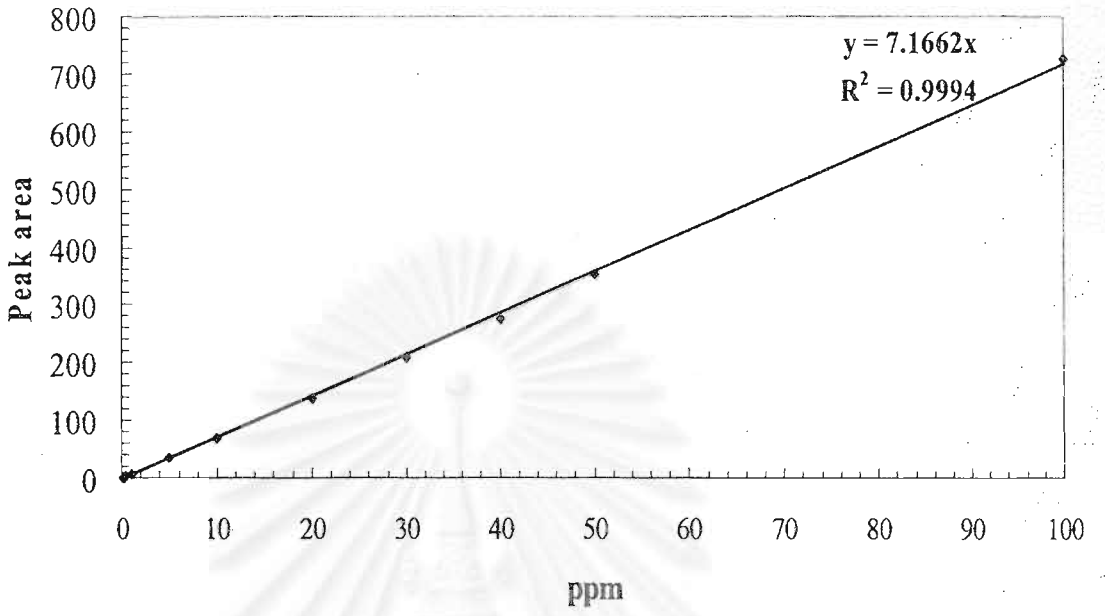

Figure B.3 Standard curve between pyrene and peak area analyzed by Gas Chromatography.

The concentration of pyrene can calculate by substitute peak area values in linear equation:

Peak area = (slope of standard curve x pyrene) – y intercept

From this graph slope = 0.9994

y intercept = 7.1662

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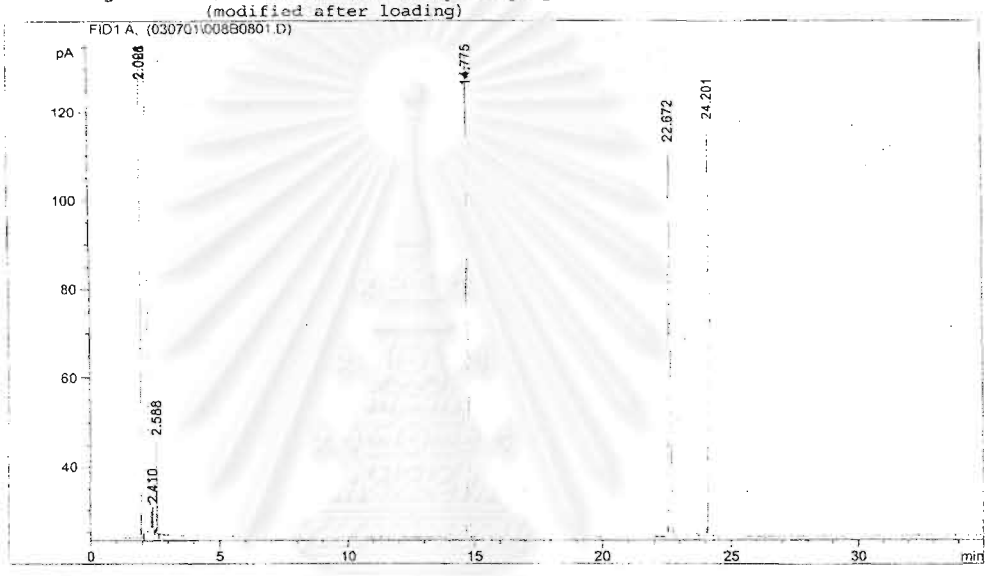
APPENDIX C

