

**ENHANCED BIODEGRADATION OF DISPERSED CRUDE OIL USING
MARINE MICROORGANISMS**

by

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LIST OF ABBREVIATION

$(\text{NH}_4)_2\text{SO}_4$	Ammonium Sulfate
ANOVA	Analysis of Variance
C.I.	confidence interval
$\text{C}_{12}\text{H}_{26}$	n-Dodecane
$\text{C}_{14}\text{H}_{30}$	n-Tetradecane
$\text{C}_{16}\text{H}_{34}$	n-Hexadecane
$\text{C}_{18}\text{H}_{38}$	n-Octadecane
$\text{C}_{20}\text{H}_{42}$	n-Eicosane
$\text{C}_{22}\text{H}_{46}$	n-Docosane
$\text{C}_{24}\text{H}_{50}$	n-Tetracosane
$\text{C}_{26}\text{H}_{54}$	n-Hexacosane
$\text{C}_{28}\text{H}_{58}$	n-Octacosane
$\text{C}_{30}\text{H}_{62}$	n-Triacontane
$\text{C}_{32}\text{H}_{66}$	n-Dotriacontane
$\text{C}_{34}\text{H}_{70}$	n-Tetratriacontane
CaCl_2	Calcium chloride
CCD	Central composite design
CCFD	Central composite face-centered design
CH_2Cl_2	Dichloromethane
COD	Chemical oxygen demand
CV	Coefficient of Variation
DCO	Dispersed crude oil
DF	Degree of Freedom
DO	Demand oxygen
DOE	Design of Experiment

ESI	Environmental Sensitivity Index
Fe	Iron
FeCl ₃	Ferric chloride
GESAMP	Group of Experts on the Scientific Aspects of Marine Environmental Protection
H ₂ SO ₄	Sulfuric acid
HABs	Harmful algal blooms
HCl	Hydrochloric acid
HEM	hexane extractable material
HNO ₃	Nitric acid
K ₂ HPO ₄	Dipotassium hydrogen phosphate
KH ₂ PO ₄	Potassium di hydrogen phosphate
Ln Residual SS	Residual sum of square Logarithm
MgSO ₄	Manganese sulfate
N ₂	Nitrogen gas
Na ₂ HPO ₄	Di potassium hydrogen phosphate
Na ₂ SO ₄	Glauber's salt
NaOH	Sodium hydroxide
NH ₄ Cl	Ammonium chloride
NH ₄ NO ₃	Ammonium nitrate
NOAA	National Oceanic and Atmospheric Administration
OTA	Office of technology assessment
Pa	Pascal
PRESS	Predicted Residual Sum of Squares
R ² _{Adj}	Adjusted R-Squared
R ² _{Pre}	Predicted R-Squared
RSM	Response surface methodology

SS	sum of squares
StD	Standard deviation
TPH	Total Petroleum Hydrocarbon
WCO	Weathered Crude Oil
WSF	Water soluble fractions

LIST OF SYMBOLS

Abbreviation		Unit
α	Alpha (axial distance from the center point which makes the design rotatable)	(-)
β_0	Constant coefficient	(-)
β_i	Coefficients for the linear effect	(-)
β_{ii}	Coefficients for the quadratic effect	(-)
β_{ij}	Coefficients for the cross-product effect	(-)
X_i, X_j	Variables corresponding to factors (independent variables)	mg/L
Y	Response (dependent variables)	mg/L
ε	Error	%
$^{\circ}\text{C}$	Celsius Degree	$^{\circ}\text{C}$
mg	Milligram	mg
μg	Micro gram	μg
g	Gram	g
ppm	parts per million	
μm	Micrometer	
Mt	Million tons	
t	Time	(min)

PENINGKATAN BIODEGRADASI MINYAK MENTAH TERSERAK MENGUNAKAN MIKROORGANISMA MARIN

ABSTRAK

Pembersihan tumpahan minyak di lautan masih menjadi cabaran utama bioteknologi disebabkan kepelbagaian sifat tumpahan serta berbagai komplikasi alam sekitar di mana tumpahan berlaku. Walaupun berbagai penyelidikan secara bioremediasi telah dilakukan dalam tempoh dua dekad yang lalu, kesesuaian minyak mentah serta kepekatan nutrien semasa aplikasi bioremediasi masih belum termuktamat. Untuk itu, penyelidikan ini bertujuan mengkaji kesan kepekatan minyak mentah terhadap biodegradasi air laut serta penentuan nutrien optimum dalam meningkatkan penyingkiran hidrokarbon pada kepekatan tinggi dan rendah minyak mentah. Langkah pertama melibatkan kajian kesan kepekatan awal minyak mentah dan bahan penyerak terhadap kadar bioremediasi hidrokarbon petroleum dalam sampel air laut pada kepekatan 100, 500, 1000 and 2000 mg/L. Dalam eksperimen minyak mentah, bioreaktor dibekalkan dengan mikroorganisma teraklamitiasi serta nutrien. KNO_3 dan K_2HPO_4 digunakan sebagai sumber nitrogen dan fosforus. Sejumlah 1.00 mL mikroorganism inokula (mengandungi 1.2×10^7 sel /mL) ditambah ke dalam setiap reaktor. Dalam eksperimen minyak mentah terserak (DCO), bahan penyerak Corexit 9500 dibekalkan dengan nisbah minyak mentah:bahan penyerak 20:1 (w/w). Pada kepekatan rendah minyak mentah, atenuasi semulajadi (tanpa penambahan nutrien) menyingkirkan 22% minyak mentah dalam 28 hari. Penyingkiran tertinggi sebanyak 68% dicapai dalam keadaan tanpa-pengoptimuman menggunakan 20 mg/L nitrogen dan 2 mg/L fosforus dalam 28 hari; manakala proses pengoptimuman berjaya meningkatkan penyingkiran kepada 69% pada kepekatan nitrogen 16.05 mg/L dan fosforus 1.34 mg/L dalam 27 hari. Oleh itu, proses pengoptimuman boleh meningkatkan kadar biodegradasi dan mengurangkan penggunaan nutrien. Untuk penyingkiran n-alkana, model menjangkakan 98% penyingkiran dalam 20 hari pada kepekatan nitrogen 13.62% dan 1.39 mg/L fosforus. Dalam eksperimen sebenar, 95% penyingkiran dicapai dalam keadaan yang sama. Pada kepekatan tinggi minyak mentah (1 g/L), dengan menggunakan 190.21 mg/L nitrogen dan 12.71 mg/L fosforus, model berjaya meramal 61% penyingkiran TPH berbanding 59% penyingkiran secara eksperimen pada hari ke 28. Degradasi dalam keadaan tanpa pengoptimuman dan atenuasi semulajadi adalah masing-masing 53 dan 23%. Selanjutnya, sebanyak 85% penyingkiran n-alkana dicapai menggunakan 188.71 mg/L nitrogen dan 18.99 mg/L fosforus. Model Reka Bentuk Eksperimen (DOE) menjangkakan 91% penyingkiran berbanding 92% penyingkiran secara eksperimen. Kajian ini menunjukkan penggunaan bahan penyerak meningkatkan biodegradasi dan lebih efektif pada kepekatan minyak mentah yang tinggi dalam 30 hari pertama. Proses pengoptimuman dapat mengurangkan penggunaan nutrien selain meningkatkan penyingkiran n-alkana dalam masa yang lebih singkat. Analisis ANOVA dan diagnostik menunjukkan bahawa RSM merupakan kaedah yang boleh diyakini untuk melakukan pemodelan dan pengoptimuman bioremediasi minyak mentah. Pengoptimuman numerik menunjukkan keupayaan yang cemerlang dalam mengurangkan penggunaan nutrien dan meningkatkan penyingkiran TPH dan n-alkana. Ujian statistik mengesahkan kebolehpercayaan dan verifikasi model pada takat optimum untuk keseluruhan eksperimen.

