

INTRINSIC BIODEGRADATION POTENTIAL OF CRUDE OIL IN SALT MARSHES

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements of the degree of
Master of Science in Civil Engineering

in

The Department of Civil and Environmental Engineering

by
Julius Enock
B. Sc. (Eng.), University of Dar-es-salaam, 1998
August 2002

To my dear wife, Demetria, for being the light of my life, and
to my baby son, Joel, for giving me new eyes to see the world

ACKNOWLEDGEMENTS

The author wish to extend profound appreciation to his Major Professor, Dr. John H. Pardue, for the relentless and intuitive guidance, which helped in sharpening the focus of the research effort. It has been a stimulating and rewarding experience to work with him.

The author is indebted to the members of his advisory committee, Dr. William M. Moe and Dr. Clinton. S. Willson, for accepting to serve in the committee and rendering generously their time and expertise.

The author expresses his gratitude to present and former Wetland Research Group members for the helping hand and sharing moments and experiences. Thanks are due to Gabriel Kassenga, Jason House, Ms Eun-Ju Lee, Dr. Cesar Gomez, Ms Lizhu Lin, Dr. Sangjin Lee, Stephen Mbuligwe and Jeff Maynor.

The author is thankful to Ms Sarah C. Jones, for facilitating the analytical work involving the Spectrophotometer. Also, the author appreciates the service provided by the Graduate School Editor, Ms Susanna Dixon.

The author gratefully acknowledges jointly the USAID (United States Agency for International Development), the Government of the United Republic of Tanzania and the Africa-America Institute (AAI), for the award of the ATLAS (African Training for Leadership and Skills) scholarship. This has provided an opportunity to benefit from the cutting-edge environmental research experience and know-how. Special thanks should go to Ms R. Caldwell, AAI (New York), for the timely service and care. Accordingly, the author would like to thank his employer, the Vice President's Office

(Division of Environment), Tanzania, for facilitating the necessary arrangements prior to the studies.

The author is thankful to his former undergraduate mentors in particular, Dr. Osmund K. Kaunde and Prof. Jamidu H. Y. Katima from the Chemical and Process Engineering Department, University of Dar-es-salaam, Tanzania, who have helped and have been case in point that have inspired his career.

The author extends special thanks to his wife, Demetria, for her unique blend of love and understanding, inspiration, encouragement and support. And to his baby son, Joel, whose presence has been a blessing with an unending joy.

Lastly, but not least, the author is grateful to his parents, Enock and Fausta Moshi, who have loved and supported him through everything.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	iii
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT.....	ix
CHAPTER 1. INTRODUCTION AND OUTLINE.....	1
1.1 Background.....	1
1.2 Research Objectives.....	4
1.3 Environmental Relevance of the Study	4
1.4 Organization of the Thesis	5
CHAPTER 2. INTRINSIC BIODEGRADATION POTENTIAL OF CRUDE OIL IN MARINE SEDIMENTS: A REVIEW.....	6
2.1 Introduction.....	6
2.2 Fractional Composition of Crude Oil.....	7
2.3 Effect of Crude Oil on Microbial Communities.....	8
2.4 The Role of Soluble Organic Carbon.....	9
2.5 Preferential Biodegradation of Crude Oil Fractions.....	10
2.6 Effect of Prior Exposure on Biodegradation Potential.....	10
2.7 Linking Flooding Effect to Microbial Activity.....	12
2.8 Summary and Implications.....	13
CHAPTER 3. EFFECT OF FLOODING ON BIODEGRADATION POTENTIAL OF CRUDE OIL IN A SALT MARSH	14
3.1 Introduction	14
3.2 Materials and Methods	16
3.3 Results	25
3.4 Discussion	28
3.5 Conclusions and Implications.....	37
CHAPTER 4. OIL SPILL RECURRENCE IN A SALT MARSH UNDER NATURAL RECOVERY.....	39
4.1 Introduction.....	39
4.2 Materials and Methods.....	41
4.3 Results.....	48
4.4 Discussion.....	51
4.5 Conclusions and Implications.....	59

CHAPTER 5. SUMMARY AND OUTLOOK	61
5.1 Experimental Findings and Implications.....	61
5.2 Future Research.....	63
LITERATURE CITED	64
APPENDIX	
A. CALIBRATION CURVES	69
B. RESIDUAL HYDROCARBON DEGRADATION DATA	70
C. MICROBIAL ACTIVITY (FDA HYDROLYSIS) DATA	74
D. SOLUBLE ORGANIC CARBON (SOC) DATA	76
E. SAMPLE STEPWISE REGRESSION OUTPUT	78
F. PEARSON CORRELATION RESULTS	80
VITA	82

LIST OF TABLES

2.1	Typical chemical composition and selected physical properties of some crude oil samples.....	8
3.1	Summary results of first-order rate constants for alkanes and PAHs in the flooding study	30
4.1	Summary results of first-order rate constants for alkanes and PAHs in the oil spill recurrence study.....	53
B1	Summary data for the degradation of alkanes for the flooding study.....	72
B2	Summary data for the degradation of PAHs in the flooding study.....	72
B3	Summary data for the degradation of alkanes in the oil spill recurrence study...	73
B4	Summary data for the degradation of PAHs in the oil spill recurrence study	73
C1	Summary data of microbial activity (FDA hydrolysis) for flooding study	74
C2	Summary data of microbial activity (FDA hydrolysis) for the oil spill recurrence study	75
D1	Summary data of SOC analysis for the flooding study.....	76
D2	Summary data of SOC analysis for the oil spill recurrence study	77
F1	The P-values from Pearson correlation for alkane degradation under the IF regime	80
F2	The P-values from Pearson correlation for PAH degradation under the IF regime.....	81
F3	The P-values from Pearson correlation for alkane degradation under the four successive oiling treatment	81
F4	The P-values from Pearson correlation for PAH degradation under the four successive oiling treatment	81

LIST OF FIGURES

3.1	Map of the study site located in the Leeville Oil Field.....	18
3.2	The effect of flooding regime on selected residual petroleum hydrocarbons.....	29
3.3	The effect of flooding on microbial activity in terms of the amount of FDA hydrolyzed.....	31
3.4	The effect of flooding on soluble organic carbon (SOC) content.....	32
4.1	The effect of oil spill recurrence on the residual petroleum hydrocarbons	52
4.2	The effect of oil spill recurrence on microbial activity in terms of the amount of FDA hydrolyzed.....	54
4.3	The effect of oil spill recurrence on soluble organic carbon (SOC) content	55
A1	The calibration curve for FDA analysis using spectrophotometer	69
A2	The calibration curve for SOC analysis using TOC analyzer.....	69
B1	The effect of flooding on degradation of phenanthrene and pyrene	70
B2	The effect of oil spill recurrence on degradation of phenanthrene and pyrene.....	71

ABSTRACT

Understanding the influence of different perturbations on the fate of spilled oil in marine ecosystems is useful in assessing the environmental impact and remedial investigation. The effect of flooding and spill recurrence on the fate of an experimental crude oil spill (2 L/m²) was investigated using salt marsh intact cores, incubated for about 3 months, by monitoring residual petroleum hydrocarbons, heterotrophic microbial activity (fluorescein diacetate assay) and soluble organic carbon (SOC).

For the flooding study, biodegradation rate of crude oil (with half-lives varying between 16.50 and 49.51 days and turnover times between 23.81 and 71.43 days) and microbial activity increased significantly ($P > 0.05$) in the order from continuously-flooded (CF), intermittently-flooded (IF) to non-flooded (NF) regime. The SOC increased significantly ($P > 0.05$) in the opposite order. The results signify the influence of flooding on microbial activity and indirectly affecting biodegradation of crude oil and decomposition and accumulation of organic matter in salt marshes.

For the oil spill recurrence study (single, two, three and four successive oilings; each totaling to 2 L/m²), biodegradation rate of crude oil (with half-lives varying between 11.95 and 69.31 days and turnover times between 17.24 and 100.00 days), microbial activity and SOC increased significantly ($P > 0.05$) with each subsequent oiling. The results suggest that, microbial degradation might not be significant in a pristine tidal marsh particularly immediate to an oil spill event as opposed to a previously contaminated one.

The lack of significant linear relationships ($P > 0.05$) among the parameters measured in both experiments, as indicated by both (forward) stepwise regression and Pearson correlation, reflects the challenge in understanding the complex interaction of environmental factors and microbial ecology in predicting the fate of spilled crude oil in the salt marshes at least under the experimental conditions.

CHAPTER 1

INTRODUCTION AND OUTLINE

1.1 Background

The coastal marshes and estuaries located in the southern United States adjacent to the Gulf of Mexico, account for over 40% of the coastal wetlands of the United States (Mitsch and Gosselink, 1993). These wetlands are remarkably productive ecosystems that provide habitat, breeding, and nursery grounds for fish and wildlife, oil and gas production, protection from shoreline erosion, and serves as a buffer from hurricanes and other storms (Fleury, 2000; Rozas *et al*, 2000).

Among the coastal wetlands are the salt marshes, characterized by saline conditions and emergent vegetation such as *Spartina alterniflora* (smooth cord grass) in areas alternately flooded and drained by tides (Penfound and Hathaway, 1938; Fleury, 2000). Notably, there is eminent threat from oil spills, due to oil shipping tankers after accidents, oil exploration and development activities, rupture or leakage from oil pipelines laid through the ocean, and even natural seeps. Oil is swept into salt marshes by tidal currents and wind and is trapped by marsh grass and the organic-rich sediments.

It is estimated that world annual oil spills into the ocean amounts to about 1.7-8.8 million metric tons, approximately equivalent to about 0.1 to 0.2% of the world annual petroleum production (National Academy of Sciences, 1985; Harayama *et al*, 1999). Worse still, experience from the *Exxon Valdez* oil spill indicates that physical recovery of the spilled oil can hardly manage to reclaim 14%

of the spilled oil (Miller, 1999). Despite many of the crude oil components being biodegradable through numerous microbial processes in the environment, some recalcitrant ones may persist for longer periods in the soil from several years to decades. Crude oil components are of environmental concern due to their toxic, mutagenic, and carcinogenic properties (Nelson-Smith, 1973; Freedman, 1995; Rozas *et al*, 2000). Consequently, crude oil is deleterious to a wide spectrum of marine plants, animals and microbial communities, through oxygen stress (from organic enrichment) and direct toxic effects (mortality) (Carman *et al*, 2000). Generally speaking, oil spills into salt marsh ecosystems imparts potential damage to their physical and ecological integrity even in minimal spill levels let alone catastrophic accidents like the *Exxon Valdez* in 1989 (36,000 tones of crude oil covered approximately 500 kilometers of shoreline) (Miller, 1999). With increasing oil and gas operations along the Louisiana coastal zone (Jackson, 1996) and in view of the ecological and economic benefits of the surrounding marshes and estuaries, then understanding the fate of occasional oil spills into these ecosystems is of significant interest, for their appropriate management in case of an oil spill.

The ultimate fate of oil spills in the marine environment is dictated by a set of biotic and abiotic processes including spreading and drifting, emulsification, evaporation, dissolution, photochemical oxidation and microbial degradation (Nelson-Smith, 1973; Lee, 1980). Previous studies have established that microbial degradation is an important process in determining the fate of spilled oil trapped in coastal marsh sediments, and is estimated to contribute in the removal of as much as 40-80% of the spilled oil (Christian and Wiebe, 1978; Lee, 1980). In addition, salt

marshes are sensitive ecosystems (even foot traffic can cause substantial damage), implying that less intrusive, biodegradation-based remedial alternatives are the suitable option since they present minimum harm to these ecosystems (Jackson, 1996). It follows that, the present work was undertaken to obtain a better understanding of the influence of selected factors on intrinsic biodegradation of crude oil in salt marshes.

Noteworthy, biodegradation of crude oil in the environment occurs at varying rates, depending on numerous physical and biogeochemical perturbations imposed onto these ecosystems. These perturbations include such factors as temperature (Atlas, 1981), sedimentation, wind, precipitation and tidal flooding (Wright *et al*, 1997). The logic follows that, one of the pressing research needs for biodegradation-based oil spill remediation strategies is determining and evaluating the biotic and abiotic factors that influence the fate of the spilled oil and devising ways to accelerate the biodegradation rate.

As it will be revealed in the following three chapters, limited information is available with regard to the effect of tidal flooding and oil spill recurrence on the biodegradation of crude oil in salt marshes. Fundamental to this, oil spills in salt marshes tend to alter the nature and extent of microbial populations and diversity and soil characteristics (Leahy and Colwell, 1990; Nyman, 1999) with potentially important effects on oil-degrading microbial processes. Further, heterotrophic microbes are known to play a major role in organic matter decomposition and assimilation and are of considerable importance in nutrient mineralization (Freedman, 1995; Hunter, 2000). In that perspective, this study sought to examine

the influence of flooding and oil spill recurrence on the biodegradation rate of crude oil, soil heterotrophic microbial activity and water-soluble organic carbon.

1.2 Research Objectives

The intent of this study in relation to the fate of crude oil in salt marshes has been indicated. The specific objectives were to:

- (i) Evaluate the influence of batch-flooding – non-flooded, continuously flooded and intermittently flooded regimes - on the biodegradation rate of crude oil in salt marsh intact cores.
- (ii) Compare the biodegradation rate of crude oil between single and multiple successive oiling of the same total volume (2 L/m²).
- (iii) Evaluate the effect of flooding and oil spill recurrence on selected soil biogeochemical parameters namely, heterotrophic microbial activity and soluble organic carbon, in artificially oil-contaminated salt marsh intact cores
- (iv) To relate intrinsic biodegradation potential of crude oil in salt marshes to microbial activity and soluble organic carbon under the influence of flooding and oil spill recurrence.

1.3 Environmental Relevance of the Study

Crude oil spills in the marine environment is one of the major pollution problems in the US and worldwide. Notably, salt marshes are inaccessible for physical remedial schemes and they are ecologically sensitive areas particularly

when impacted by oil spills and can trap large quantities of oil and therefore, they may provide a challenge in the clean up.

Microbial biodegradation is one of the principal processes for removal of non-volatile crude oil components from oil-contaminated marine sediments. Clearly, environmental restoration from oil spills focuses on the need for environmental benign strategies. Therefore, gaining a better understanding of the factors influencing the biodegradation of spilled oil and soil physico-chemical and biological functions is an important step in the assessment of the environmental impact of oil spills and in developing and/or improving existing biodegradation-based remediation strategies.