Chromatogram(s) of samples

Data File C:\HPCHEM\1\DATA\030701\008B0801.D Sample Name: 100ppm(2)

Injection Date : 7/1/2003 3:57:15 PM Seq. Line : 8
Sample Name : 100ppm(2) Location : Vial 8
Acq. Operator : vorapong Inj : 1
Inj Volume : 1 µl
Different Inj Volume from Sequence ! Actual Inj Volume : 2 µl
Acq. Method : C:\HPCHEM\1\METHODS\VOR_FID.M
Last changed : 6/27/2003 9:36:42 AM by vorapong
Analysis Method : C:\HPCHEM\1\METHODS\VOR_FID.M
Last changed : 7/1/2003 11:13:50 AM by vorapong
(modified after loading)



Area Percent Report

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: FID1 A,

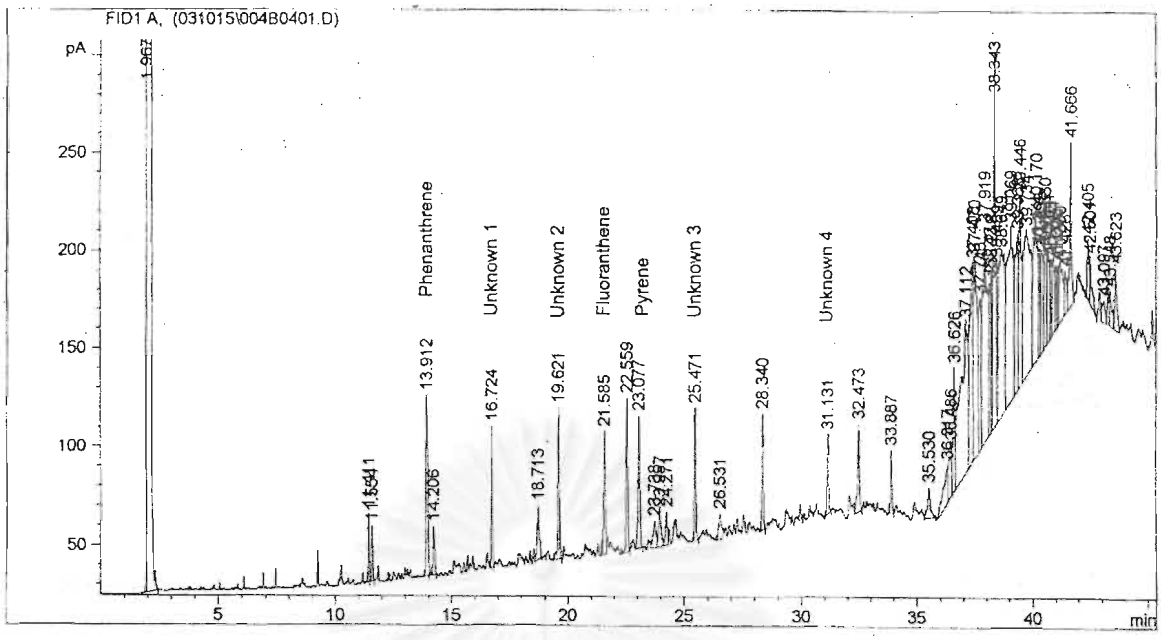
Peak #	RetTime [min]	Type	Width [min]	Area [pA*s]	Height [pA]	Area %
1	2.021	BP S	0.0205	1.01947e5	7.66920e4	5.22161
2	2.066	VB S	0.0547	1.84901e6	5.63539e5	94.70419
3	2.410	BP	0.0312	8.07655	4.24616	0.00041
4	2.588	PP	0.0304	39.00702	21.27850	0.00200
5	14.775	BB	0.0651	458.27356	108.96110	0.02347
6	22.672	BB	0.0749	444.80362	88.06681	0.02278
7	24.201	BP	0.0774	498.38123	93.11125	0.02553
Totals :				1.95241e6	6.40547e5	

Results obtained with enhanced integrator!