ENHANCED BIODEGRADATION OF DISPERSED CRUDE OIL USING MARINE MICROORGANISMS

ABSTRACT

Cleanup of oceanic oil spills continues to be an important biotechnological challenge due to the highly variable nature of spills and the large number of complicating factors introduced by the environment in which they occur. Although much research in bioremediation has been conducted in the past two decades, appropriate crude oil and nutrient concentration for application of bioremediation in the marine environment is still inconclusive. Therefore this study aims to investigate the effects of crude oil concentration on its biodegradation in seawater as well as optimization of nutrient concentration for improved hydrocarbon removal in high and low initial crude oil concentrations. In the first part of study, the effects of initial oil concentration and dispersant on the rate of bioremediation of petroleum hydrocarbons in seawater samples were investigated for four concentrations i.e. 100, 500, 1000 and 2000 mg/L. In crude oil experiments, bioreactors were supplemented with microorganisms and nutrients. KNO_3 and K_2HPO_4 were used as nitrogen and phosphorus sources respectively. A total of 1.00 mL microorganism inocula (containing 1.2×10^7 cell /mL) were added to each bioreactor. In dispersed crude oil (DCO) experiments, bioreactors were supplemented with the dispersant Corexit 9500 at a ratio of 20:1 (w/w), crude oil-to-dispersant. In low crude oil concentrations, natural attenuation (no nutrient addition) removed 22% of crude oil in 28 days. The highest removal of 68% was observed in un-optimized condition by using nitrogen 20 mg/L and phosphorus 2 mg/L in 28 days; while process optimization exhibited a crude oil removal of 69% with nitrogen 16.05 mg/L and phosphorus 1.34 mg/L in 27 days. Thus, optimization process can improve biodegradation rate and reduce nutrient consumption. For n-alkanes removal, the model predicted 98% n-alkanes removal for a 20-day trial using nitrogen and phosphorus concentrations of 13.62 and 1.39 mg/L, respectively and 95% removal was observed under these conditions. For high crude oil concentrations (1 g/L), by using 190.21 mg/L nitrogen and 12.71 mg/L phosphorus, the model predicted 61% TPH removal while a TPH removal of 59% was observed in a 28-day experiment. Degradation under conditions with no optimization and by natural attenuation was 53 and 23%, respectively. Furthermore a maximum of 85% total n-alkanes removal was observed using 188.71 mg/L nitrogen and 18.99 mg/L phosphorus. Design of experiment (DOE) model predicted 91% removal and a removal of 92% were observed experimentally. This study shows that the use of dispersant enhanced biodegradation and is more effective for higher crude oil concentration within the first 30 days. Optimization process could reduce nutrient consumption as well as increase n-alkanes removal in shorter time. ANOVA and diagnostic analysis indicated that RSM is a reliable tool for modeling and optimization of crude oil bioremediation. Numerical optimization demonstrated excellent ability to decrease nutrients use and increase both TPH and n-alkanes removals. Statistical tests confirmed model validation and verification at optimum point for all experiments.

CHAPTER 1

INTRODUCTION

1.1 Marine environment

Marine environments cover approximately two-thirds of the Earth's surface. They serve as a source of food and valuable minerals and a means of transportation, provide a place for recreation, and have a profound effect upon the world's climate, rainfall, and water cycles. Unfortunately, marine environments have been used for waste disposal as well. Hydrocarbons, particularly crude oil, heavy metals, chlorinated compounds and radioactive wastes are the major marine pollutants. Among these, hydrocarbon contamination has been addressed as the main critical challenge in aquatic ecosystems (Abolhasani Soorki and Ebrahimipour, 2009).

1.2 Oil pollution at sea

Oil and oil products are a group of pollutants that have complex and diverse composition and various impacts on living organisms - from physical and physicochemical damage to carcinogenic effects (Patin, 1999). Hydrocarbon pollution in marine ecosystems has potentially dangerous effects on organism and human health through bioaccumulation and biomagnification via food chains. Consequently, oil removal from contaminated environments is highly recommended.

1.3 Cleanup methods

Crude oil spill cleanup involves various physical/mechanical, chemical, and biological methods and strategies. Biodegradation is the main mechanism for the removal of contaminants from the environment. Bioremediation is the use of living

organisms to accelerate biodegradation of contaminants into non-toxic or less toxic forms. In most marine ecosystems heavily contaminated with hydrocarbons, nitrogen and phosphorus are limiting factors in oil biodegradation (Zhu et al., 2001). Bioremediation of oil spills has therefore focused on overcoming this limitation by adding fertilizers to petroleum-contaminated marine environments (Swannell et al., 1996; Da Silva et al., 2009).

Several laboratory experiments have shown that the addition of nutrients might be effective for increasing the biodegradation of organic compounds because such supplementation effectively stimulates bacterial growth (Jean et al., 2008; Das and Mukherjee, 2007). Much is still unknown to the organisms involved in biodegradation. However, it has been shown that successful biodegradation is often a result of the actions of multiple organisms (Atlas, 1995). Natural attenuation, biostimulation and bioaugmentation were emphasized in our study.