1.4 Organization of the Thesis

Chapter 2 reviews selected aspects on the fate of spilled oil in marine sediments. Chapter 3 presents results of the flooding effect on biodegradation of crude oil, heterotrophic microbial activity and soluble organic carbon (SOC). Succeeding Chapter 4, details the results of a study on the influence of oil spill recurrence on biodegradation of crude oil in salt marshes in relation to residual petroleum hydrocarbons, microbial activity and SOC. Following this, Chapter 5 summarises the results from this work and highlights some areas for future research.

CHAPTER 2

INTRINSIC BIODEGRADATION POTENTIAL OF CRUDE OIL IN MARINE SEDIMENTS: A REVIEW

2.1 Introduction

While the amount of annual oil spills in the marine environment is significant, the coastal marshes and estuaries are constantly at risk from oil pollution from accidents, leakage or rupture of oil pipelines, oil and gas exploration, and even natural seeps. The adverse environmental impact of oil contamination in these marine ecosystems can not be overemphasized. As a result, increasing attention is being focused on understanding the fate of oil spills in the environment and the weathering mechanisms (Lee, 1980; Atlas, 1981; Berry *et al*, 1987; Leahy and Colwell, 1990; Harayama *et al*, 1999). The unique features of these coastal marshes such as organic-rich sediments and anoxic conditions favour the accumulation and penetration of oil in the soil. Oil penetration through soil reduces aeration and upsets the carbon/inorganic nutrient balance for the indigenous microbial communities (Riser-Roberts, 1998), which indirectly affects the fate of the oil trapped in the sediments. Although the microbial degradation of petroleum hydrocarbons in the environment is well established (Edwards and Grbic-Galic, 1994; Long *et al*, 1995; Lovley *et al*, 1995; Coates *et al*, 1996; Vroblesky *et al*, 1997; Caldwell *et al*, 1998; Shin, 1998; Phelps and Young, 1999; Nyman, 1999; Pardue *et al*, 2001; El-Tarabily, 2002), however, the importance of indigenous microbial activity and processes has only recently attracted considerable interest due to the increased incidence of major oil accidents.

Since the degradation of trapped oil in marine sediments is mainly mediated by microbes, biodegradation rates are therefore, dependent on the microbial activity and

environmental factors influencing microbial processes (Atlas, 1981; Leahy and Colwell, 1990; Freedman, 1995; Sugai *et al*, 1997). As a result, gaining fundamental knowledge of the interaction of environmental factors and microbial activity may be of interest in an attempt to improve existing oil-spill remediation processes and developing novel ones.

The primary effort of this chapter is to provide a brief overview on the impact of oil spills on microbial activity and functions and the influence of selected environmental and physical factors on the biodegradation of petroleum hydrocarbons in marine sediments.

2.2 Fractional Composition of Crude Oil

Crude oil is a complex mixture of hydrocarbons, varying widely in both physical and chemical properties depending on the source (Atlas, 1981; Leahy and Colwell, 1990). Crude oil may be characterized in terms of four primary fractions, namely saturates, aromatics, resins and asphaltenes with an average density of 850 kg/m³ (Connell and Miller, 1981; Harayama *et al*, 1999). Saturates include straight or branched chain *n*-alkanes, and the cycloalkanes with one or more saturated rings, while aromatics include compounds with one or more fused aromatic rings, each of which may be attached saturated side chains (alkyl-substituents). In contrast to the saturated and aromatic fractions, both the resins and asphaltenes consist of non-hydrocarbon polar compounds, with trace amounts of nitrogen, sulfur and/or oxygen in addition to carbon and hydrogen, and often forming complexes with heavy metals. For the sake of clarity, asphaltenes consist of high-molecular-weight compounds, which are not soluble in a solvent such as *n*-heptane, while resins are *n*-heptane-soluble molecules, principally containing

heterocyclic compounds, acids and sulfoxides (Harayama *et al*, 1999). Typical chemical composition of different crude oils is presented in Table 2.1.

Table 2.1: Typical chemical composition and selected physical properties of some crude oil samples (Connell and Miller, 1981)

Component	% Composition (w/w)	
	“Sweet” Louisiana crude oil	Kuwait
Saturates (<i>n</i> -alkanes and cycloalkanes)	56.30	34.00
Aromatics	35.10	44.60
Resins (polar and insoluble substances)	8.60	21.40
Sulfur	0.25	2.44
Nitrogen	0.069	0.14
Nickel (ppm)	2.2	7.70
Vanadium (ppm)	1.9	28.00
API gravity	34.50 (15.5°C)	31.40 (15.5°C)
Specific gravity	0.8524	0.8686

2.3 Effect of Crude Oil on Microbial Communities

While it is important to understand and identify environmental and other constraints that affect biodegradation of trapped oil in marine sediments and soils, it is necessary to assess and quantify microbial activity and functions of the impacted soils.

Crude oil is known to reduce microbial diversity in marine sediments and enrichment of oil-degrading microbes has been widely reported (Pfaender and Buckley, 1984; Nyman, 1999). Several studies have found little or no effect of oil on abundance of soil microbial community (DeLaune *et al*, 1979; Nyman, 1999) while others have noted adverse effects on oil microorganism populations (Jackson, 1996; El-Tarabily, 2002). Similarly, Li *et al* (1990) found that high levels ($33.3 \text{ g C m}^{-2} \text{ day}^{-1}$) of a mixture of 10 petroleum hydrocarbons inhibited microbial respiration and nutrient re-mineralization in salt marsh soils, but low levels ($3.33 \text{ g C m}^{-2} \text{ day}^{-1}$) of the hydrocarbon mixture stimulated microbial activity.

It can be assumed that, the exposure of microorganisms to petroleum hydrocarbons may be stimulatory, inhibitory or neutral and the degree and duration of the impact is a function of the concentration and chemical composition and environmental factors (Pfaender and Buckley, 1984)

2.4 The Role of Soluble Organic Carbon (SOC)

Heterotrophic microorganisms are important in soil nutrient mineralization and decomposition of organic matter (Nyman, 1999). Noteworthy, the microbial population size and activity in soil is influenced by the quantity and quality of organic matter (i.e. readiness for utilization by microorganisms). Soluble organic carbon (SOC) is one of the labile fractions of dissolved organic matter considered to be a critical carbon and energy source for heterotrophic microbes (Hunter, 2000). Although SOC consists of a mixture of simple substances such as sugars, fatty acid and alkanes and relatively small fraction of complex polymeric molecules, it is well known that not all SOC is labile (Marschner and Bredow, 2002).

Microbial populations in soils rely on organic matter as a source of carbon, other nutrients and growth factors. Therefore, the availability of SOC may be a significant factor in determining the fate of crude oil in marine sediments and it may well compete with petroleum hydrocarbons as a substrate for the oil-degrading microbes. For instance, Hebert *et al* (1993) observed pyrene partitioning to SOC to be significant depending on its concentration in soil solution and its lability. This implies that, the SOC may decrease the aqueous concentration of a sorbed hydrocarbon while providing additional source of carbon and energy to microbes and this may enhance or inhibit degradation rate of the hydrocarbon depending on the importance of one process over the other. However, there is no available information on the competitive metabolism of SOC and petroleum hydrocarbons and this may be worth exploring.

2.5 Preferential Biodegradation of Crude Oil Fractions

Many genera of microbes are able to completely oxidize alkanes and to a lesser extent, aromatic hydrocarbons (Jackson, 1996). Based on previous studies and reviews on biodegradation of petroleum hydrocarbons in the marine environment, several generalizations can be made (Atlas, 1988; Jackson, 1996; Harayama *et al*, 1999):

- Straight chain aliphatic hydrocarbons are easier to be degraded than branched chain aliphatic hydrocarbons.
- Aliphatic hydrocarbons are degraded more easily than aromatic hydrocarbons.
- Saturated hydrocarbons are more easily degraded than unsaturated hydrocarbons.

- Long chain aliphatic hydrocarbons are more easily degraded than short chain (<C₁₀) hydrocarbons, with few exceptions, since the latter are essentially toxic to microorganisms.
- Asphaltenes (and resins) are the most recalcitrant fractions in the crude oil.

2.6 Effect of Prior Exposure on Biodegradation Potential

It is known that prior exposure of petroleum hydrocarbons may result in accelerated biodegradation of the subsequent additions. A brief summary on the effect of prior exposure and microbial adaptation to petroleum hydrocarbons on biodegradation potential is included in the review paper by Leahy and Colwell (1990). Microbial communities adapt to contaminants such as crude oil by enrichment of those microorganisms that are either resistant to the toxic effects of the contaminant, or capable of utilizing the contaminant as a nutrient source (Atlas, 1981). Also, microbial adaptation has been associated with changes in specific metabolic enzymes and genetic alterations resulting in enhanced metabolic capabilities (Leahy and Colwell, 1990).

Since the marine ecosystems are constantly at risk from oil spills, repetitive spills on the same location, due to tidal effects or simply repeated spills, may have a range of effects on the biodegradation potential of crude oil and ultimately on the functional recovery of these ecosystems. However, limited information is available as regards to the influence of spill recurrence especially of complex hydrocarbon mixtures such as crude oil on biodegradation potential in marine sediments and soils.

2.7 Linking Flooding Effect to Microbial Activity

In an ecological context, tidal flooding may be considered as a major physical disturbance that can result in large changes in soil aeration status and sediment biogeochemical characteristics such as redox potential, pH (Gambrell and Patrick Jr., 1979; Hambrick, 1979) and organic matter decomposition (Christian and Wiebe, 1978; Nyman and DeLaune, 1991). As a result, Sugai *et al* (1997) observed that sediment chemistry data alone could not predict the persistence of petroleum hydrocarbons following the *Exxon Valdez* oil spill and emphasized the need for studies of the abiotic and biotic factors influencing biodegradation in the coastal marsh ecosystems.

Flooding is known to be associated with anaerobic conditions (Gambrell and Patrick Jr., 1979; Nyman and DeLaune, 1991). The anaerobic biodegradation of petroleum hydrocarbons in anoxic marine sediments has been reported to occur with ferric iron (Beller *et al*, 1992), nitrate (Berry *et al*, 1987; Rockne *et al*, 2000), sulfate (Berry *et al*, 1987; Beller *et al*, 1992; Lovley *et al*, 1995; Coates *et al*, 1996) and carbon dioxide (Berry *et al*, 1987; Edwards and Grbic-Galic, 1994) acting as alternative electron acceptors to oxygen.

Hambrick (1979) observed that by varying Eh from +130 mV (aerobic) to -220 mV (anaerobic), the degradation of [¹⁴C] naphthalene decreased from 22.6% to only about 0.62% for a period over 35 days. Similarly, Bauer and Capone (1985) observed that anoxic sediments were more sensitive than aerobic sediments to anthracene and naphthalene additions based on d-[U-¹⁴C]glucose metabolic activity and [methyl-³H]thymidine incorporation activity. Both of these studies reflect possible decrease in

microbial population and/or activity under anaerobic conditions, which typically prevails in coastal marine sediments primarily due to flooding.

2.8 Summary and Implications

The environmental impact of oil contamination in coastal marshes and estuaries is potentially serious. The microbial degradation of petroleum hydrocarbons in marine sediments is a rapidly growing research area with focus to develop a better understanding of the fate of spilled oil in marine environment and devising remedial measures that fully utilize the indigenous microbial assimilative capacity.

A great deal of information available recognizes the significance of microbial degradation on the fate of trapped oil in marine sediments and acknowledges the influence of a variety of biotic and abiotic factors. However, the linkage of tidal flooding to overall microbial activity which in turn may influence the intrinsic biodegradation of spilled oil in coastal marsh sediments is not well established. On the other hand, there is an emerging question regarding the impact of oil spill recurrence on biodegradation potential of complex mixtures of petroleum hydrocarbons such as crude oil in marine sediments.

CHAPTER 3

EFFECT OF FLOODING ON BIODEGRADATION POTENTIAL OF CRUDE OIL IN A SALT MARSH

3.1 Introduction

The approximately 12.5 million hectares of salt marshes are a major and important component of the coastal wetlands in the southern United States (Mitsch and Gosselink, 1993). However, these salt marshes are vulnerable to oil spills from a variety of sources including marine vessels after accidents, oil pipelines, oil and gas exploration and natural seeps. Oil is swept into salt marshes by tidal currents and wind and is trapped by marsh grass and organic-rich sediment. By and large, oil spills into coastal marshes and estuaries can present potential damage and disruption of their physical and ecological functions even in minimal spill levels. Salt marshes are known to have a significant inherent capacity to degrade crude oil components (Jackson and Pardue, 1999). However, our ability to utilize the indigenous microbial diversity and genetic versatility for successful application of remedial measures, still relies on our understanding of the interaction between environmental and ecological features within the marine ecosystems and their influence on the fate of spilled oil.

As one of the oil-spill remediation strategies, nutrient (fertilizer) amendment is being advocated and has been demonstrated to enhance oil biodegradation in experimental and actual oil-contaminated marine ecosystems. For instance, Jackson and Pardue (1999) reported the application of ammonium sulfate in Louisiana salt marsh microcosms, doubling the biodegradation rate of crude oil. Similar laboratory results have been widely reported in salt marshes (Wright *et al*, 1997; Shin, 1998), as well as in

other marine ecosystems such as mangroves (Scherrer and Mille, 1990). Also, a number of successful full-scale bioremediation projects have been reported (Bragg *et al*, 1994; Rozas *et al*, 2000) particularly the well known case of *Exxon Valdez*. However, in a recent field trial in a salt marsh (Shin *et al*, 1999) and in some of the mangrove soils (Scherrer and Mille, 1990), the addition of fertilizers did not show statistically significant effect on the biodegradation rate of crude oil components. Among many factors, it was hypothesized that the discrepancy in findings between field and laboratory studies may be attributed to the influence of tidal flooding under field conditions. Tidal flooding may directly or indirectly influence oxygen availability (De and Bose, 1938; Gambrell and Patrick, 1979; Wright *et al*, 1997), and as hypothesized by Shin *et al* (1999), limiting aerobic degradation of the trapped oil in marsh sediments and/or encouraging anaerobic degradation pathway(s) that are not nutrient limited.

Typically, tidal flooding is a dominant physical feature of the salt marshes. Flooding essentially restricts gaseous exchange between soil and air, increases pH, and reduces redox potential (Hambrick, 1979; Nyman and DeLaune, 1991; Taylor III, 1995). As a result, flooding influences the dynamics of nutrient exchange (Taylor III, 1995), sedimentation (Adam, 1990), soil biogeochemical processes (DeLaune *et al*, 1979) and vegetation development (Penfound and Hathaway, 1938; Christian and Wiebe, 1978). This reflects that, tidal flooding is of ecological significance in salt marshes, and therefore may have an important role on the physical and functional recovery of the salt marshes following oil spills.