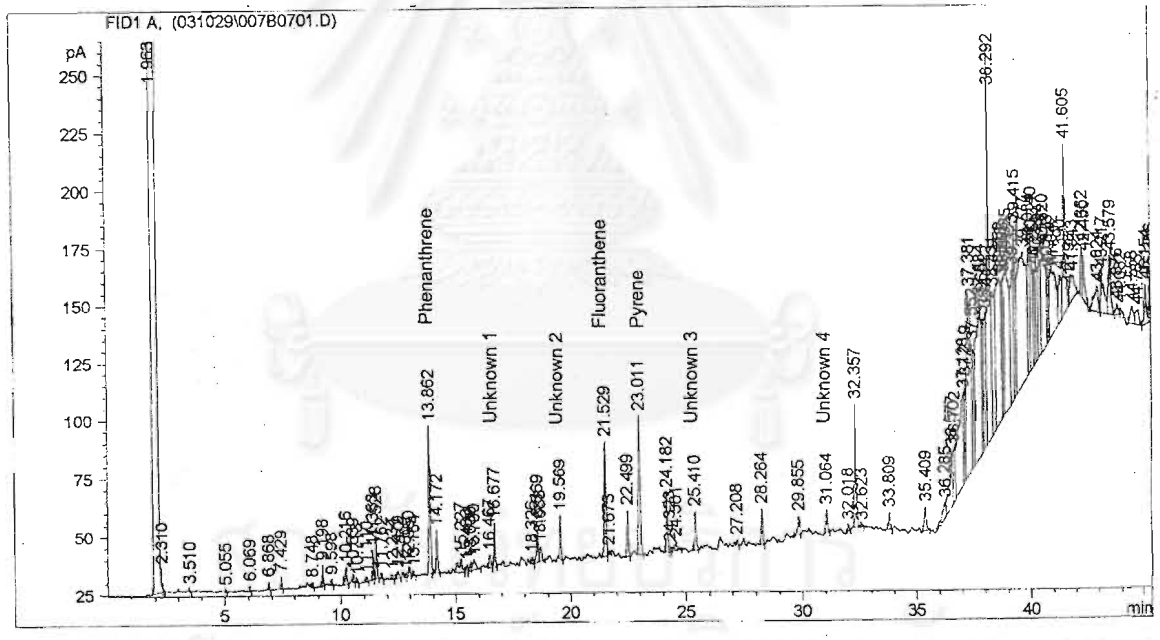
*** End of Report ***

Instrument 1 7/1/2003 5:07:31 PM vorapong Page 1 of 1

Figure C.1 Gas chromatogram of standard PAHs i.e. phenanthrene (RT = 14.775), fluoranthrene (RT = 22.672), pyrene (RT = 24.201).