1.4 Problem statement

It is estimated that 26.4×10^5 tones of oil enters marine environments annually from ships and other sea-based activities (GESAMP, 1997). It is physically, chemically and biologically harmful to the marine ecosystems because crude oil contains many toxic compounds. The dangerous consequence of hydrocarbon on marine biota is widely investigated (Hasanuzzaman et al., 2007; Knezevich et al., 2006; API, 2001; Delille et al., 1998).

After a major spill, physical/mechanical methods are primary response option but recover no more than 10-15% of the crude oil (OTA, 1991). Physical strategies

are included Usage of booms, Skimmers, washing, Low pressure flushing, Cutting vegetation and burning.

Chemical techniques have been commonly used in aquatic ecosystems. Application of dispersants, demulsifiers, surface film chemicals and cleaners were extensively investigated. Often, the bioavailability of crude oil increased after application of chemical methods, however the toxicity of compounds are also important.

Malaysian marine environments have been subjected to numerous oil spills in the past ten years (Mohajeri et al., 2009) including the following:

- a) A Chinese cargo ship sank at the Tanjung Po anchorage point at the mouth of the Sarawak River on September 2, 2000.
- b) An oil tanker carrying approximately 67 tonnes of fuel (including diesel and 1,500 tons of bitumen) sank after impact with a super tanker about 7.5 nautical miles off Pulau Undan (near Malacca) on May 28, 2001.
- c) An Indonesian tanker loaded with toxic chemicals capsized off Malaysia's southern coast in Johor State, just across from Singapore, on June 13, 2001.

Oil production in Sarawak and tanker transport of crude oil in the Strait of Malacca and South China Sea provide an ongoing potential for a catastrophic oil spills. Although crude oil bioremediation has been frequently investigated in the last three decades, there is still lack of knowledge about the suitable initial oil concentration for bioremediation as well as critical factors such as the influence of oil composition and specification and sensitivity of the contaminated environment. Furthermore, interactions between major variables affecting oil biodegradation have

not been established well enough to achieve optimum process parameters. Therefore, a statistical design technique has been applied to improve understanding of crude oil bioremediation.

1.5 Research Objectives

The overall goal of this research is to determine the highest concentration of crude oil that is amenable to bioremediation and to evaluate optimum process parameters at high and low crude oil concentrations. Results will provide the basis for suggestions to be made to the Malaysian government as to when and how bioremediation can be efficiently applied in the case of an oil spill in the marine environment. A deeper understanding of the relationships among effective factors is expected to provide improved tools for optimization of bioremediation strategies for crude oil contaminated seawater. This main goal is divided into the following more specific objectives:

1. To investigate the effect of crude oil concentrations on its biodegradation in seawater
2. To study the effectiveness of dispersant on crude oil bioremediation at different oil concentrations
3. To evaluate the effect of different nitrogen and phosphorus concentrations on the rates and extent of biodegradation of total petroleum hydrocarbons (TPH) and selected n-alkanes
4. To optimize nutrient concentration for improved hydrocarbon removal in high and low initial crude oil concentrations

5. To study the interaction among process parameters to maximize biodegradation of hydrocarbons
6. To obtain mathematical models that could be used for prediction of crude oil removal by studying the laboratory-scale bioremediation

1.6 Scope of Study

Since there is still a gap of knowledge about appropriate crude oil and nutrient concentration for application of bioremediation in the marine environment, this research focused on these topics. In a preliminary study, the effects of initial oil concentration and dispersant on the rate of bioremediation of petroleum hydrocarbons in seawater samples were investigated in range of 100 to 2000 mg/L. Based on the results of this study, bioremediation of dispersed crude oil using supplementation with nitrogen and phosphorus, was optimized by the application of response surface methodology (RSM) and central composite design (CCD) for two initial concentrations of crude oil, i.e. 100 and 1000 mg/L. The removal of TPH and selected n-alkenes ($C_{12}H_{26}$ to $C_{34}H_{70}$) was investigated. The experimental data obtained were fitted to a second-order polynomial mathematical model with multiple regressions. The adequacy of the models was checked by statistical methods. Numerical optimization based on the desirability function was applied to maximize removal of hydrocarbons.

1.7 Thesis Organization

This section provides an overview of the thesis and describes how each chapter contributes towards meeting the study objectives:

Chapter 1 (introduction) is a brief introduction to marine pollution, crude oil at sea, biodegradation and bioremediation as well as a problem statement provides a basis and the underlying principles for the research directions taken in this study. Overall and detailed objectives are given in this chapter as well. The organization of the contents is also provided (this section).

Chapter 2 (Literature Review) is an overview of the literature related to this study and the main basic knowledge about this work including biodegradation mechanism, factors affecting bioremediation of crude oil in aquatic environments, and application of response surface methodology for biodegradation study.

Chapter 3 (Materials and Methods) describes the experimental approach, including sample collection, design of the experiments, and steps of experiential set up. Analytical techniques for nutrients and hydrocarbons as well as quality control and quality assurance (QA/QC) procedures are listed.

Chapter 4 (Results and Discussion) comprises the main part of this thesis and explains the three major parts of the research; the results of first part of the study are explained; the percent removal for abiotic control, natural attenuation, crude oil bioremediation and dispersed crude oil (DCO) bioremediation are compared for crude oil concentrations of 100, 500, 1000 and 2000 mg/L. Application of RSM for design, modeling and optimization of TPH removal and selected n-alkanes removal in low crude oil concentration of 100 mg/L are discussed. High initial oil concentration of 1000 mg/L was used for bioremediation using the same method, and

the effects and interactions of process parameters, numerical optimization and method verification are examined.

Chapter 5 (Conclusions and recommendations) summarizes the knowledge gained from the study and how it can be used in practical applications for optimization of bioremediation for the cleanup of crude oil contaminated seawater. It also provides recommendations for future studies in the field that are based on the results, conclusion and data generated in this research.

CHAPTER 2

LITERATURE REVIEW

2.1 **Maine pollution**

Pollution is defined as “The introduction by man into the environment of substances or energy liable to cause hazards to human health, harm to living resources and ecological systems, damage to structure or amenity, or interference with legitimate uses of the environment” (EEAC, 2009).

Marine pollution is defined as pollution of any of the world's oceans, seas, or other saltwater systems. Focus on the oceans of the world is critical, because - not being owned by any individual nation - environmental protection of them is simply not regulated (ALCOA, 2009).