Limited studies have been undertaken to assess the influence of tidal flooding on the biodegradation of crude oil and associated physico-chemical and biological function

of oil-contaminated marine ecosystems. A handful of nutrient-amendment laboratory-based studies have demonstrated statistically significant effect of flooding on the biodegradation of crude oil but not on the sulfate reduction rate (indirectly linked to crude oil biodegradation) (Shin, 1998) neither on the number of heterotrophic bacteria (Wright *et al*, 1997). Respectively, these results reflect that, other factors besides sulfate reduction process may have contributed to the decrease in biodegradation rate observed and oil pollution may be associated with possible increase in microbial metabolic activity per cell rather than substantial increase in number of bacteria. Therefore, this merit further scrutiny to explore other possible factors that may have contributed in limiting the biodegradation rate of crude oil under flooding conditions.

Evidently, reliable prediction of the fate of spilled oil in coastal marsh sediments requires knowledge on the influence of the various perturbations on both soil geochemical and biological factors. Monitoring and exploring the interplay between these processes is useful in assessing the environmental impact and recovery of oil-contaminated marine ecosystems. In the present work, greenhouse experiments were conducted using salt marsh intact cores growing *Spartina alterniflora* to investigate the effect of batch-flooding on the biodegradation rate of crude oil, soil heterotrophic microbial activity and soluble organic carbon (SOC).

3.2 Materials and Methods

3.2.1 Site Description

The study site is located near Port Fourchon at the southwestern end of the Barataria Basin in Louisiana, as shown in Figure 3.1. While catering to several other business sectors, the primary purpose of the port is to support offshore oil-and-gas

activities throughout the central Gulf of Mexico. The site is situated in the Leeville oil field in the Lafourche Parish at approximately 29°14' 52" N latitude and 90° 12' 27" W longitude. The climate is sub-tropical, with annual temperature averaging 15°C with a mean annual low of 10 °C and a mean annual high of 30 °C. Average yearly precipitation is about 157 cm/year.

The marsh site is flooded with diurnal tides of approximately 0.07-0.67 m in magnitude, which are predominantly influenced by seasonal winds. The marsh site is dominated by uniform stands of *Spartina alterniflora* plants.

3.2.2 Sample Collection

Sediment cores were collected using thin-walled aluminium core tube to minimize compaction and then transferred into 15-cm i.d., 30-cm long thick-walled glass cores before transporting to a greenhouse. Approximately 20-cm long sediment columns were taken between the culms of *Spartina alterniflora*.

3.2.3 Testing Crude Oil

The 'sweet' South Louisiana crude oil (SLCO) was used in the present work. The PAHs content of the SLCO was modified by adding pyrene and phenanthrene by about 0.2 g of each/ mL of crude oil. The modification of the testing oil chemical composition was meant to increase the amount of the selected model PAHs to levels comparable to other crude oil samples, since SLCO has its name 'sweet' for having relatively lower amount of PAHs. Technically, the PAHs represent the more recalcitrant fraction of crude oil, and therefore, this would help evaluate 'fairly' the biodegradation potential of crude oil in the salt marshes.

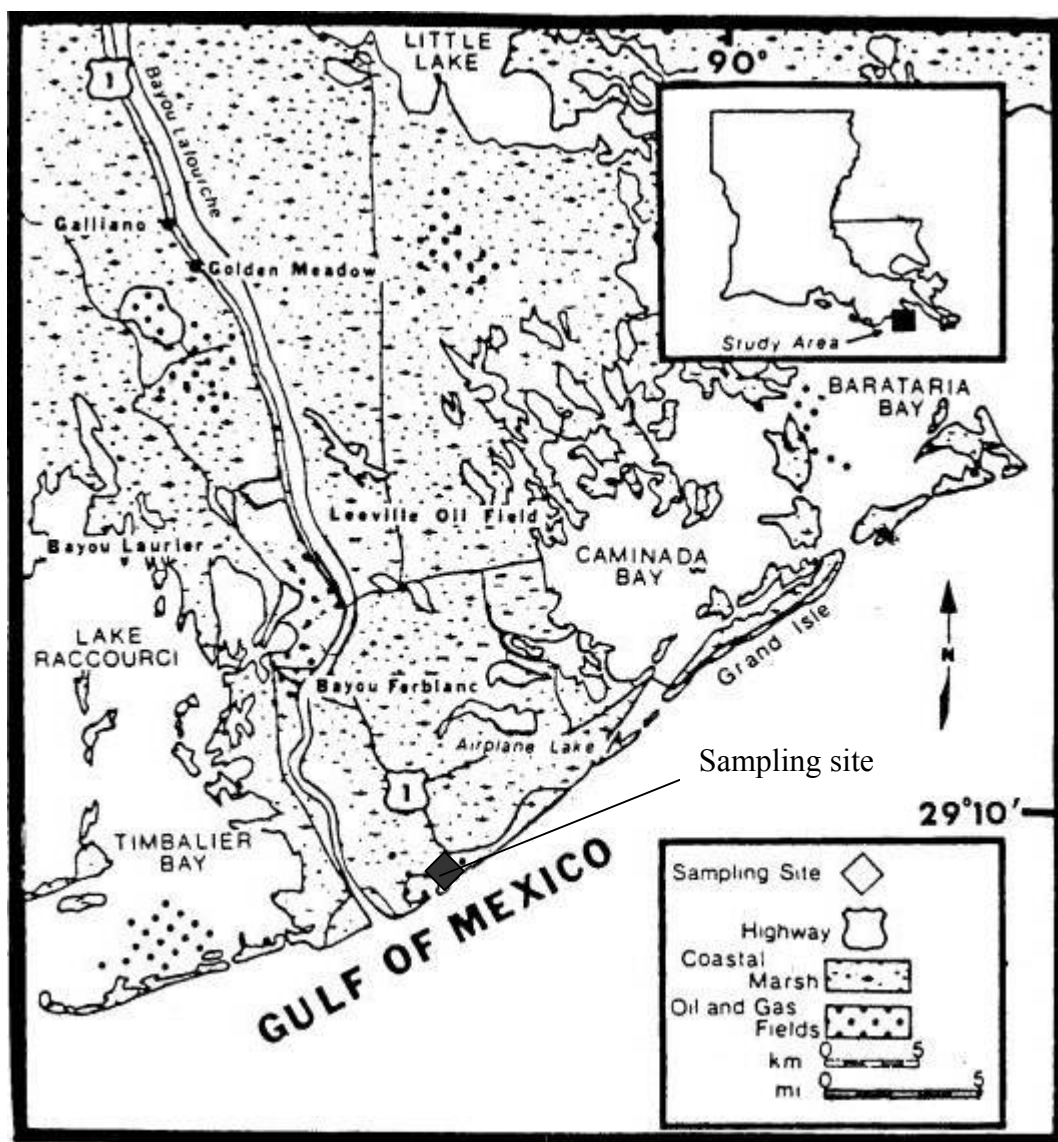


Figure 3.1: Map of the study site located in the Leeville Oil Field

The degradation profiles of phenanthrene and pyrene are presented in Figure B1 (Appendix B). The crude oil was artificially weathered before spiking into the sediment cores by flushing with nitrogen for about 48 hours to minimize the amount of volatile hydrocarbon components so that oil loss due to volatilization is minimized during the biodegradation study. A loss of about 15% of the initial crude oil weight was observed.

3.2.4 Experimentation

Fifteen cores (16-cm diameter x 35-cm long) were placed in a greenhouse and each spiked with 35 mL (2 L/m²) of "sweet" Southern Louisiana Crude Oil (SLCO). Cores were left for 7 days to initiate contact of oil with marsh soil. Crude oil was applied directly to the surface of each core using a pipette. The air temperature of the greenhouse was 22 ± 4°C during the experimental work. The cores were wrapped in aluminium foil to prevent algae from growing below the soil surface on the sides of the cores. The cores were subjected, in triplicate, to continuously-flooded (CF), intermittently-flooded (IF), non-flooded (NF) regimes and control with no oil. The CF cores were flooded with sea-water for the whole period of the experiments, having approximately a 10-cm deep-water column. The IF cores were flooded with sea-water for 2 days and drained for 2 days, alternately. The water was drained from the cores by siphoning with a small diameter tube. The NF cores were left with water just flush with the sediment surface, only enough to have the cores saturated. Water evaporation from both cores was compensated by adding sea-water. Samples were taken after every 20 days.

Sediment samples were taken from the intact cores by scooping with a knife approximately 5-cm deep, removing sediment sample weighing about 30-g. Then, the sampled cavities in the cores were refilled with sand and marked to prevent resampling in the same location. Care was taken not to sample subsequent samples too close from previously sampled spots. Each sample was homogenized by manually mixing and cutting with a serrated knife.

3.2.5 Extraction and Analysis of Crude Oil from Core Sediments

A 4-g sub-sample was apportioned from each sample collected from the intact greenhouse cores. The 4-g sub-sample was placed in a Teflon tube, to limit adsorption of any petroleum fraction, and 20 mL of a hexane: acetone solvent mixture (50/50 v/v%) was added and the solution incubated on a shaker for 48 hours. After 48 hours, the suspension was centrifuged at 3,000 rpm for about 20 minutes at room temperature. The supernatant was transferred into a separatory funnel.

Using the separatory funnel, the petroleum-laden solvent was decanted into scintillation vials, through sodium sulfate, to remove any remaining traces of water. The petroleum-laden solvent was then evaporated to 5 mL using nitrogen gas to minimize further oxidation. The samples were stored at 5°C until GC-MS analysis was performed.

Preparation for the GC-MS analysis included transferring 1-mL from the scintillation vial into an amber GC-MS vial and adding 10- μ L of internal standard (2000 μ g/mL in methylene chloride containing the following components: 1,4-dichlorobenzene- d_4 , naphthalene- d_8 , acenaphthene- d_{10} , phenanthrene- d_{10} , chrysene- d_{12} and perylene- d_{12}) (SUPELCO Chemical Co.). The sample was then analyzed on GC-MS using 17 α (H), 21 β (H)-hopane as a normalizing compound (i.e. ratio of compound to hopane concentration), allowing only biodegradation to be monitored.

The analysis of the extracted hydrocarbon analytes was patterned after the US EPA Method 8270 using GC-MS. A GC-MS (Hewlett Packard 5890 Series II Plus) was utilized to analyze the samples for selected petroleum hydrocarbon components. The HP 5890 was outfitted with a HP-5 high-resolution capillary column (30-m x 0.250-mm i.d., 0.25- μ m film thickness) which was directly interfaced to a quadrupole mass spectrometer

(HP 5972 Mass Selective Detector). The carrier gas was high purity helium at flow rate of 1.0 mL/min, the injector temperature was 300 °C, and the column temperature was 300°C. The column temperature was programmed from 55 to 310°C at 8°C/min rate with initial 3 minutes delay and 15 minutes hold at the end. The interface to the mass selective detector was maintained at 280 °C.

Prior to sample analysis, a five-point calibration was established to demonstrate the linear range of the analysis and to determine the relative response factors for individual compounds.

Degradation data for alkanes and PAHs under the influence of flooding are presented in Table B1 and B2 (Appendix B) respectively. The degradation data were fitted using non-linear regression to the following first order kinetic equation:

$$\frac{C}{C_0} = e^{-kt} \quad (3.1)$$

where C = substrate's hopane ratio

C₀= initial substrate's hopane ratio

k = first order rate constant (day⁻¹)

t = time (days)

The half-lives of the crude oil fractions (i.e. alkanes (C₁₀-C₃₆) and PAHs) were determined using the following relation for first-order kinetics:

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \quad (3.2)$$

where $t_{1/2}$ = half life (days)

k = first order rate constant, (day^{-1})

The turnover times were determined using the following relationship:

$$\text{Turnover time (days)} = \frac{1}{k} \quad (3.3)$$

where k = first order rate constant (day^{-1})

3.2.6 Microbial Activity Analysis: Fluorescein Diacetate (FDA) Assay

Fluorescein Diacetate (FDA) assay was used to quantify microbial activity in the oil-contaminated sediment intact cores. The FDA assay has been used to measure total heterotrophic soil microbial activity in a variety of ecosystems (Hunter, 2000; El-Tarabily, 2002). The FDA assay does not quantify microbial biomass, but it is useful for comparing microbial hydrolytic activity in similar soil ecosystems (Schnurer and Rosswall, 1982).

The determination of FDA consists of incubating a soil sample in a buffer solution in the presence of FDA, which acts as an electron acceptor that is reduced to a coloured fluorescein, and the colour intensity is determined spectrophotometrically. The amount of absorbance of fluorescein is indicative of the hydrolytic activity of the heterotrophic microbial population within the soil sample. To obtain a constant production rate of fluorescein and to avoid extensive growth of microorganisms, a short incubation time of 1 hour is commonly used. Also, phosphate buffers are used to minimize the influence of pH which exerts a significant effect on FDA hydrolytic activity.

An FDA standard solution was made by dissolving 0.0399 g FDA in acetone and bringing the volume to 100 mL. Standards were made by adding 50 mL phosphate buffer and 10 g of each set of soil samples to each of seven flasks and then adding 0, 0.1, 0.2, 0.3, 0.5, 1.0 and 1.5 mL of fluorescein standard to the flasks. The resulting solutions contained the equivalent of 0, 50, 100, 150, 250, 500 and 750 μg FDA converted to fluorescein/flask. Standards were incubated on a rotary shaker (120 rpm) for 1 hour and then 50 mL of acetone added. The solution was centrifuged for 10 minutes at 6000 rpm, filtered and filtrate absorbance values were measured spectrophotometrically (SHIMADZU UV-1201, 1-cm path length) at 490 nm. The absorbance values were plotted to obtain a regression equation as presented in Figure A1 (Appendix A).

An FDA stock solution was made by dissolving 0.200 g fluorescein diacetate (ALDRICH® Chemical Co.) in acetone and bringing the volume to 100 mL with deionized water. Ten grams of soil was weighed and placed in a Teflon tube. Then, 50 mL 0.1 M sodium phosphate buffer (pH 7.6) and 0.5 mL FDA stock solution was added and the tube capped and incubated on a rotary shaker at 120 rpm for 1 hour. After one hour, 50 mL acetone was added to terminate the FDA hydrolysis reaction. The solution was swirled by hand and 40 mL decanted into a centrifuge tube. The solution was centrifuged for about 10 minutes at 6000 rpm and then filtered (using 0.45 μm polysulfone membrane filters) into scintillation vials and finally the filtrate absorbance was measured spectrophotometrically (SHIMADZU UV-1201, 1-cm path length cell) at 490 nm. Absorbance values were converted to μg fluorescein produced/g soil/ hour by using a standard absorbance curve created from a random selected oiled intact core before the start of flooding.