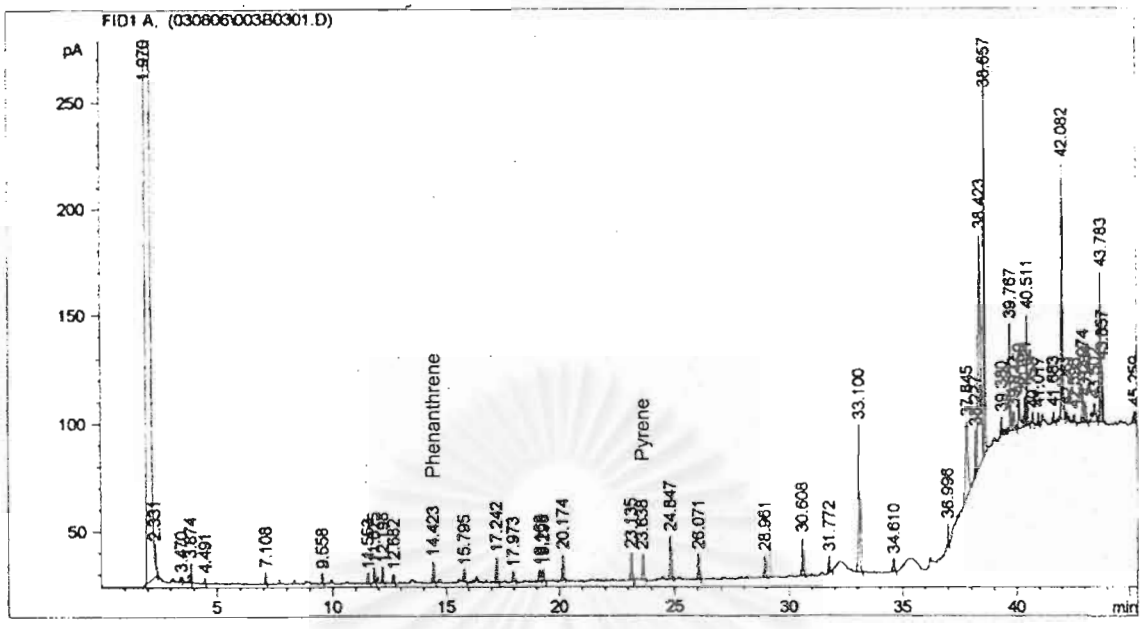


(A)

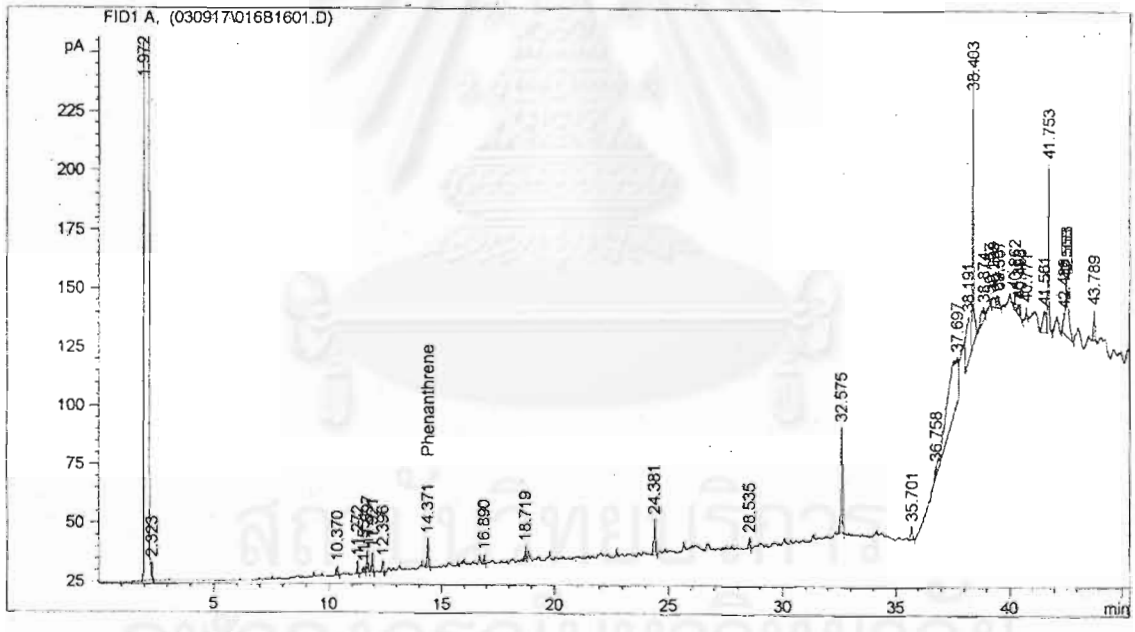


(B)

Figure C.2 Gas chromatograms of petroleum-contaminated soil collected from Phrachulachomklao Royal Navy Dockyard (A) and Bangkoknoi Railway Station (B) showing the amount of phenanthrene, fluoranthrene, pyrene, and four unknown hydrocarbons before treatment.