2.2 **Sources of pollution in aquatic ecosystems**

Natural and anthropogenic organics (xenobiotics) enter and are dispersed in aquatic ecosystems by various routes, including direct discharge, direct use, land runoff, atmospheric deposition, in situ production, abiotic and biotic movement, and food-chain transfer (Gagnaire et al., 2006; Livingstone, 1998).

Human activities have led to a variety of water pollution and disturbance to aquatic ecosystem (Zhang et al., 2008). Regulation of the aquatic environment has generally been based on the assessment of physical and chemical attributes of anthropogenic inputs and their concentration in the receiving water (Pennington et al., 2001). However, to fully assess the impact on aquatic environment, i.e.

disturbance of normal material circulation and energy flow maintained by constituents of an ecosystem, evaluation of the status of ecological functions should also be included in the regulation.

Different amount of the total input of petroleum have been documented. It is estimated that totality effort of petroleum to the oceans through man's activities and sources such as atmospheric fallout and natural seepage is at 2.37×10^6 t per year. Out of these, about 65.2% is discharged through municipal and industrial wastes, urban and river runoffs, oceanic dumping and atmospheric fallout; 26.2% derives from discharges during transportation, dry docking, tanker accidents, de-blasting, etc.; and remaining 8.5% comes from fixed installations like coastal refineries, offshore production facilities, marine terminals, etc. Though a considerable fraction of petroleum hydrocarbons entering the marine environment is removed by evaporation, a portion gets distributed in water, accumulated in sediment and transferred to biota (Chouksey et al., 2004). On the other hand in 2007, the IMO/FAO/UNESCO/IOC/UNIDO/WMO- /IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) estimated that oil enters marine environments from ships and other sea-based activities at a rate that is in the order of metric tonnes per year (tonnes/year). Table 2.1 summarizes this data along with data from other records for atmospheric fallout for total hydrocarbon pollution in marine environments worldwide.

Table 2.1 Annual hydrocarbon contamination in marine environments worldwide (GESAMP, 2007)

<i>Source</i>	<i>Amount (1000 tons/year)</i>	<i>P ercent</i>
Land-based	1200	45.45
Oil transportation and shipping	457	17.31
Offshore production discharge	20	0.76
Small craft activity	53	2.01
Atmospheric fallout	300	11.36
Natural seeps	600	22.73
Other	10	0.38
Total	2640	100.00

As can be seen in the table, main source of marine pollution are tanker operations, land based discharges and natural seeps (GESAMP, 2007).

2.3 Fate and effect of oil in the aquatic ecosystem

Crude oil is highly unstable in the marine environment, quickly losing the volatile fractions through weathering and becoming both photo- and biodegraded. Prolonged turbulent mixing by wind and currents results in the release of a water accommodated (or soluble) fraction mainly composed of naphthalene. Hydrocarbons may bioaccumulated in biota and affect organisms higher in the food chain in marine systems. The water soluble fraction (WSF) has been shown to be harmful to marine biota (Noyo et al., 2008; Faksness and Brandvik 2008; Anderson et al., 2009). WSF is highly toxic to the embryos of fish and starfish and crustaceans from pristine environments (Lewis et al., 2008; Law, 2000)

The alteration of petroleum by fresh water mainly involves three processes: inorganic oxidation, water washing, and biodegradation. Inorganic oxidation (by molecular oxygen) is restricted largely to near-surface reservoirs. Water washing has

occurred where formation waters have dissolved the more soluble lighter hydrocarbons.

Impacts from oil pollution depend on the amount and type of oil discharged as well. Lighter crude oils (higher API degree crude oils) disappear rapidly from the water column. And heavier oils may sink and persist in coastal ecosystems. Seabirds can be harmed when their feathers are coated with crude oil moreover toxicity of oil components kill animals and damage their reproduction.

When pollutants are released into aquatic habitats, direct (toxic) effects on aquatic biota are possible. Direct effects vary with the intensity and duration of exposure to a toxicant, and they are frequently studied, in part, because predictive criteria to estimate risk and establish permissible levels of contamination are based on species responses to contaminants (Fleeger et al., 2003).

These criteria are derived from laboratory toxicity tests that usually employ model species in single-toxicant exposures. The direct effects of toxicants typically reduce organism abundance (by increased mortality or reduced fecundity). Biota from a given habitat often exhibit a wide range of tolerance to specific toxicants (e.g. insecticides and herbicides target specific organisms in an interacting community) with the consequence that a toxicant may exert lethal effects on some species, but cause no observable effects on others. Direct sublethal effects, e.g. behavioral impairment or physiological stress, are also possible (Fleeger et al., 2003).

Oil spills in the sea can have wide spread impact and long-term consequences on wildlife, fisheries, coastal and marine habitats, human health and livelihood, as well as recreational resources of shoreline communities (ASMA, 2009; Mishra et al., 2004). The degree of the damage caused by an oil spill event depends primarily upon the quantity of oil spilt, the chemistry and characteristics of the oil and the sensitivity of the biological resources impacted. However, restoration of contaminated sites has been a significant challenge for marine scientists (Steliga et al., 2010; Pala et al., 2006; Pelletier et al., 2004). Impacts of spills on foreshores also depend heavily upon the form and exposure of the coastline (for example protected mangroves and salt marshes) are extremely sensitive to oil spills yet rocky exposed coastlines naturally self clean and are less sensitive to oil spills (AMSA, 2009).

The deleterious effects of spilt crude oil have been investigated for fish resources (Jung et al., 2009; Martinho et al., 2009; Carls et al., 2008), coral reefs (Feary et al., 2009) and marine mammals (NRC, 2002). Melville and co-authors (2009) studied the impact of oil on mangrove forests and concluded that petroleum hydrocarbons can adversely affect mangroves by coating stems, roots and leaves or adsorbing into the mangroves through the sediment. The mangrove ecosystems of the Persian Gulf have also been affected by oil spill during the Iraq–Kuwait War in 1991. Marine oil pollution presents a high risk for coastal salt marshes (Vega et al., 2009). Salt marshes provide the primary source of organic carbon and habitat for coastal fisheries and wildlife; furthermore they provide a protective mechanism against shoreline erosion (Sabeen et al., 2009). Part of the deleterious effects from

spilled oil stranded on tidal flats is due to the lack of oxygen caused by organic enrichment or by direct toxic or carcinogenic effects (Chung et al., 2004).