3.2.7 Soluble Organic Carbon Analysis

One hundred mL of de-ionized water was added to 10-g moist soil sample and then the solution was incubated on a shaker at 120 rpm for about 1 hour and allowed to stand for approximately 18 hours (overnight). The solution was shaken by hand and 40 mL was poured into a centrifuge tube and centrifuged at 6000 rpm for 10 minutes. Twenty mL of the supernatant was filtered through 0.45 µm polysulfone membrane filter into a scintillation vial and refrigerated at 4 °C prior to analysis.

The four-point calibration of the TOC analyzer for SOC analysis was performed using Potassium hydrogen phthalate (C₈H₅O₄K) (SIGMA Chemical Co.). The calibration curve is presented in Figure A2 (Appendix A).

Samples were analyzed for non-purgable organic carbon using a Total Carbon Organic Analyzer (SHIMADZU TOC-5050A). Non-purgable organic carbon concentration in each sample was measured by acidifying the sample with 40 µL of HCl and then purging for 8 minutes with TOC grade compressed air. Acidification reduces inorganic carbon to primarily CO₂ in these samples and purging volatilizes CO₂ out of solution. Samples were then analyzed for soluble organic carbon (SOC) concentration. Results were corrected for soil moisture so that the final results were expressed as mg SOC/g soil on a dry weight basis.

3.2.8 Statistical Analyses

In both experiments three replicates per treatment were used. Data were analyzed using SIGMASTAT[®] version 1.0. One way Analysis of variance (ANOVA) were performed at the significance level of 5% to detect significant differences among the

flooding regimes. Both stepwise regression and Pearson correlation techniques were used to determine significant linear relationships among the parameters measured.

3.3 Results

3.3.1 Biodegradation of Alkanes and PAHs

The residual alkane and PAH concentration profiles over time relative to hopane were used to account for biodegradation and were used to detect statistically significant effects of flooding regime. Degradation profiles for both alkanes (C₁₀-C₃₆) and PAHs are presented in Figure 3.2 while the degradation data are presented in Table B1 and B2 (Appendix B) respectively. The alkanes (*n*-C₁₀ to *n*-C₃₆) decreased by 94.6%, 92.4%, and 90.9% in the NF, IF and CF regime, respectively whereas PAHs decreased by 87.8%, 78.6% and 75.6% in the NF, IF and CF regime, respectively. This demonstrates that some biodegradation of the crude oil was occurring.

The degradation data were fitted to both zero-order and first-order kinetics to confirm the common practice of using the first-order kinetics in fitting oil degradation data, however, only the results for the former are presented in detail. This is because first-order kinetics was found to fit the data better based on the correlation of coefficient (R²)

and some form of the coefficient of variation determined as $\left(\frac{\text{Standard Error}}{\text{Rate constant } (k)} \right) \times 100$.

For the different flooding regimes, the zero-order kinetics had numerically lower R² values (from 0.82 to 0.94) for alkanes though statistically comparable (paired t-test; P = 0.15) to those of first-order kinetics (from 0.96 to 0.99). Similarly, zero-order kinetics had numerically higher coefficient of variation values (from 14.21% to 27.17%) for

alkanes though statistically comparable (paired t-test; $P=0.210$) to those of first-order kinetics (from 4.76% to 14.81%).

The comparison of the PAHs indicated zero-order kinetics having statistically comparable (paired t-test; $P=0.184$) R^2 values (from 0.96 to 0.98) from those of first-order kinetics (from 0.92 to 0.97). However, zero-order kinetics had numerically lower coefficient of variation values (7.94% to 11.17%) though comparable (paired t-test; $P=0.057$) to those of first-order kinetics (from 11.11% to 17.39%).

From the above results, on the basis of R^2 values and coefficients of variation, it was concluded that the degradation data were better fitting first-order kinetics.

No significant differences in biodegradation of crude oil were detected for both among the flooding regimes except for CF regime against NF regime in terms of both total alkanes ($P=0.005$) and total PAHs ($P=0.02$). The first order rate constants and other statistical results are summarized in Table 3.1. The rate constants for NF regime were significantly greater than the IF regime which are greater than CF cores. The results corresponds to half-lives of 16.50, 20.39, 25.67 days for alkanes as compared to 30.14, 38.51 and 49.51 days for PAHs. Further, the degradation results correspond to turnover times of 23.81, 47.62 and 62.50 for alkanes ($n-C_{10}$ to $n-C_{36}$) in comparison to 43.48, 55.56 and 71.43 days for PAHs. However, no significant differences ($P = 0.095$) were detected among replicate cores for the same flooding regime. This reflects a satisfactory reproducibility with individual treatments.

Also, it was determined from Figure 3.2 that, beyond day 60, there were no significant differences in both residual alkanes ($P=0.933$) and PAHs ($P=0.933$) among the flooding regimes.

3.3.2 Microbial Activity

The FDA hydrolytic activity assay was used to measure microbial activity, determined as the rate of hydrolysis of FDA to fluorescein which was detected using spectrophotometer at a wavelength of 490 nm. The time profile of microbial activity (the amount of FDA hydrolyzed) and mean values for each flooding regime are presented in Figure 3.3 while the data are presented in Table C1 (Appendix C). The mean FDA hydrolyzed was about 15.43, 18.65, 22.67 and 24.73 μg FDA hydrolyzed/g dry soil/hr for control, CF cores, IF cores and NF cores respectively. The amount of FDA hydrolyzed in NF cores were found to range from 1.2 to 1.7 times those in CF cores. No significant differences ($P > 0.05$) were detected in microbial activity among the flooding regimes with the exception of NF cores against the control (with no oil with intermittent flooding to mimic the tidal flooding) ($P = 0.0393$).

3.3.3 Soluble Organic Carbon

The time profile of SOC under the influence of flooding is presented in Figure 3.4 while the data are presented in Table D1 (Appendix D). The mean SOC values were found to be 0.98, 1.38, 1.09 and 2.7 mg C/ g-oven-dry-soil for control, NF regime, IF regime and CF regime respectively. The values of SOC for CF regime were consistently higher than NF regime ranging from about 1.2 to 4 times. No significant differences were detected among the flooding regimes, with the exception of IF regime against the control and NF regime ($P = 2.62 \times 10^{-4}$).

3.3.4 Regression and Correlation of Measured Parameters

The stepwise regression and Pearson correlation techniques were performed to establish relationships between hydrocarbon hopane ratio, SOC concentration and microbial activity (amount of FDA hydrolyzed).

A sample output for stepwise regression is presented in Appendix E while the P-values for Pearson correlation are presented in Table F1 and F2 (Appendix F) for alkanes and PAHs respectively. The analysis utilized data from the IF regime only since this regime mimic the tidal flooding typically experienced by salt marshes. Both stepwise regression and Pearson correlation techniques revealed no significant linear relationships ($P > 0.05$) among these variables for both alkanes and PAHs. This suggests complex interaction between soil geochemical and microbiological properties in determining the significance of biodegradation of crude oil in salt marshes at least under the experimental conditions.

3.4 Discussion

Significant differences were detected in biodegradation of crude oil fractions among the flooding regimes. The non-flooded (NF) regime had the highest inherent biodegradation rates compared to intermittently-flooded (IF) and continuously-flooded (CF) regime, in that order. These results contradict those previously reported by Wright *et al* (1997), in which it was reported that the CF regime had higher inherent biodegradation rate than the IF regime. The discrepancy between the two studies may be attributed to nutrient amendment used in the latter study, which may have lead to difference in microbial diversity and associated metabolic activity.

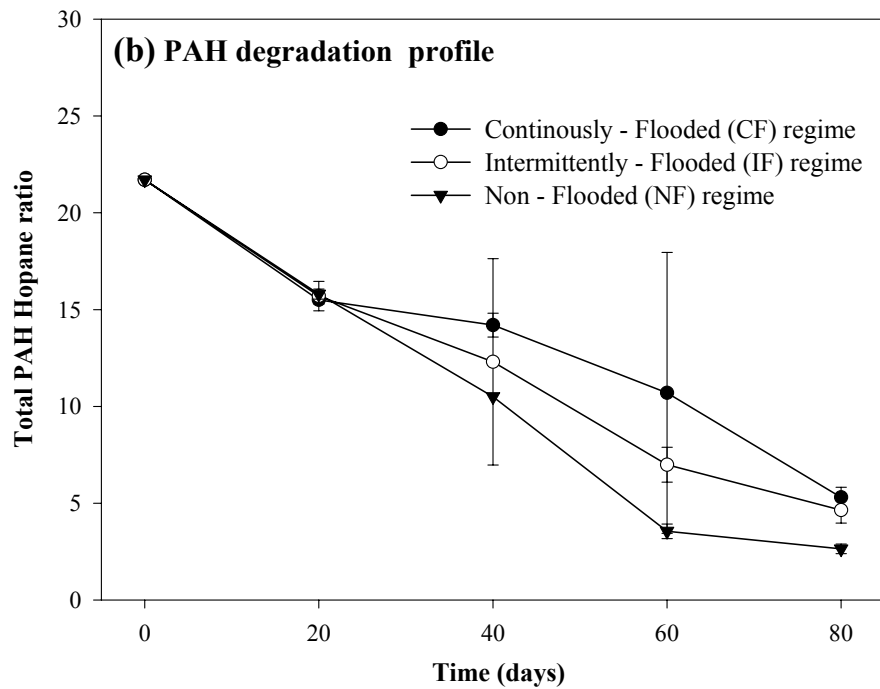
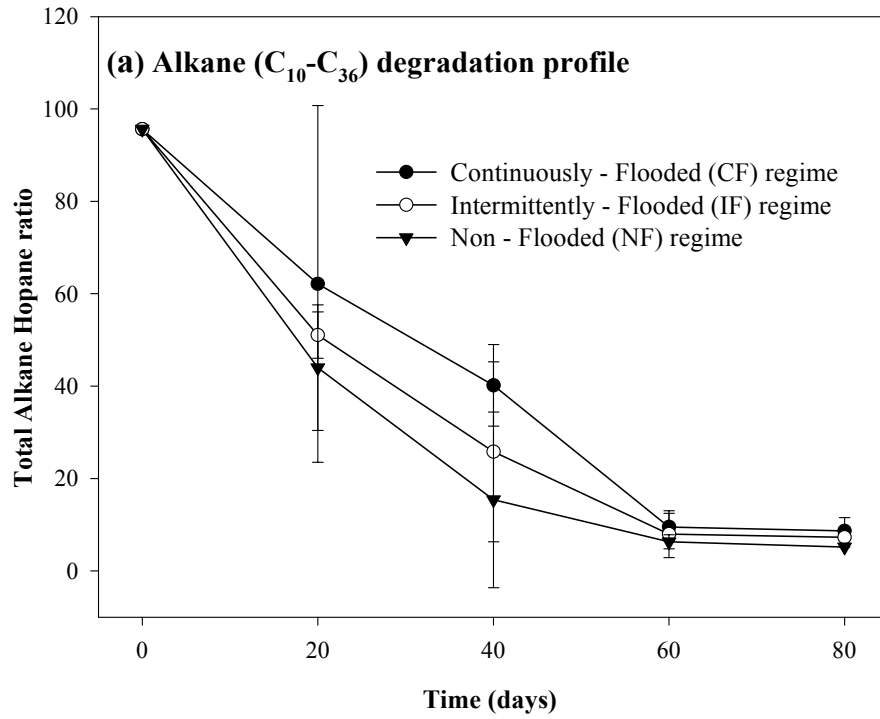


Figure 3.2: The effect of flooding regime on selected residual petroleum hydrocarbons

Table 3.1: Summary results of first-order rate constants for alkanes and PAHs under the influence of flooding

Treatment	Alkanes (C ₁₀ -C ₃₆)					PAHs				
	k (day ⁻¹)	Std error	t _{1/2} (days)	Turnover time (days)	R ²	k (day ⁻¹)	Std error	t _{1/2} (days)	Turnover time (days)	R ²
Non-flooded (NF)	0.042	0.002	16.50	23.81	0.99	0.023	0.004	30.14	43.48	0.95
Intermittently-flooded (IF)	0.021	0.002	33.01	47.62	0.99	0.018	0.002	38.51	55.56	0.97
Continuously-flooded (CF)	0.016	0.004	43.32	62.50	0.96	0.014	0.002	49.51	71.43	0.92

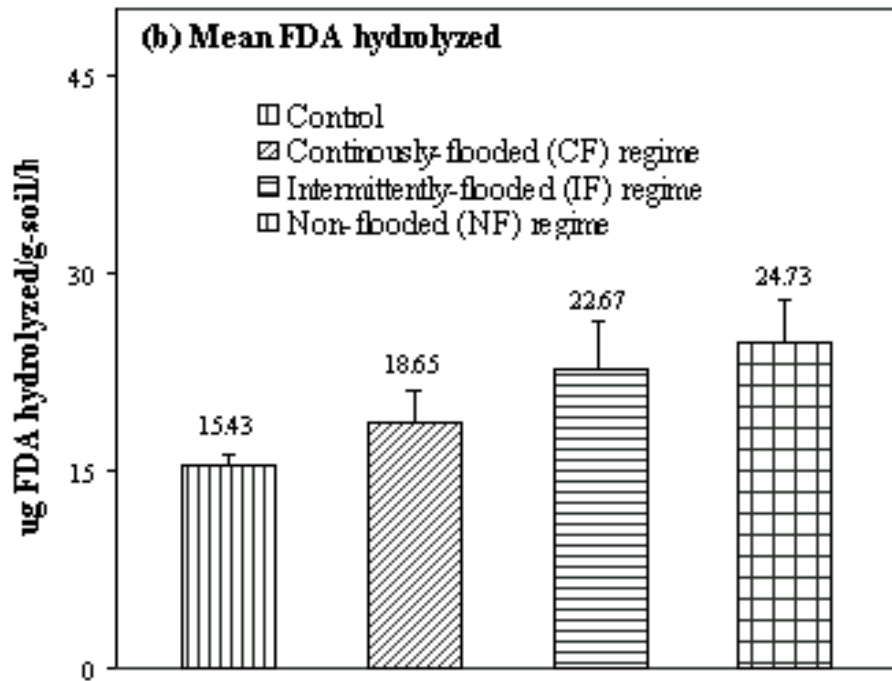
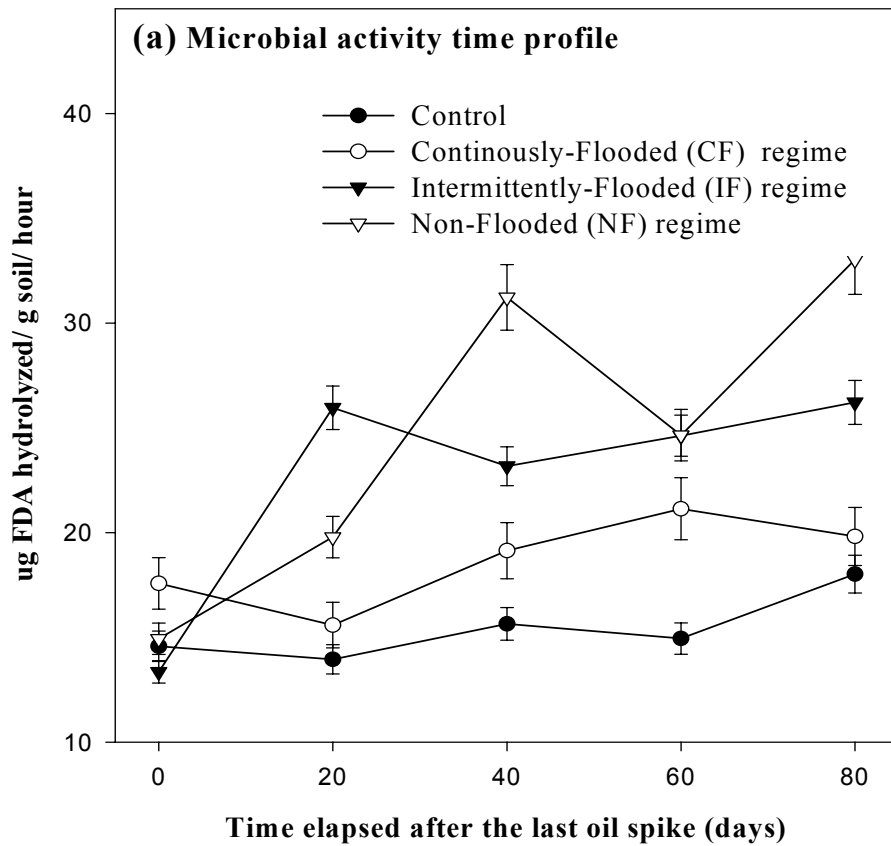