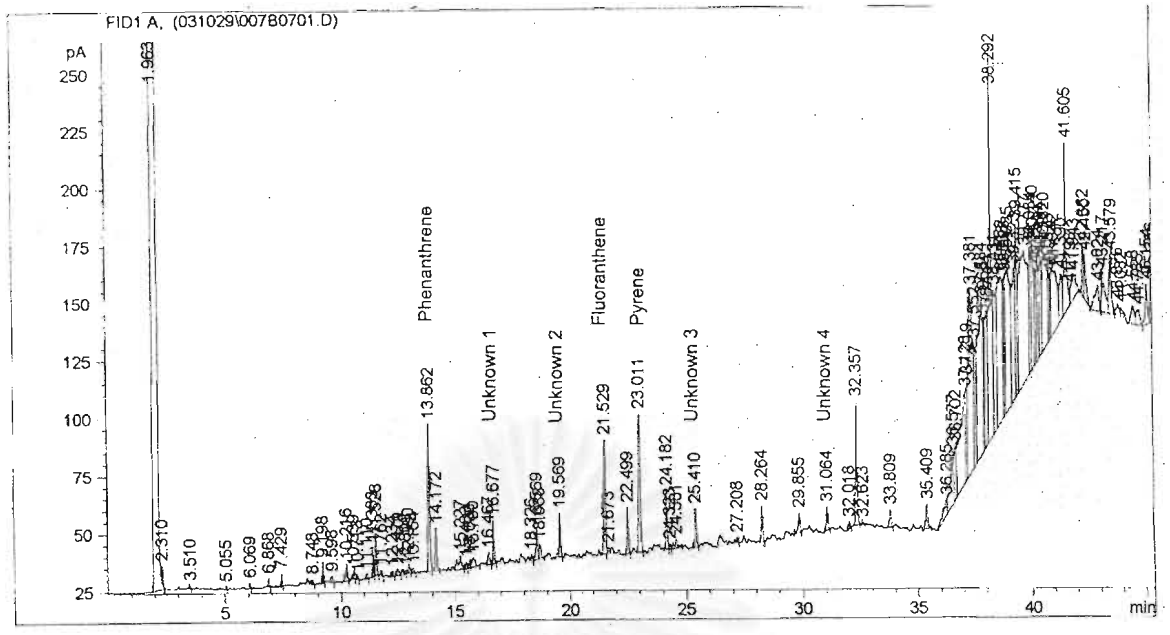


(C)

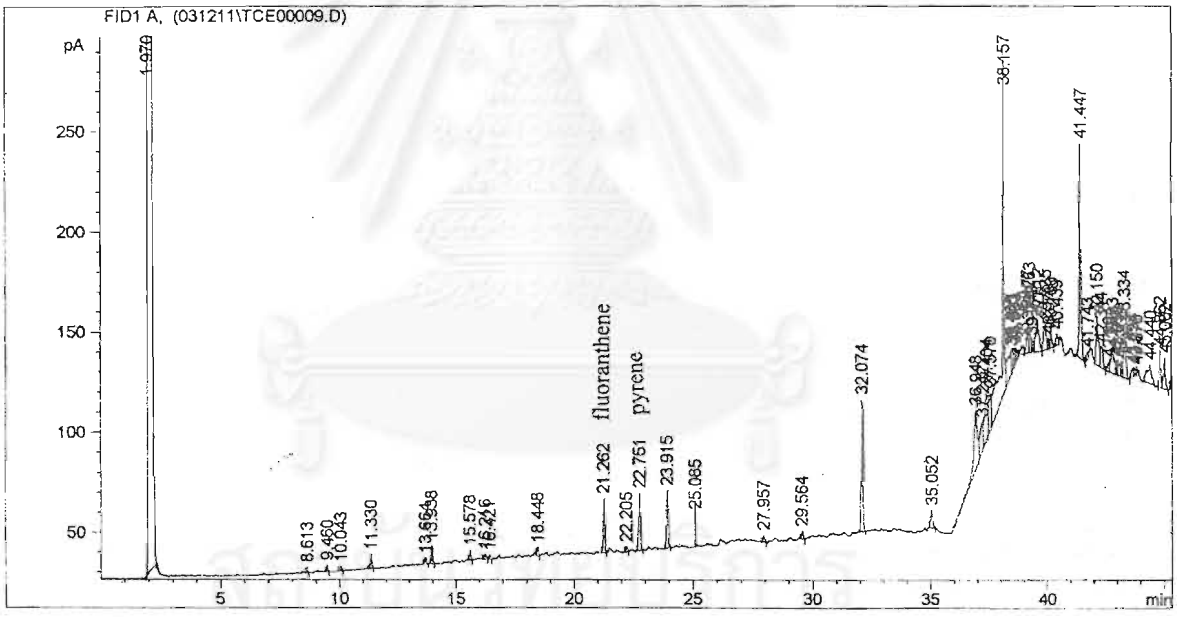


(D)

Figure C.3 Gas chromatograms of tamarind leaves (C) and yard waste (D).