2.4 Classes of Oil Spills

Classification of oil spill is an important topic for the control and protection of aquatic habitats. Oil spills in marine ecosystems can be divided into three broad classes. Firstly, small oil spills which are associated with bunkering, berthing and other terminal operations, and unintentional discharges of oily water mixtures from the bilges of machinery spaces. Generally these kinds of spills occur in port and tend to result from accidents, equipment failure or poor work practices; it is usual in most of ports in third world countries because of using old technology as the results of political sanction as well as mismanagement. The second class of oil spills occur as the consequences of major shipping incidents like collisions, groundings, fires or other forms of damage. Undersea pipelines, drilling rigs and platforms are also potential sources of oil pollution by damage or accident e.g. severe weather events like cyclones, blow outs or fires on off shore rigs. Spills resulting from damage to an oil tanker, off shore structures or pipelines tend to produce the isolated intensive/large scale spill, whereas port spills tend to be slight to moderate in size but can still result in extensive environmental and economic damage. The third class of spills referred to as the "dishonorable discharges" or "midnight dumpers" who intentionally dump waste oil and oily waters into our marine environment to save money or time. Maritime accidents are understandable but these intentional spills are essentially criminal actions and are not characterized as "operational discharges" by vessel or rig personnel (AMSA, 2009). Other sources of hydrocarbon contamination in the marine environments are classified as non point source (NPS) pollution.

2.5 History of oil spill in the world and South East Asia region

2.5.1 The major oil spills in history

Although every major oil spill from a tanker or a rig, hitting coastal areas and beaches and killing marine life and seabirds, is a tragedy and causes much damage, it has been estimated that oil spills in conjunction with tanker accidents or oil platform blowouts account for a minor part, approximately 10-15 per cent, of the total annual input of oils to the marine environment. History of major oil spills (over 100,000 tons) sorted by volume is presented in Table 2.2.

Table 2.2 History of oil spill in the marine ecosystems (GESAMP, 2007)

<i>Spill / Tanker</i>	<i>Location</i>	<i>Date</i>	<i>*Tons</i>
Persian Gulf War oil spill	Persian Gulf	January 23, 1991	1,500,000
Ixtoc I oil well	Gulf of Mexico	June 3, 1979	480,000
Atlantic Empress	Trinidad and Tobago	July 19, 1979	287000
Fergana Valley	Uzbekistan	March 2, 1992	285000
Nowruz oil field	Persian Gulf	February 1983	260000
ABT Summer	Angola	1991	260000
Castillo de Bellver	Saldanha, South Africa	August 6, 1983	252000
Amoco Cadiz	Brittany, France	March 16, 1978	223000
Amoco Haven tanker	Mediterranean Sea, Italy	1991	144000
Odyssey	Nova Scotia, Canada	1988	132000
Exxon Valdes	Prince William Sound, Alaska	March 24, 1989	119000
Sea Star	Gulf of Oman	1972	115000
Torrey Canyon	Scilly Isles, UK	March 18, 1967	110,000
Irenes Serenade	Navarino Bay, Greece	1980	100000
Urquiola	A Coruña, Spain	May 12, 1976	100000

*One tonne of crude oil roughly equals to 308 US gallons, or 7.33 barrels

2.5.2 Oil spill in southeast Asia

In the period 1988-1997 in GESAMP Region 17 (East Asian/Southeast Asian Seas), an area of high commerce, the estimated annual spill number increased. However, it was not as high as it had been in the period 1974-1980. Spill amounts were also slightly higher during this period, although again not as high as it had been

in the period 1974- 1980. This region experienced nine tanker spills of at least 5,000 tonnes between 1988 and 1997. These were: the Century Dawn spill of 10,700 tonnes in Singapore in 1988; the Vishru spill of 5,280 tonnes in the Philippines in 1989; the Nagasaki Spirit spill of 13,000 tonnes in the Malacca Strait, Malaysia, in 1992; the Maersk Navigator spill of nearly 25,000 tonnes in the Malacca Strait, Malaysia, in 1993; the Frontier Express spill of 7,800 tonnes in the Yellow Sea off South Korea in 1993; the Thanassis A. spill of 37,000 tonnes in the South China Sea off Hong Kong in 1994; the Nakhodka spill of 6,200 tonnes in Japan in 1997; the DaQing 243 spill of 17,000 tonnes in the estuary of the Yangtze River, China, in 1997; and the Evoikos spill of 28,600 tonnes in the Singapore Strait in 1997 (GESAMP, 2007). Major oil spills in Malaysian seawaters in recent years are as follows:

October 10, 1997 - An oil spill happened in Northern side of Penang Island two ships collided. The collision occurred approximately 20 miles off the Malaysian north coast above Penang Island. A light wind of 5 knots was blowing from the North West. One was a bulk crude oil carrier of 55,000 tons, the other a coastal freighter. The crude oil was a medium density of API 25. The smaller coastal freighter had no running lights and apparently the watch knew the local waters and was slowly moving away from the Malaysian coast. The tanker proceeding south at 18 knots struck the bow of the freighter. The collision struck the front port side of the tanker below the water line and then scraped along the side of the oil carrier ripping a wide scar along the port side. Oil started to pour out of the side of the tanker. About 40 percent of the tanker compartments were within the damaged area with a potential of releasing approximately 20,000 tons of the medium heavy crude oil from the China Sea. The wind started to move the oil toward the Malaysian coast and the

beautiful beaches of north Penang. The wind started the physical spreading and evaporation, and the waves started to break the oil into streaks and patches. The wave action was not enough to physically emulsify the oil.

September 2, 2000 - Oil spill from a sunken Chinese cargo ship at Tanjung Po anchorage point at the Sarawak River mouth. The ill-fated 5,000 ton Kingston registered vessel Double Brave was loaded with about 116 ton of diesel oil when it sank after a collision with a barge being towed by a tugboat. About 60 workers from the Marine Department, Department of Environment, and the Kuching Port Authority helped in the clean-up operation.

May 28, 2001 - An oil tanker with some 67 ton of fuel, including diesel and 1,500 ton of bitumen, sunk after it was crashed from behind by a super tanker about 7.5 nautical miles off Pulau Undan, near Malacca. Officials said the crash caused MT Singapore Timor to take in water, and remained half-submerged in the sea floating southwards. Diesel and bitumen started to spill into the sea, and spreader to about one nautical mile from the collision spot.

June 13, 2001 - An Indonesian tanker laden with a toxic chemical capsized off Malaysia's southern Johor state, just across from Singapore. The 533 ton MV Endah Lestari was on its way to East Kalimantan in Indonesia with some 600 ton of the poisonous industrial chemical phenol, and 18 ton of diesel. Newspaper reports said the toxic spill had killed thousands of fish and cockles reared in 85 offshore cages, and Singapore authorities also warned its citizens to stay away from nearby

waters. Officials said it would be tough to mop up the phenol, as it is soluble in water.