Figure 3.3: The effect of flooding on microbial activity in terms of the amount of FDA hydrolyzed

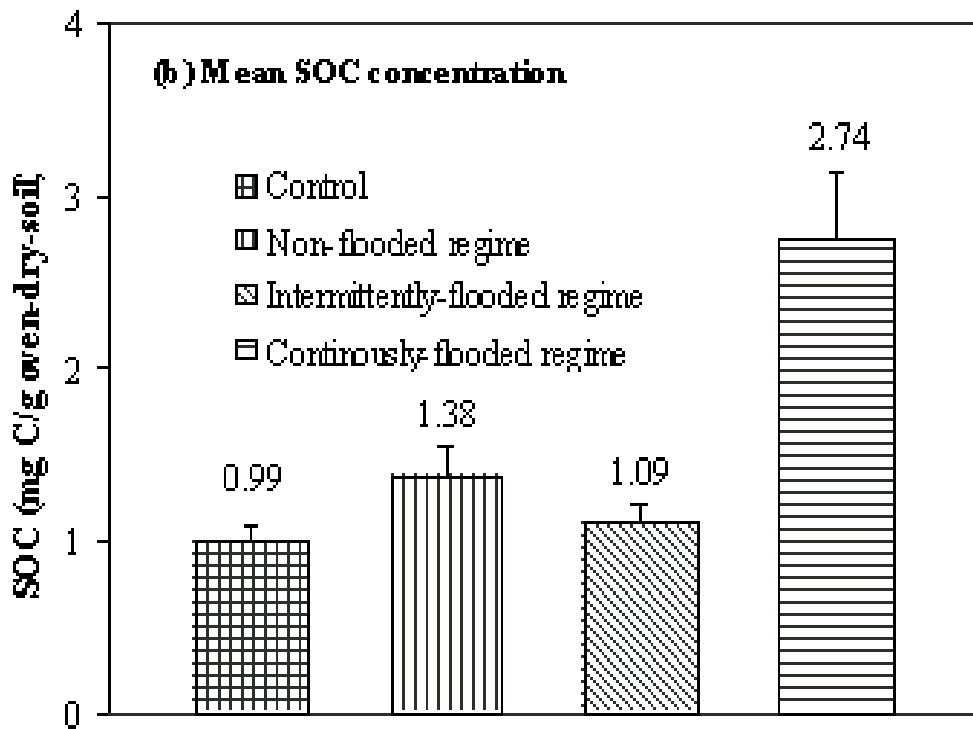
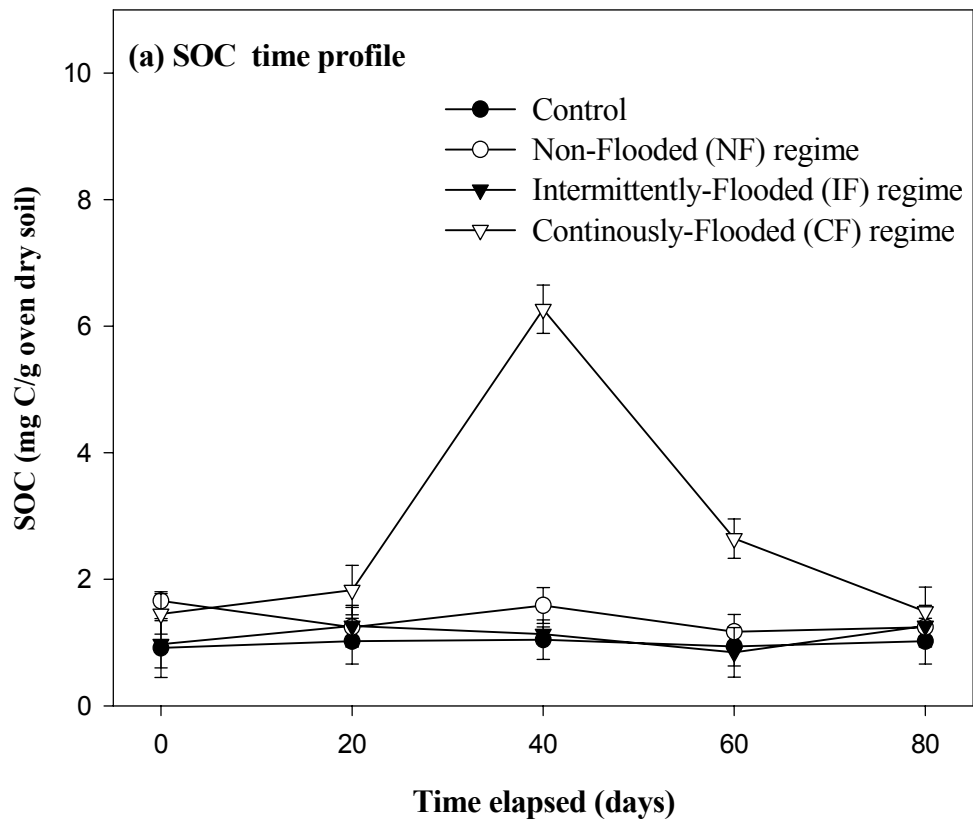


Figure 3.4: The effect of flooding on soluble organic carbon (SOC) content

Since there was poor correlation between SOC concentration against soil microbial activity and biodegradation of crude oil, this suggests that other factors could account for the lower biodegradation observed under flooding conditions possibly oxygen limitation. It is known that, tidal flooding in salt marshes may prevent oxygen from diffusing to the soil, increase nutrient inputs, dilute salinities and may well affect the soil pH (Taylor III, 1995). Lack of oxygen to support aerobic metabolism is probably one of the important factors that influences microbial activity and this may affect biodegradation of crude oil under flooding conditions. This is based on the assumption that the NF cores had higher oxygen availability (redox potential) due to more oxygen being able to reach the soil, which is more exposed to the air as opposed to IF cores and CF cores characterized by a water column over the soil surface. The highest redox potentials in wetland soils are found in the top 0-2 cm layer (Taylor III, 1995; Shin, 1998), therefore, this is the location where oil biodegradation is expected to occur faster, but less oxygen is available during a flooding event. It is estimated that, with water as the oxygen carrier from the air to the soil, air will supply about 8 mg O₂/L of water and about 400 kg of water would be required to degrade 1 g of hydrocarbon (Riser-Roberts, 1998). Alternatively, Johnston (1970) estimated that amounts of oil greater than 100 g/m² (with about 2000 g oil/m² in the present work) would initiate the onset of anaerobic conditions. This indicates that oxygen might have been limited during flooding of the intact cores, with anaerobic conditions prevailing.

The preceding discussion introduces the role of anaerobic biodegradation of crude oil in the natural recovery of oil-contaminated salt marsh intact cores. The significance of the anaerobic degradation of petroleum hydrocarbons was previously thought to be slow

as to be negligible or not to occur at all (Shelton and Hunter, 1975) since oxygen was assumed to naturally diffuse from the atmosphere into the soil during periods of low tides. However, several studies have established the significance of anaerobic biodegradation of petroleum hydrocarbons (Hambrick, 1979; Berry *et al*, 1987; Coates *et al*, 1996; Phelps and Young, 1999; Rockne *et al*, 2000). In a recent study, Pardue *et al* (2001) observed increase in sediment oxygen demand (from 2,000 to 11,000 mg O₂/m²-day) and sulfate reduction rate (indicator of anaerobic conditions in salt marshes) (from ~2000 to 4,000 mg SO₄²⁻/m²-day) following an experimental crude oil spill (1.42 L/m²). This demonstrates the importance of both aerobic and anaerobic processes during natural recovery of an oiled salt marsh. Therefore, the degradation of the crude oil components, can no longer be considered a defining characteristic of aerobic biodegradation processes alone (Caldwell *et al*, 1998), and particularly so in the coastal marshes, with the spilled oil trapped in the essentially anoxic sediments.

The higher microbial activity and biodegradation of crude oil in NF regime may be related to possible difference in microbial population among the flooding regimes as suggested by the FDA assay. A case in point, De and Bose (1938) found that bacterial and fungal numbers were markedly reduced on flooding rice soil and that the decrease became more pronounced with time under laboratory conditions. This may be explained of the fact that in non-flooded soils, a wide range of microorganisms assisted by the microfauna of the soil participates in organic matter and nutrient mineralization as opposed to flooded soils involving mostly anaerobic bacteria (Christian and Wiebe, 1978). Therefore, the processes of both decomposition and assimilation of organic matter and nutrient mobilization in flooded soils are comparatively much slower. In addition,

decomposition of organic matter and nutrient mobilization in flooded soils differ from those in non-flooded soils due to differences in the decomposition end-products (Gambrell and Patrick Jr., 1979). Typically, ethanol and hydrogen sulfide are commonly produced in flooded marsh soils (Riser-Roberts, 1998) which can be toxic to some microbial species, resulting in slowed overall microbial activity.

Further, higher biodegradation rate and microbial activity in NF cores may have been facilitated by attachment of the petroleum hydrocarbons onto the soil sediments which assist their availability to microbes (de Jonge *et al*, 1997; Carlsson, 1998). The adsorption of crude oil components onto the salt marsh sediments may be of significance as it has been observed that generally soil-attached bacteria are 2-3 times more metabolically active than the freely-suspended water column bacteria (Nyman, 1999). Though not well understood, it has been proposed that once bacteria become attached, microcolonies and associated extracellular material grow on the particle surface forming a biofilm.

The inherent lower biodegradation rate of crude oil in CF cores may as well be ascribed to changes in soil biogeochemistry such as sediment-water mineral exchange. In unstirred flooded sediment cores spiked with crude oil (0-30 L/m²) over 5-cm overlying water column, DeLaune *et al* (1979) observed the release of iron, manganese and ammonium ions from salt marsh sediments to the overlying water column possibly due to lack of oxygen because of the oil barrier on the water surface. In addition, it is believed that reduced soils and sediments result in different degradative microbial populations and breakdown pathways of organic pollutants and possibly different behavior in adsorption to soil and sediment solids (Gambrell and Patrick Jr., 1979).

The decomposition of organic matter was monitored by measuring the soluble organic carbon (SOC) concentration. The SOC represents the fraction of organic compounds in a soil matrix that is water soluble at room temperature and is mainly comprised of sugars, amino acids, fulvic and humic acids, that are readily utilized by microbes (Hunter, 2000). Significant differences were observed in SOC values among flooding regime, with comparatively higher SOC values observed in CF cores. However, it was hypothesized that lower amounts of SOC would exist in CF regime as compared to NF regime since SOC production is dependent on microbial population and metabolic activity in the soil. In support of this hypothesis, the rate of decomposition of organic matter in flooded soils is generally estimated to be only about half that in non-flooded soil (Riser-Roberts, 1998). Therefore, the observation made in this study raises an important question: why did CF cores contain comparatively higher SOC level than other flooding regimes despite showing lower microbial activity? This may be attributed to changes in solution chemistry or starvation and lysis of microbes possibly due to substrate depletion which can contribute to SOC release from the sediments (Marschner and Bredow, 2002).

Alternatively, the relatively higher SOC content observed in the CF regime may be related to distribution and inherent microbial activity between sediment-attached and freely-suspended microbial population. As a guideline, Bekins *et al* (1999) determined that in an anaerobic portion of an aquifer contaminated by crude oil, only about 15% of the total microbial population were freely-suspended. In addition, it is known that microbes attached to soil sediments are associated with higher growth rates as compared to those in the water column since nutrients tend to concentrate at sediment surfaces

(Carlsson, 1998), therefore, resulting into accumulation of SOC. Otherwise, the relatively high SOC concentration in CF regime can be interpreted as a result of lower consumption of the produced SOC supposedly due to lower microbial activity as indicated by the fluorescein diacetate (FDA) assay.

3.5 Conclusions and Implications

Tidal flooding is of ecological significance in salt marshes, and therefore may have an important role on the physical and functional recovery of the salt marshes following oil spills. The effect of batch-flooding - continuously-flooded (CF), intermittently-flooded (IF) and non-flooded (NF) regimes - was investigated using salt marshes intact cores growing *Spartina Alterniflora* spiked with south Louisiana crude oil (2 L/m²). Residual petroleum hydrocarbons, heterotrophic microbial activity and soluble organic carbon (SOC) were monitored for about 3 months.

The biodegradation rate of both alkanes (n -C₁₀ to n -C₃₆) and PAHs and microbial activity essentially increased in the order from the CF regime, IF regime to NF regime. The SOC concentration increased significantly ($P < 0.05$) in the opposite order. Both stepwise regression and Pearson correlation revealed no significant linear relationships ($P > 0.05$) among the parameters investigated, suggesting for complex interaction among the measured parameters in predicting the fate of spilled oil in salt marshes at least under the experimental conditions.