(E)



(F)

Figure C.4 Gas chromatogram of PAHs and unknown hydrocarbon compounds in the Bangkoknoi Railway Station soil sample mixed with seed culture at day 0 (A) and day 28 (B).

APPENDIX D

1. Soil sieve.

Figure D.1 soil sieve.

Figure D.1 showed the soil sieve that was used for sieved the soil and tamarind leaves to a particle size of 2.36 mm.

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2.. Standard Triangle Diagram.

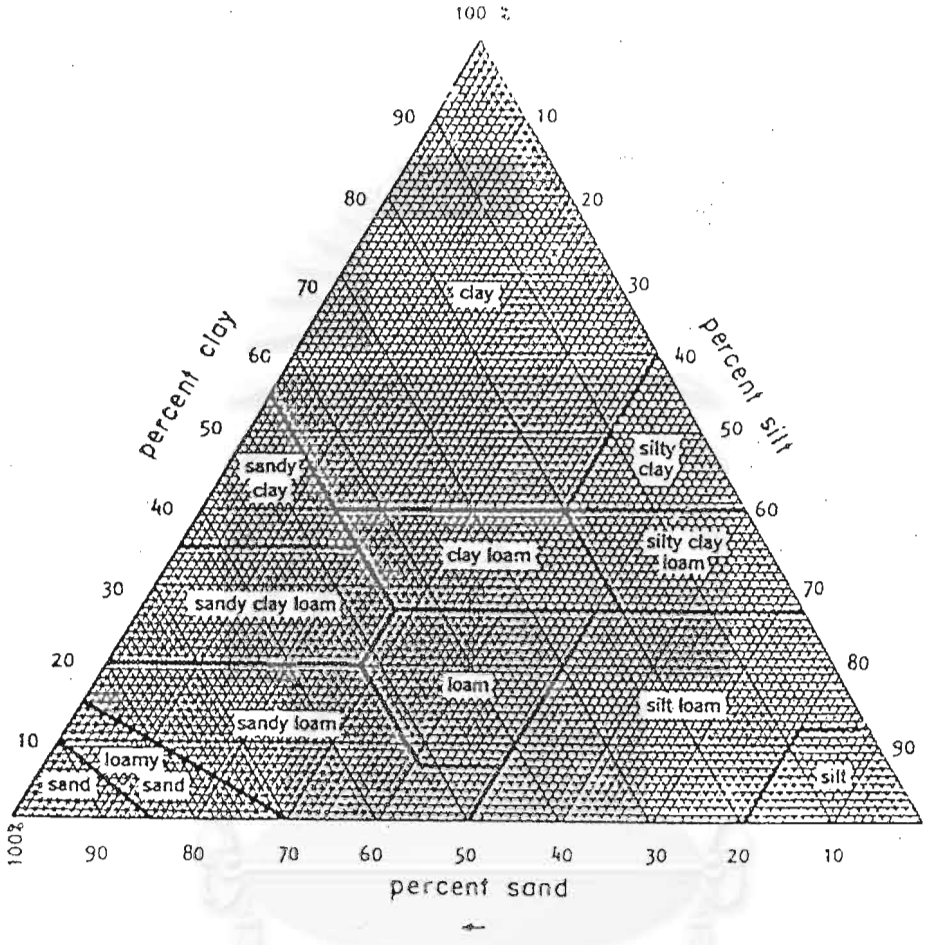

Figure D.2 Standard Triangle Diagram.

Figure D.2 showed standard triangle diagram that was used for translate and find out the type of soil sample from analytical result by compare the percentages of clay, sand and silt with value in the sides of standard triangle diagram.

BIOGRAPHY

Mr.Waurapong Lertthamrongsak was born on July 15, 1980 in Bangkok, Thailand. He attended Satitbansomdejchoapraya School and graduated in 1997. He received his Bachelor's Degree in Department of Microbiology from Faculty of Science, Chulalongkorn University in 2002. He pursued his Master Degree study in the International Postgraduate Programs in Environmental Management, Inter-Department of Environmental Management, Chulalongkorn University, Bangkok, Thailand in May 2002. He was awarded Master Degree of Science in Environmental Management in April 2004.

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