2.5.3 Coastal Oil Refinery Effluent Output in Malaysian seawaters

Entering petroleum hydrocarbon in to the sea from Coastal Oil Refinery Effluent Output was estimated in the period 1988-1997 by Etkin, (1999). Estimates represent maximum values based on assumptions that refineries operate at full capacity year-round, effluents are 5 ppm or 25 ppm oil depending on national laws and practices, and refineries produce 4.5 units of wastewater per unit refining capacity; actual discharges may be as low as 25-50% of these amounts if refineries operation on less than full-time schedule. The results for Malaysia are summarized in Table 2.3:

Table 2.3 Estimated petroleum hydrocarbon entering in to the sea from Malaysian Coastal Oil Refinery (Etkin, 1999),

<i>Daily Crude Capacity</i>	<i>Effluent Output</i>	<i>Oil Discharge in Effluent</i>
263,000 bpd (37,571 tpd)	17,336,696,999 gal/y (65,619,595 m ³ /y)	433,397 gal/y (1,474.14 t/y)

Bpd=Barrels per day, tpd=Tonnes per year, gal= US Gallons

2.6 Methods for oil clean up

Major oil spills highlight the need for environmentally responsible and cost-effective mitigation technologies. Crude oil spill clean up includes various physical/mechanical, chemical, and biological methods and strategies. After a major spill, physical/mechanical methods are primary response option but recover no more than 10-15% of the crude oil (OTA, 1991).

Physical strategies include usage of booms, skimmers washing low pressure flushing cutting vegetation and burning. Chemical techniques have been used as a response option in aquatic ecosystems (Tam et al., 2009). Application of dispersants, demulsifiers, surface film chemicals and cleaners has been extensively investigated. Often, the bioavailability of crude oil increased after application of chemical methods, however the toxicity of compounds are also important. Biological treatment is based on the idea that a large percentage of petroleum compounds are biodegradable (Radwan et al., 2002; Obuekwe and Al-Zarban, 1998).

Bioremediation has been defined as “the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes” (OTA, 1991; Zhu et al., 2001). The need for alternative and additional responses led to bioremediation that has emerged as one of the most promising secondary treatment options for oil removal since its successful application after the 1989 Exxon Valdez oil spill (Zhu et al., 2001). Bioremediation can offer a less ecologically damaging alternative by taking advantage of the oil degrading microbes (Kuhan and Gupta 2009) and by establishing and maintaining the physical, chemical and biological conditions that favour enhanced oil biodegradation rates in the contaminated environment (Nikolopoulou and Kalogerakis, 2008; Zhu et al., 2001; Fedorak and Westlake, 1981).

Recently, combinations of these techniques have been documented. Alamri, (2009) showed that the combination of chemical tools and different classes of biological technologies would be more successful in monitoring hydrocarbon bioremediation than any single approach. Gertler et al., (2009) removed the heavy

fuel oil from the water body by combination of mechanical and biotechnological techniques.

2.7 Biodegradation

Biodegradation is breaking down or decomposition of organic materials by organisms. It is the most important process in the environment (D'Auria et al., 2009). A term related to biodegradation is biomineralisation, in which organic matter is converted into minerals. Mineralization is the complete conversion of organic molecules into inorganic substances. Biotransformation is the transformation of a parent compound into other metabolites, which may be more or less toxic than the parent compound.

In 1946, Zobell from Scripps Institution of Oceanography discovered that many microorganisms are capable of utilizing hydrocarbons as the sole source of energy for metabolism.

Biodegradation of petroleum hydrocarbons is a complex process that depends on several parameters. Enhancing biodegradation processes to assist in the cleanup of oil spills in marine environments has been suggested several times, with much emphasis on the treatment of oil on the sea (Tam et al., 2009; Rahman et al., 2003). Several approaches have been discussed and debated. For example, the idea of accelerating oil biodegradation rates by increasing the availability of oil to bacteria through the use of dispersants (bacteria degrade oil primarily at oil-water interfaces) has been tested with mixed results, giving a confusing picture as to the ultimate effectiveness of this treatment (Ostberg et al., 2006).

The concept is complicated by potential problems of dispersant toxicity to both bacteria and other aquatic life. Seeding oil-contaminated areas with hydrocarbon-degrading bacteria has also been considered as a possible bioremediation option in both aquatic and terrestrial environments, but again the results have been mixed. The simplest approach for enhancing petroleum hydrocarbon biodegradation is the addition of nutrients in a well-oxygenated environment (Zhu et al., 2001).

It is well known that enrichments of oil degrading microorganisms occurs rapidly following oil spills in most environments. But in the face of a large amount of degradable oil carbon, biodegradation quickly becomes limited by oxygen and nutrient availability and attempts to overcome these limitations usually lead to successful degradation (Atlas, 1995).

2.8 Bioremediation

2.8.1 Theory and application

Bioremediation is the act of adding materials to cause an acceleration of the biodegradation processes (OTA, 1991). Municipal wastewater treatment plants have been using this technology for decades. Bioremediation is an application of the same principles in a different setting.

Bioremediation has several advantages over conventional technologies. First, the application of bioremediation is relatively inexpensive. For example, during the cleanup of the Exxon Valdez spill, the cost of bioremediating 120 km of shoreline

was less than one day's cost for physical washing (Zhu et al., 2001). Bioremediation of an oil spill on the shoreline of Delaware Bay, United States is also reported by Venosa et al., (1996). Bioremediation is also a more environmentally benign technology since it involves the eventual degradation of oil to mineral products (such as carbon dioxide and water), while physical and chemical methods typically transfer the contaminant from one environmental compartment to another. Since it is based on natural processes and is less intrusive and disruptive to the contaminated site, this "green technology" may also be more acceptable to the general public (Zhu et al., 2001).

2.8.2 Types of bioremediation

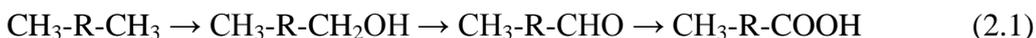
Economical strategies for bioremediation include natural attenuation, biostimulation, bioventing, bioaugmentation, landfarming, composting, and phytoremediation (Bento et al., 2005). This study focused on natural attenuation, biostimulation, and bioaugmentation.