The results from this work suggest that the pattern and frequency of flooding markedly influenced the biodegradation of crude oil, heterotrophic microbial activity and SOC concentration. This reflects that tidal flooding is not only important in terms of ecological function of salt marshes but also has a key role in determining the nature and

level of microbial activity and indirectly affects the biodegradation of spilled oil and decomposition and accumulation of organic matter.

CHAPTER 4

OIL SPILL RECURRENCE IN A SALT MARSH UNDER NATURAL RECOVERY

4.1 Introduction

Crude oil spills in the marine environment is one of the major pollution problems in the United States and worldwide (Jackson, 1996; Shin, 1998; Wright *et al*, 1997). It is estimated that world annual oil spills into the ocean amounts in the range between 1.7 and 8.8 million metric tons, equivalent to about 0.1 to 0.2 % of the world annual petroleum production (National Academy of Sciences, 1985; Harayama *et al*, 1999). Oil spills involve numerous spill sources including oil-shipping tankers after accidents, oil exploration and development activities, rupture or leakage from oil pipelines laid through the ocean, and even natural seeps. Oil is swept into salt marshes by tidal currents and wind and is trapped by marsh grass and the organic-rich sediments. The intermittent nature of the tidal flooding reflects the potential for repetitive spillage of the salt marshes and the consequences in terms of biodegradation potential can be important in assessing the environmental impact. Comparatively, much less is known as to the intrinsic biodegradation potential of complex mixture of petroleum hydrocarbons such as crude oil in marine such as the salt marshes following oil spill recurrence.

A vast majority of available literature suggest that, the fate of petroleum hydrocarbons in the environment is dependent on the characteristics and pollution history of the sediments (Leahy and Colwell, 1990; Freedman, 1995). For instance, Hayes *et al* (1999) observed that naphthalene and phenanthrene were oxidized without a lag phase in marine harbor sediments that were previously contaminated with petroleum while pristine

sediments showed no significant degradation. In a parallel effort, Phelps and Young (1999) examined the extent of biodegradation of BTEX (benzene, toluene, ethylbenzene, and xylenes) as a mixture and from gasoline in pristine and previously polluted marine harbor sediments. Similarly, higher biodegradation rates were observed for the previously polluted sediments. On the other hand, Edwards and Grbic-Galic (1994) observed inhibition of degradation of toluene and *o*-xylene under anaerobic conditions (lag phase ranging between 100-255 days). This was presumed to be due to presence of natural organic substrates or contaminants in the sediments that were collected from historically-known contaminated sites. Reflecting on these studies, a question emerges about the effect of oil spill recurrence on the natural restorative ability of the salt marshes especially of a complex mixture of petroleum hydrocarbons such as crude oil.

There are a handful of laboratory-based studies that have assessed the effect of repeated application of crude oil in salt marshes with emphasis on recovery of vegetative plants (Baker, 1973; Li *et al*, 1990) and microbial activity in terms of CO₂ production (total respiration), acetylene reduction activity, denitrification and methanogenesis (Li *et al*, 1990). Noteworthy, the focus of these studies was to determine the levels of oil that can impair the selected marsh ecosystem receptors, therefore, different amounts of crude oil were compared among the treatments. That is to say, these results can only provide qualitative information as to the biodegradation potential of crude oil in salt marshes following the oil spill recurrence.

While microbial degradation is an important fate process for the trapped oil in salt marsh sediments, it is affected by soil chemical, physical and biological factors. In that perspective, the present work examined residual petroleum hydrocarbons, soil

heterotrophic microbial activity and soluble organic carbon to determine the influence of oil spill recurrence on the fate of an experimental crude oil spill (2 L/m²) in salt marsh intact cores. Specifically, single oiling was compared to multiple successive oiling for the same total volume of crude oil.

4.2 Materials and Methods

4.2.1 Site Description

The study site is located near Port Fourchon at the southwestern end of the Barataria Basin in Louisiana. While catering to several other business sectors, the primary purpose of the port is to support offshore oil and gas activities throughout the central Gulf of Mexico. The site is situated in the Leeville oil field in the Lafourche Parish at approximately 29°14' 52" N latitude and 90° 12' 27" W longitude. The climate is sub-tropical, with annual temperature averaging 15°C with a mean annual low of 10 °C and a mean annual high of 30 °C. Average yearly precipitation is about 157 cm/year.

The marsh site is flooded with diurnal tides of approximately 0.07-0.67 m in magnitude, which are predominantly influenced by seasonal winds. The marsh site is dominated by uniform stands of *Spartina alterniflora* plants.

4.2.2 Sample Collection

Sediment cores were collected using thin-walled aluminium core tube to minimize compaction and then transferred into 15-cm i.d., 30-cm long thick-walled glass cores before transporting to the laboratory. Approximately 20-cm long sediment columns were taken between the culms of *Spartina alterniflora*.

4.2.3 Testing Crude Oil

The 'sweet' South Louisiana crude oil (SCLO) was used in the present work. The PAHs content of the SLCO was modified by adding pyrene and phenanthrene for about 0.2 g of each compound /mL of crude oil. The modification of the testing oil chemical composition was meant to increase the amount of the selected model PAHs to levels comparable to other crude oil samples, since SLCO has its name 'sweet' for having relatively lower amount of PAHs. Technically, the PAHs represent the more recalcitrant fraction of crude oil, and therefore, this would help evaluate 'fairly' the biodegradation potential of crude oil in the salt marshes. The degradation profiles of phenanthrene and pyrene are presented in Figure B2 (Appendix B). The crude oil was artificially weathered before spiking into the sediment cores by flushing with dry nitrogen gas for about 48 hours to minimize the amount of volatile hydrocarbon components so that oil loss due to volatilization is minimized during the biodegradation study. A loss of about 15% of the initial crude oil weight was observed.

4.2.4 Experimentation

A total of fifteen sediment intact cores (16-cm diameter x 35-cm long) were set up (in triplicate for each treatment) as follows:

- Treatment 1: Single oil spike of 40 mL
- Treatment 2: Two oilings @ 20 mL oil , at 5-day interval
- Treatment 3: Three oilings @ 13.3 mL oil, at 5-day intervals
- Treatment 4: Four oilings @ 10 mL, at 5-day intervals
- Treatment 5: Control with no oil

Artificially weathered crude was applied directly to the surface of each core (2 L/m²) using a pipette as per treatment shown above. The cores were wrapped with aluminium foil to avoid light penetration and growth of algae. After 25 days (5 days after the last spike in treatment 4), the cores were flooded for 2 days and drained for 2 days alternately, to mimic the tidal effect. The water was drained from the cores by siphoning with a small diameter tube. Water evaporation from cores was compensated by adding seawater. Samples were taken after every 20 days.

Sediment samples were taken from the intact cores by scooping with a knife (about 5-cm deep), removing approximately 30-g samples. Then, the sampled cavities in the cores were refilled with sand and marked to show previously sampled locations. Care was taken not to sample subsequent samples too close from previously sampled spots.

4.2.5 Extraction and Analysis of Crude Oil from Core Sediments

A 4-g sub-sample was apportioned from each sample collected from the intact greenhouse cores. The 4-g sub-sample was placed in a Teflon tube, to limit adsorption of any petroleum fraction, and 20 mL of a hexane: acetone solvent mixture (50/50 v/v %) was added and the solution incubated on a shaker for 48 hours. After 48 hours, the suspension was centrifuged at 3,000 rpm for about 20 minutes at room temperature. The supernatant was transferred into a separatory funnel.

Using the separatory funnel, the petroleum-laden solvent was decanted into scintillation vials, through sodium sulfate, to remove any remaining traces of water. The petroleum-laden solvent was then evaporated to 5 mL using nitrogen gas to minimize further oxidation. The samples were stored at 5°C until GC-MS analysis was performed.

Preparation for the GC-MS analysis included transferring 1-mL from the scintillation vial into an amber GC-MS vial and adding 10- μ L of internal standard (2000 μ g/mL in methylene chloride containing the following components: 1,4-dichlorobenzene- d_4 , naphthalene- d_8 , acenaphthene- d_{10} , phenanthrene- d_{10} , chrysene- d_{12} and perylene- d_{12}) (SUPELCO Chemical Co.). The sample was then analyzed on GC-MS using 17 α (H), 21 β (H)-hopane as a normalizing compound (i.e. ratio of compound to hopane concentration), allowing only biodegradation to be monitored.

The analysis of the extracted hydrocarbon analytes was patterned after the US EPA Method 8270 using GC-MS. A GC-MS (Hewlett Packard 5890 Series II Plus) was utilized to analyze the samples for selected petroleum hydrocarbon components. The HP 5890 was outfitted with a HP-5 high-resolution capillary column (30-m x 0.250-mm i.d., 0.25- μ m film thickness) which was directly interfaced to a quadruple mass spectrometer (HP 5972 Mass Selective Detector). The carrier gas was high purity helium at flow rate of 1.0 mL/min, the injector temperature was 300 °C, and the column temperature was 300°C. The column temperature was programmed from 55 to 310°C at 8°C/min rate with initial 3 minutes delay and 15 minutes hold at the end. The interface to the mass selective detector was maintained at 280 °C.

Prior to sample analysis, a five-point calibration was established to demonstrate the linear range of the analysis and to determine the relative response factors for individual compounds.

The degradation data for alkanes and PAHs are presented in Table B3 and B4 (Appendix B) respectively. The degradation data were fitted using non-linear regression to the following first order kinetic equation:

$$\frac{C}{C_0} = e^{-kt} \quad (4.1)$$

where C = substrate's hopane ratio

C₀ = initial substrate's hopane ratio

k = first order rate constant, day⁻¹

t = time, days

The half-lives of the crude oil fractions (i.e. alkanes and PAHs) were determined using the following relation for first-order kinetics:

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \quad (4.2)$$

where t_{1/2} = half life (days)

k = first order rate constant, (day⁻¹)

The turnover times were determined using the following relationship:

$$\text{Turnover time (days)} = \frac{1}{k} \quad (4.3)$$

where k = first order rate constant (day⁻¹)

4.2.6 Microbial Activity Analysis: Fluorescein Diacetate (FDA) Assay

Fluorescein Diacetate (FDA) assay was used to quantify microbial activity in the oil-contaminated sediment intact cores. The FDA assay has been used to measure total

heterotrophic soil microbial activity in a variety of ecosystems (Hunter, 2000; El-Tarabily, 2002). The FDA assay does not quantify microbial biomass, but it is useful for comparing microbial hydrolytic activity in similar soil ecosystems (Schnurer and Rosswall, 1982).

The determination of FDA consists of incubating a soil sample in a buffer solution in the presence of FDA, which acts as an electron acceptor that is reduced to a coloured fluorescein, and the colour intensity is determined spectrophotometrically. The amount of absorbance of fluorescein is indicative of the hydrolytic activity of the heterotrophic microbial population within the soil sample. To obtain a constant production rate of fluorescein and to avoid extensive growth of microorganisms, a short incubation time of 1 hour is commonly used. Also, phosphate buffers are used to minimize the influence of pH which exerts a significant effect on FDA hydrolytic activity.

An FDA standard solution was made by dissolving 0.0399 g FDA in acetone and bringing the volume to 100 mL. Standards were made by adding 50 mL phosphate buffer and 10 g of each set of soil samples to each of seven flasks and then adding 0, 0.1, 0.2, 0.3, 0.5, 1.0 and 1.5 mL of fluorescein standard to the flasks. The resulting solutions contained the equivalent of 0, 50, 100, 150, 250, 500 and 750 μg FDA converted to fluorescein/flask. Standards were incubated on a rotary shaker (120 rpm) for 1 hour and then 50 mL of acetone added. The solution was centrifuged for 10 minutes at 6000 rpm, filtered and filtrate absorbance values were measured spectrophotometrically (SHIMADZU UV-1201, 1-cm path length cell) at 490 nm. The absorbance values were plotted to obtain a regression equation as shown in Figure A1 (Appendix A).

An FDA stock solution was made by dissolving 0.200 g fluorescein diacetate (ALDRICH® Chemical Co.) in acetone and bringing the volume to 100 mL with deionized water. Ten grams of soil from each sample was weighed and placed in a teflon tube. Then, 50 mL 0.1 M sodium phosphate buffer (pH 7.6) and 0.5 mL FDA stock solution was added and the tube was capped and incubated on a rotary shaker at 120 rpm for 1 hour. After one hour, 50 mL acetone was added to terminate the FDA hydrolysis reaction. The solution was swirled by hand and 40 mL decanted into a centrifuge tube. The solution was centrifuged for about 10 minutes at 6000 rpm, filtered (using 0.45 µm polysulfone membrane filters) and filtrate absorbance was measured spectrophotometrically (SHIMADZU UV-1201, 1-cm path length cell) at 490 nm. Absorbance values were converted to µg fluorescein produced/g soil/ hour by using a standard absorbance curve created from a selected oiled core before the start of flooding.

4.2.7 Soluble Organic Carbon Analysis

One hundred mL of deionized water was added to 10-g moist soil from each sample and the solution was shaken at 120 rpm for 1-hour and allowed to stand for approximately 18 hours (overnight). The solution was shaken by hand and 40 mL was poured into a centrifuge tube and centrifuged at 6000 rpm for 10 minutes. Twenty mL of the supernatant was filtered through 0.45-µm polysulfone membrane filter into a scintillation vial and refrigerated at 4 °C prior to analysis.

The four-point calibration of the TOC analyzer for SOC analysis was performed using Potassium hydrogen phthalate (C₈H₅O₄K) (SIGMA Chemical Co.). The calibration curve is presented in Figure A2 (Appendix A).

Samples were analyzed for nonpurgable organic carbon using a Total Organic Analyzer (SHIMADZU TOC-5000A). Nonpurgable organic carbon concentration in each sample was measured by acidifying the sample with 40 μL of HCl and then purging for 8 minutes with TOC grade compressed air. Acidification reduces inorganic carbon to primarily CO_2 in these samples and purging volatilizes CO_2 out of solution. Samples were then analyzed for organic carbon concentration. Results were corrected for soil moisture so that the final results were expressed as mg SOC/g soil on a dry weight basis.

4.2.8 Statistical Analyses

In both experiments three replicates per treatment were used. Data were analyzed using SIGMASTAT[®] version 1.0. Analysis of variance (ANOVA) at significance level of 5% was used to detect significant differences among the oil spill recurrence treatments. Stepwise regression and Pearson techniques were used to determine linear relationships among the parameters measured.