Natural attenuation is a variety of processes that naturally act to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in the environment, and includes biodegradation, dispersion, sorption, dilution, volatilization, and chemical or biological stabilization, transformation, or destruction of contaminants (US-EPA, 1999). Natural crude oil bioremediation can be supported by increasing the number of available indigenous microorganisms by supplementing the ecosystem with nutrients (biostimulation) or by the addition of microbial consortium (bioaugmentation) (Bento et al., 2005; Atlas 1995).

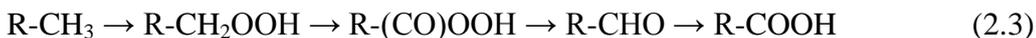
2.8.3 Mechanism of bioremediation

When the water content reaches about 80% by weight, the consistency of the emulsion changes - it becomes a thick semi-solid mass with a grease-like consistency. It is often called "chocolate mousse" at this stage because of both its consistency and its brown color. This process does not readily occur with fuel oils such as diesel oil and kerosene, but occurs easily with light and heavy crude oils - the usual cargoes of oil tankers (Mayfield, 2008).

Numerous mechanisms were documented in the literature in support of hydrocarbon biodegradation (Harayama et al., 1999). Equations (2.1) and (2.2) explain transformation of hydrocarbons to aldehyde and fatty acids.



Degradation of n-alkane via alkyl hydroperoxides mechanism is shown in Equation 2.3.



As examples, biotransformation mechanisms of Toluene are presented in Figure 2.1. Degradation mechanism of Benzo (a) pyrene shown in Figure 2.2 and mineralization of phenanthrene is presented in Figure 2.3.

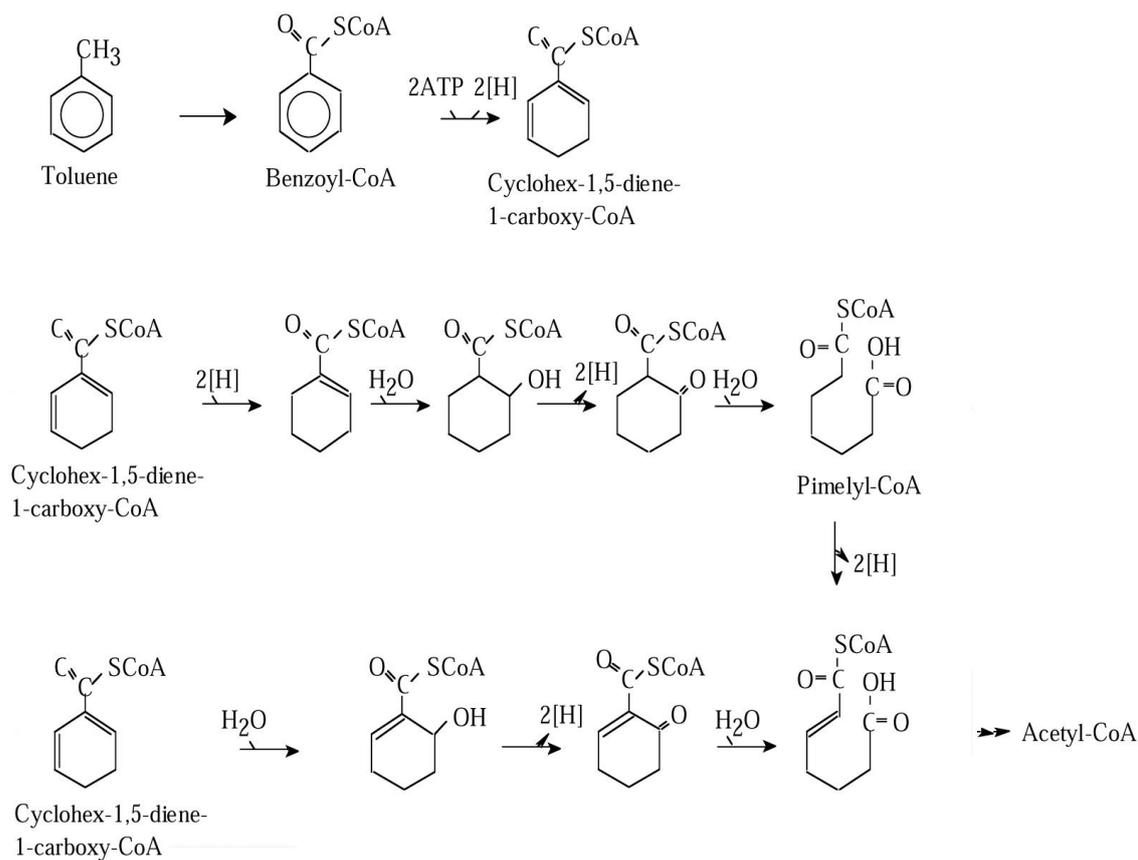


Figure 2.1 Toluene biotransformation mechanisms (Harayama et al., 1999)

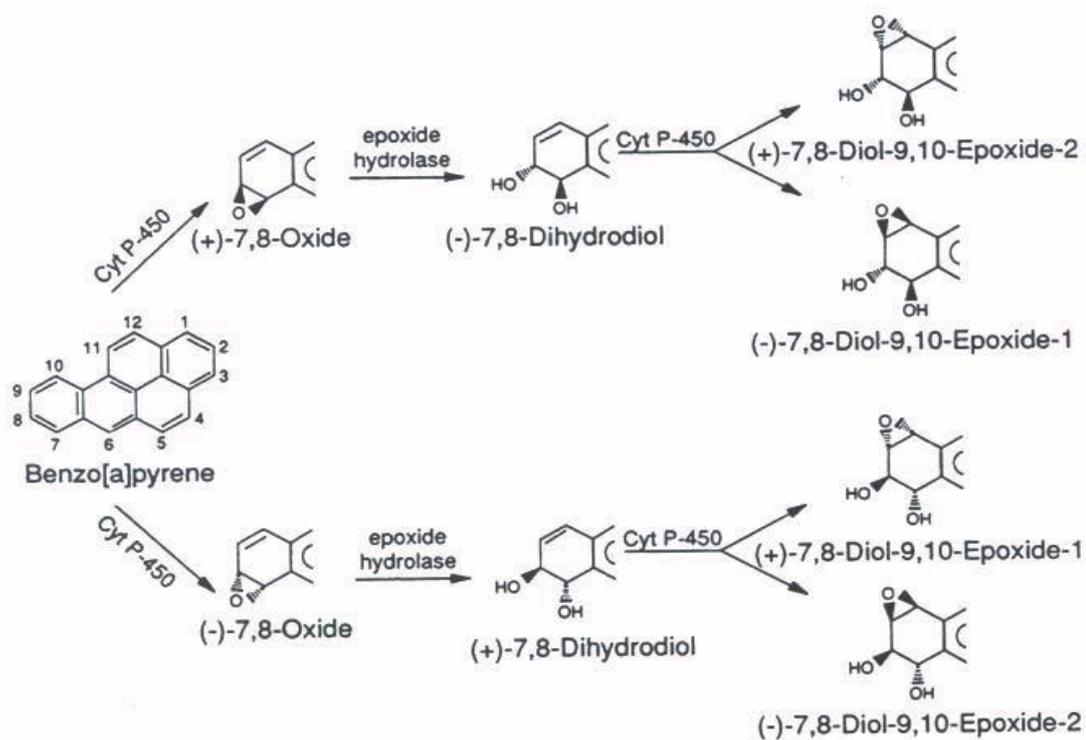


Figure 2.2 Degradation of benzo[a]pyrene (Sutherland et al., 1995)

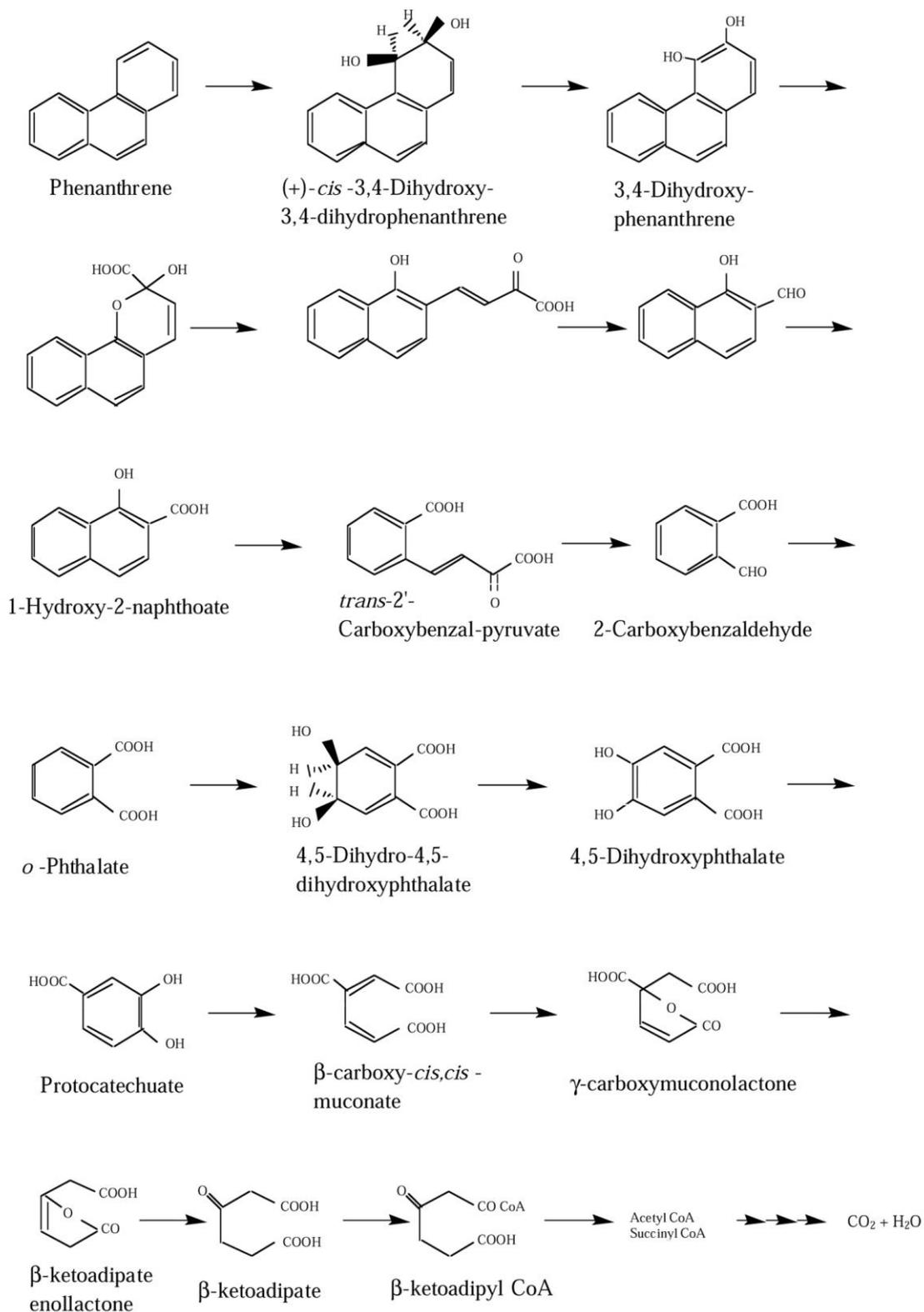


Figure 2.3 Phenanthrene mineralization mechanisms (Harayama et al., 1999)

2.9 Factors affecting hydrocarbon bioremediation

Several factors can affect petroleum hydrocarbons bioremediation in the marine environments (Pelletier et al., 2004; Pala et al., 2006; Mishra et al., 2004; Melville et al., 2009; Knezevich et al., 2006; Hçhener et al., 2003). This section details the parameters with specific emphasis on initial oil and nutrient concentrations.

2.9.1 Oil

Petroleum hydrocarbons can typically be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and resins (pyridines, quinolines, carbazoles, sulfoxides, and amides). Biodegradation rates have been shown to be highest for saturated alkanes, followed by light aromatics, with high-molecular-weight aromatics and polar compounds exhibiting extremely low rates of degradation (Reisinger, 1995; Rowland et al, 2000; IMO, 2004). In contrast, higher rates of biodegradation were reported for naphthalene than for hexadecane in a freshwater lake (Cooney et al., 1985). Fast biodegradation of aromatics has also been reported for a petroleum bioremediation process (Fedorak and Westlake, 1981). Additional dissimilar degradation rates have been reported in literature (Kuhan, and Gupta, 2009; Gentili et al., 2006; IMO, 2004; Namkoong et al., 2002).

Rontani and co authors (1985) investigated the trends in n-alkane removal and showed that the disappearance of low molecular-weight alkanes is stimulated by the addition of nutrients. It is generally thought that n-alkanes of shorter chain length are more easily used as an energy source than the longer-chain ones (Riser-Roberts,