4.3 Results

4.3.1 Biodegradation Potential of Alkanes and PAHs

The residual alkanes ($n\text{-C}_{10}$ to $n\text{-C}_{36}$) and PAHs concentrations were monitored and the results are normalized with hopane concentration to account for biodegradation only. The degradation profiles for both alkanes and PAHs are presented in Figure 4.1 while the degradation data are presented in Table B3 and B4 (Appendix B). The alkanes decreased by 88.4%, 89.1%, 94.8 and 95.5% for single, two, three and four oilings, respectively whereas PAHs decreased by 60.3%, 63.9%, 75.9% and 85.2% for the single, two, three and four successive oilings, respectively.

The degradation data were fitted to both zero-order and first-order kinetics to confirm the common practice of fitting oil degradation data to first-order kinetics, however, only the results of the former are presented and in detail. This is because first-order kinetics was found to fit the data better based on the correlation of coefficient (R^2) and some form of coefficients of variation determined as $\left(\frac{\text{Standard Error}}{\text{Rate constant } (k)}\right) \times 100$.

For the different spill recurrence treatments, the zero-order kinetics for had statistically significant lower (paired t-test; $P=0.001$) R^2 values (from 0.70 to 0.78) for alkanes from those of first-order kinetics (from 0.98 to 0.99). Similarly, zero-order kinetics had statistically significant higher (paired t-test; $P=0.004$) coefficients of variation (from 31.11% to 37.54%) for alkanes from those of first-order kinetics (from 6.90% to 13.16%).

The comparison of the PAHs indicated zero-order kinetics having statistically comparable (paired t-test; $P=0.532$) R^2 values (from 0.85 to 0.98) from those of first-order kinetics (from 0.95 to 0.97). However, zero-order kinetics had numerically higher coefficient of variation values (7.83% to 24.27%) though statistically insignificant (paired t-test; $P=0.383$) from those of first-order kinetics (10.00% to 13.64%).

In view of the above results, on the basis of R^2 and coefficient of variation values, it was concluded that the oil degradation data were better fitting first-order kinetics.

Significant differences ($P = 0.0002$) were detected in biodegradation rate of crude oil among the spill recurrence treatments with the exception of single against two successive oilings as well as three against four successive oilings. The first order rate constants of both alkanes (from $n\text{-C}_{10}$ to $n\text{-C}_{36}$) and PAHs under oil spill recurrence

treatment and other statistical results are summarized in Table 4.1. The rate constants essentially increased in the order from single oiling to four successive oilings. The results correspond to half-lives of 18.24, 17.77, 13.59 and 11.95 days for alkanes as compared to 69.31, 69.31, 38.51, 31.51 days for PAHs. Further, these results correspond to turnover times of 26.32, 25.64, 19.61 and 17.24 days for alkanes as compared to 100.00, 100.00, 55.56 and 45.45 days for PAHs.

4.3.2 Microbial Activity

The profile of microbial activity (as expressed in terms of the amount of FDA hydrolyzed) under the influence of oil spill recurrence is presented in Figure 4.2 while the data are presented in Table C2 (Appendix C). The mean values of the microbial activity were obtained as 12.76, 30.08, 34.75 and 15.43 μg FDA hydrolyzed/ g-soil/hr for single oiling, two oilings, four oilings and control. The amount of FDA hydrolyzed was consistently higher for four oilings as compared to single oiling ranging from about 1.9 to 3 times. Significant differences ($P = 2.25 \times 10^{-9}$) in microbial activity (FDA hydrolyzed) were detected among the oil spill recurrence treatments with the exception of the single oiling against the control treatment ($P > 0.05$).

4.3.3 Soluble Organic Carbon (SOC)

The profile of SOC concentration under the influence of oil spill recurrence is presented in Figure 4.3 while the data are presented in Table D2 (Appendix D). The mean SOC concentrations were 1.40, 1.63, 1.83 and 0.98 mg C/g-oven-dry-soil for single oiling, two oilings, four oilings and control treatments respectively. The SOC values for the four oilings treatment were consistently higher than single oiling ranging from about

1.2 to 1.5 times. Significant differences ($P = 0.00028$) in SOC concentration were detected among the oiling treatments with the exception of four oilings against single and two oilings ($P > 0.05$).

4.3.4 Regression and Correlation of Measured Parameters

Stepwise regression was conducted using data from the four-oiling treatment, which had the highest degradation of crude oil and microbial activity, suggesting existence of relatively minimal or no limitation in nutrients and/or other microbial growth factors. Both stepwise regression and Pearson correlation were performed to determine linear relationships among hydrocarbon hopane ratio, SOC concentration and microbial activity (amount of FDA hydrolyzed). A sample output for stepwise regression is presented in Appendix E while the P-values for Pearson correlation are presented in Table F3 and F4 (Appendix F) for alkanes and PAHs respectively. Both techniques revealed no significant linear relationships ($P > 0.05$) among the measured parameters for both alkanes and PAHs. This suggests complex interactions between soil biogeochemical and microbiological properties in determining the significance of increased biodegradation of crude oil in salt marshes at least under the experimental conditions.

4.4 Discussion

The results indicated that there were significant differences in biodegradation of crude oil among the spill recurrence treatments with the exception of single against two oilings as well as three against four oilings. The results demonstrate that biodegradation of crude oil essentially increased with each subsequent oiling.

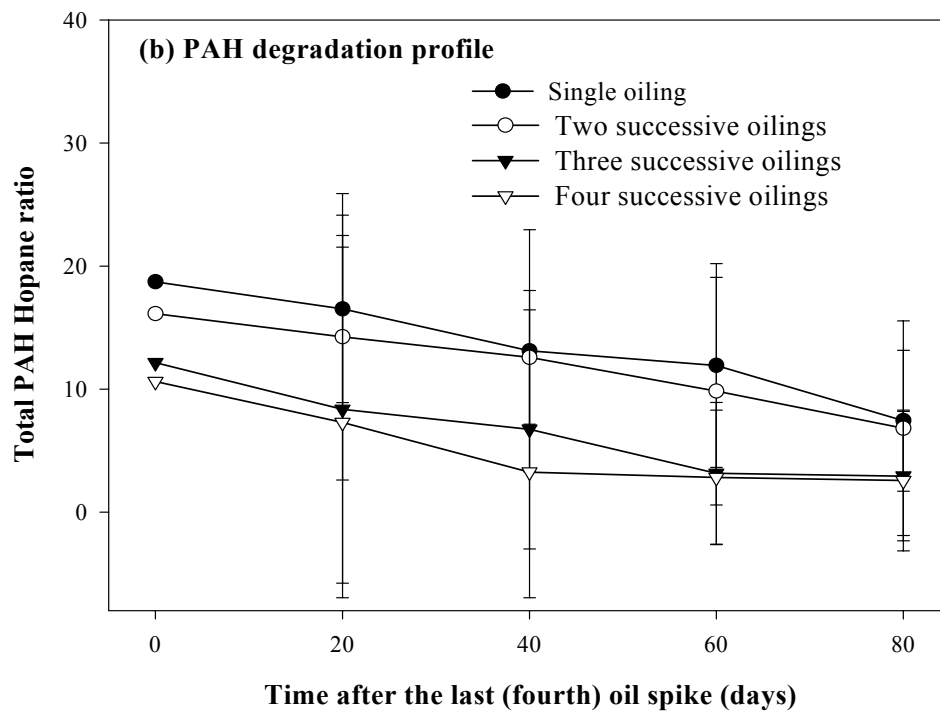
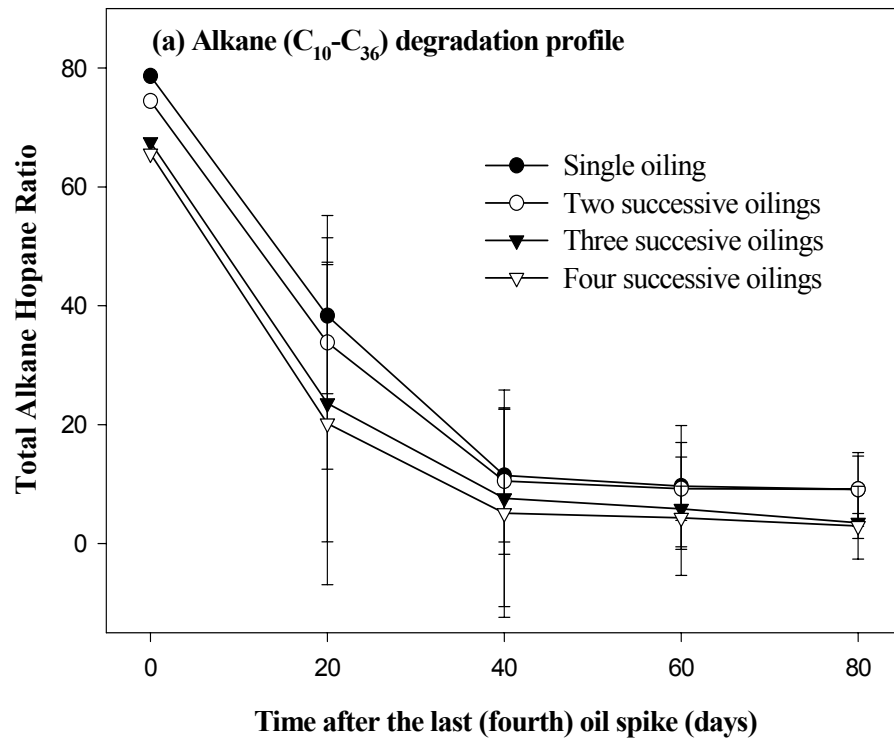


Figure 4.1: The effect of oil spill recurrence on residual petroleum hydrocarbons

Table 4.1: Summary results of first-order rate constants of alkanes and PAHs following oil spill recurrence

Treatment	Alkanes (<i>n</i> -C ₁₀ to <i>n</i> -C ₃₆)					PAHs				
	k	Std	t _{1/2}	Turnover	R ²	k	Std	t _{1/2}	Turnover	R ²
	(day ⁻¹)	error	(days)	time (days)		(day ⁻¹)	error	(days)	time (days)	
Single oiling	0.038	0.005	18.24	26.32	0.98	0.010	0.001	69.31	100.00	0.95
Two successive oilings	0.039	0.005	17.77	25.64	0.98	0.010	0.001	69.31	100.00	0.95
Three successive oilings	0.051	0.004	13.59	19.61	0.99	0.018	0.002	38.51	55.56	0.97
Four successive oilings	0.058	0.004	11.95	17.24	0.99	0.022	0.003	31.51	45.45	0.96

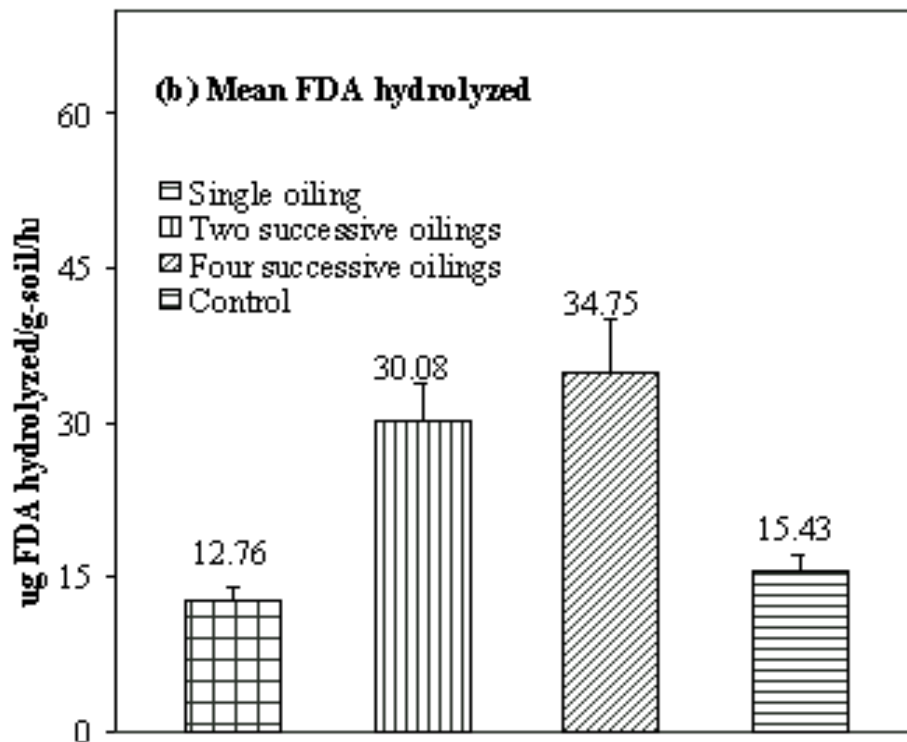
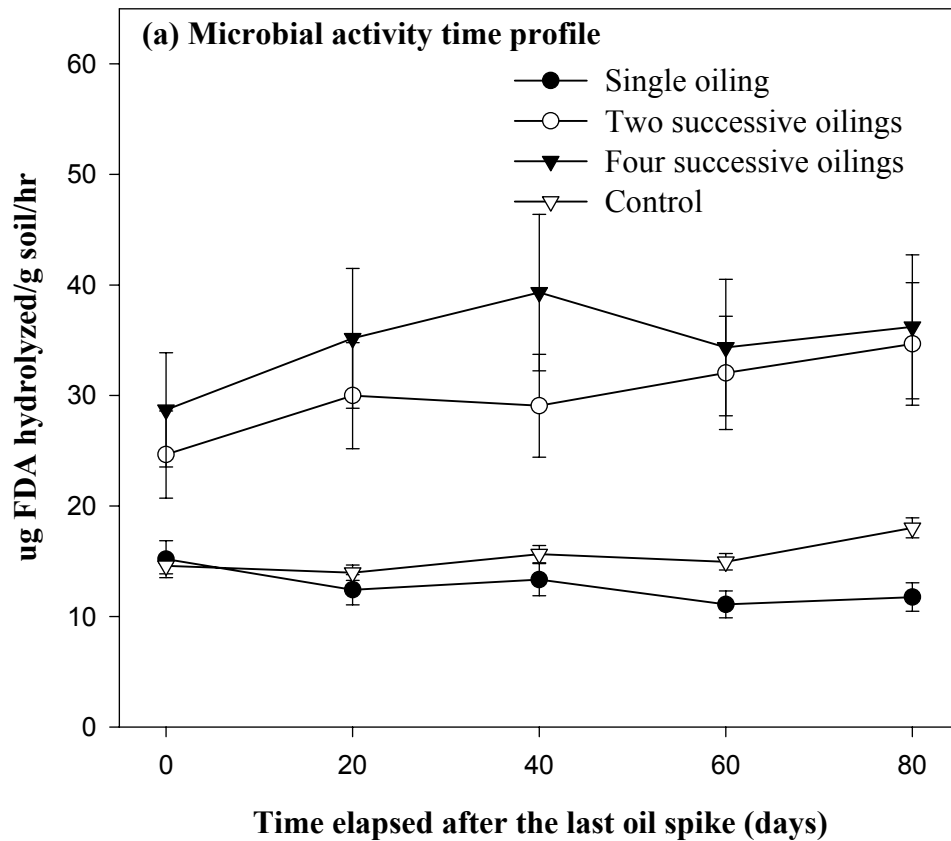


Figure 4.2: The effect of oil spill recurrence on microbial activity in terms of the amount of FDA hydrolyzed

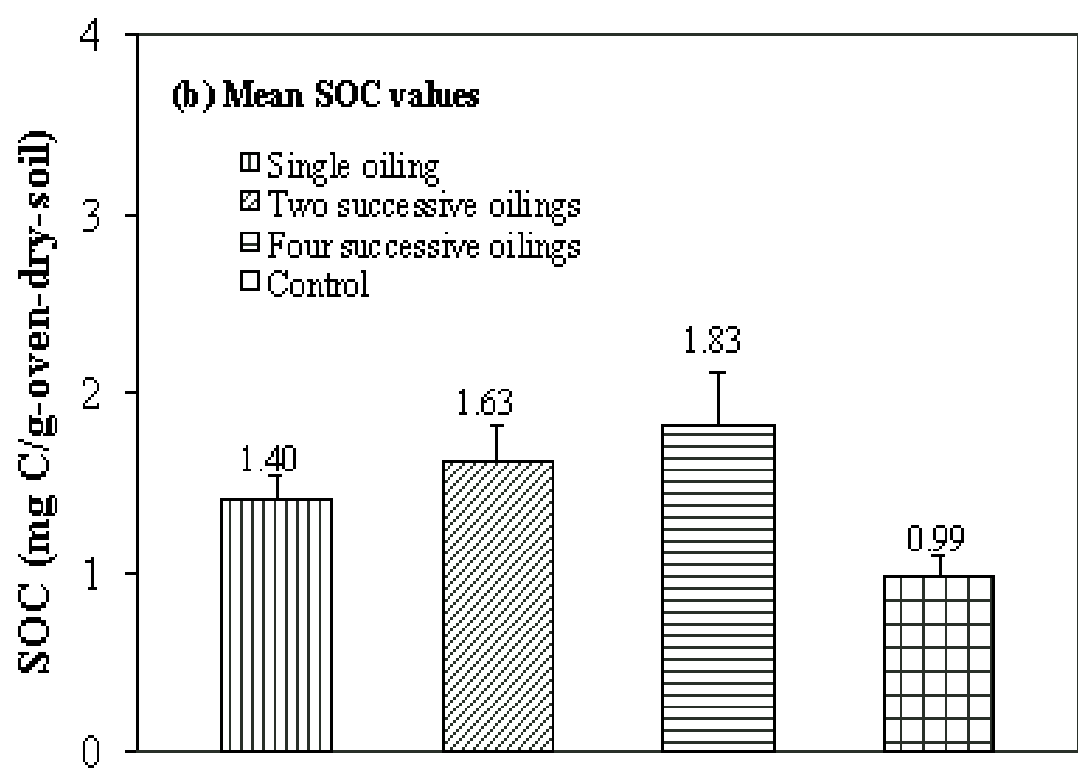
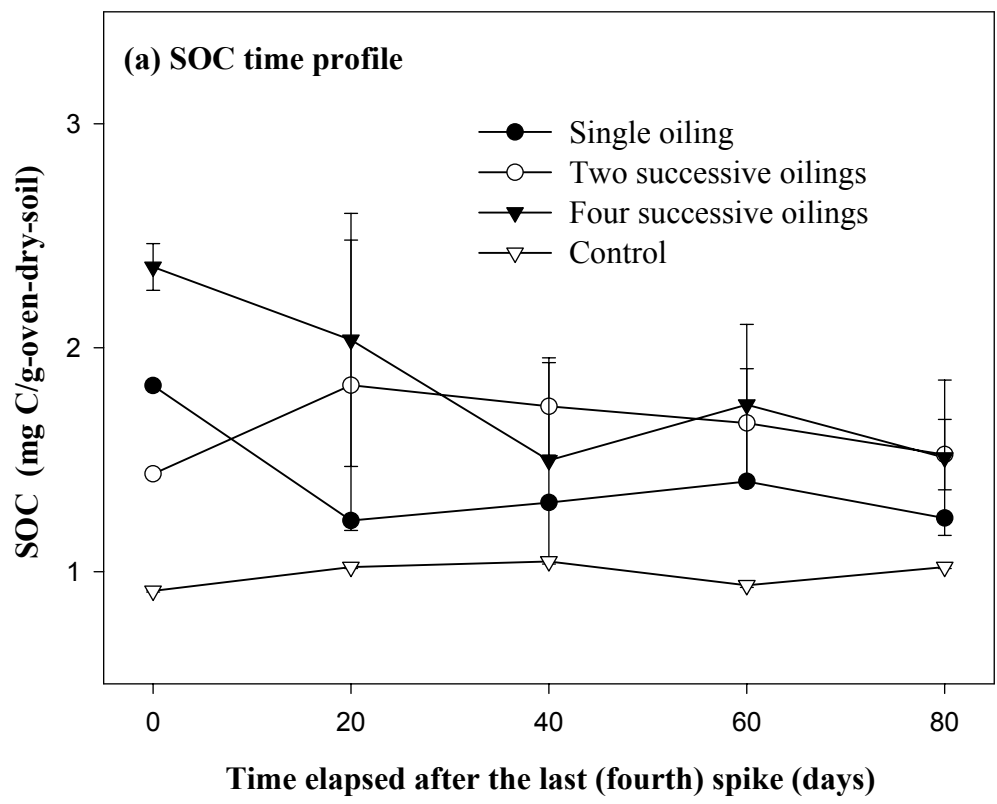


Figure 4.3: The effect of oil spill recurrence on SOC concentration

The possible hypothesis that might explain the effect of subsequent oil application is that, the increased degradation rate is primarily due to microbial adaptation resulting into stimulatory effect on microbial metabolic activity and possibly, an increase in number of microorganisms on each subsequent oiling. On the other hand, there is a possibility for increase in metabolic activity per cell upon repeated exposure to crude oil rather than a substantial increase in number of microbes. For instance, Al-Hadhrami *et al* (1996) observed that, separate additions of surfactants and molasses resulted in significant biodegradation of the *n*-alkane fraction of crude oil, however, there were no significant differences in bacterial counts at the end of the experiments from those at the beginning. It can be seen that further work is needed to explore the linkage between microbial metabolic activity and growth aspects associated with oil spill recurrence.

Alternatively, the higher biodegradation rate and microbial activity observed for three and four oilings may be related to shift in importance of one metabolic pathway over another possibly as a result of microbial adaptation and/or competition. This is based on the assumption that, more than one hydrocarbon compound degradation pathway exists in different microbial species and therefore, it is possible that individual bacteria able to degrade more than one aromatic substrate will have more than one pathway for their metabolism (Stringfellow and Aitken, 1995).

The results indicated a relatively lower biodegradation rate and microbial activity in single and two oilings suggesting that the slowed biodegradation may be due to adverse impact from relatively higher initial loading of crude oil. This is important in view of the microbial survival and adaptation in terms of suppressing effect on the synthesis of enzymes involved in crude oil metabolism or by changes in the genetic

capacity of microbial species to maintain their ability to degrade crude oil (Leahy and Colwell, 1990). For instance, Long *et al* (1995) observed that PAHs exerted toxic effects on the active microbial community at concentrations above their solubility concentrations while they noticed enrichment of specific degraders at their (PAHs) solubility concentration levels. In relation to this, Leahy and Colwell (1990) in their review paper cited that, microbial activities were generally enhanced in a contaminated soil containing up to 5% hydrocarbon mass per dry weight of soil while oil concentrations over 10% may result in inhibition of microbial activity by toxic components and/or by-products of the oil. However, comparison to the present work may be difficult using the oil-to-soil ratio as this may mislead on the extent of pollution in the intact sediment cores used, since the spiked oil is believed hardly to have penetrated beyond the top 10-cm.

The lower biodegradation rate in the single and two oilings may be related to competition between sulfate reducing microbes and methanogens in the salt marsh soil. Vroblesky *et al* (1996) observed that when BTEX concentration was low in a contaminated aquifer, sulfate reduction microbes outcompeted methanogenic microbes for the available BTEX at a lower concentration of sulfate (< 1 mg/L) than when BTEX concentration was higher. Although sulfate measurements were not taken in this study, the relatively higher initial crude oil loading for single and two oilings presumably may have limited sulfate reduction, which is known to be linked to biodegradation of crude oil in salt marshes, therefore resulting in relatively lower biodegradation rate. Alternatively, the lower biodegradation of crude oil in single and two oilings may be related to growth of competing microbial population incapable of degrading crude oil but which deprives

the oil-degrading population of nutrients or else other growth factors may also be involved.

On the other hand, the increased biodegradation rates and microbial activity observed in the order from single to four oilings may be related to altered sorption potential with each subsequent oiling. In other words, the biodegradation was controlled by the desorption rate of the sorbed fraction of the petroleum hydrocarbons presuming that the subsequent oilings had the role of “conditioning” the marsh soil.

The results obtained in this work can be explained in terms of competitive inhibitory and enhancement effect among the petroleum hydrocarbons within the crude oil as a function of concentration. In a study by Arcangeli and Arvin (1995) as cited by Riser-Roberts (1998), a toluene concentration of > 1 to 3 mg/L reduced the *o*-xylene degradation rate and concentration of *o*-xylene above 2 to 3 mg/L in turn inhibited toluene biodegradation. With different initial loading of crude oil among the spill recurrence treatments such inhibitory and/or enhancement effect may have existed.

Significant differences were detected in SOC values among the oiling treatments. The highest mean SOC values were observed in the four oilings, which had the highest biodegradation rate of crude oil and heterotrophic microbial activity. Then, why do we see higher SOC values in the four oilings irrespective of higher microbial activity assumed to utilize the SOC pool? One possible explanation may be accumulation of the recalcitrant fraction of the SOC assuming that labile compounds within the SOC fraction are preferentially utilized by microbes (Marschner and Bredow, 2002). Alternatively, the supply of SOC supposedly due to higher microbial activity may have exceeded the demand possibly due availability of a variety of organic substrates.

4.5 Conclusions and Implications

The potential for oil spill recurrence in marine ecosystems such as the salt marshes is significant in view of a variety of spill sources that may be involved. The present work was carried out to explore the effect of spill recurrence on intrinsic biodegradation of crude oil in salt marsh intact cores growing *Spartina alterniflora*. Specifically, single oil was compared to two, three and four successive oilings, each totaling to 40-mL (2 L/m²). Residual petroleum hydrocarbons, heterotrophic microbial activity and soluble organic carbon were monitored for about 3 months.

The results indicate that the biodegradation rate of crude oil, microbial activity and SOC essentially increased with each subsequent oiling. This suggests that single oiling exerted relatively toxic effect on the active microbial community as compared to multiple successive oilings of the same total volume. However, the mechanism by which the biodegradation rate is accelerated as a result of oil spill recurrence is still uncertain. Previous studies suggest this to be associated with increased number of oil degrading microorganisms and/or increased microbial activity, however, the consequence of the specific oil-degrading enzyme activity is not known.

Both stepwise regression and Pearson correlation indicated no linear relationship ($P > 0.05$) among the variables measured. This suggests complex interaction among soil geochemical and microbiological properties in determining the significance of increased biodegradation of crude oil in salt marshes following oil spill recurrence at least under the experimental conditions.

The results from this work suggest that, microbial degradation might not be significant in a pristine tidal marsh particularly immediate to an oil spill event as opposed to a previously contaminated one. However, a caution need to be made here in that, the

results obtained may be limited by the experimental design used in terms of the shorter time interval between successive oil additions, which was only 5 days.

CHAPTER 5

SUMMARY AND OUTLOOK

5.1 Experimental Findings and Implications

The environmental and economic impact of oil contamination in the coastal marine ecosystems is potentially serious. The microbial degradation of the spilled oil petroleum hydrocarbons in marine sediments is an important fate process. As a result, it is a rapidly growing research area with focus to gain fundamental knowledge of the fate of spilled oil and devising remedial measures that make use of the indigenous microbial assimilative capacity. Therefore, understanding of the influence of different perturbations on the fate of spilled oil in marine environment is useful in the assessment of the environmental impact of oil spills and remedial investigation. The present work monitored residual petroleum hydrocarbons, heterotrophic microbial activity and soluble organic carbon (SOC) to determine the effect of flooding and spill recurrence on the biodegradation of south Louisiana crude oil (SLCO) (2 L/m^2) in salt marsh intact cores incubated for about 3 months.

The results for the flooding study indicate that, the biodegradation of crude oil fractions (i.e alkanes and PAHs) (with half-lives from 16.50 to 49.51 days and turnover times from 23.81 to 71.43 days) and microbial activity essentially increased in the order from the continuously-flooded (CF) regime, intermittently-flooded (IF) regime to non-flooded (NF) regime. The SOC concentrations increased significantly but opposite to the trend for crude oil biodegradation and microbial activity. Both stepwise regression and Pearson correlation revealed no linear relationship among the parameters investigated

($P > 0.05$) implying for complex interaction among the parameters measured at least under the experimental conditions. Notably, tidal flooding is a natural disturbance occurring in salt marshes. This reflects that tidal flooding is of vital importance in defining the ecological characteristics of the salt marshes and indirectly can affect the biodegradation of spilled oil and decomposition and accumulation of organic matter in salt marshes.

The results for the oil spill recurrence study (single, two, three and four successive oilings; each treatment totaling to 2 L/m^2) indicate that, the biodegradation of alkanes and PAHs (with half-lives from 11.95 to 69.31 days and turnover times from 17.24 to 100.00 days), heterotrophic microbial activity and SOC concentration essentially increased with each subsequent oiling. These results suggest that single oiling may have exerted relatively toxic effect on the active microbial community and associated soil biogeochemical processes as compared to multiple successive oiling of the same total volume. Both stepwise regression and Pearson correlation methods revealed no linear relationship ($P > 0.05$) among the parameters investigated reflecting for complex interplay among the parameters at least under the experimental conditions. The results from the spill recurrence study reflect that, microbial degradation might not be significant in a pristine tidal marsh particularly immediate to an oil spill event as opposed to a previously contaminated one. However, a caution need to be made here in that, the results obtained may be limited by the experimental design used in terms of the shorter time interval between successive oil additions, which was only 5 days.

The lack of correlation among the parameters measured for both experiments reflect the challenge in understanding the interrelationship between environmental factors and microbial ecology in determining the fate of oil spills in salt marshes at least under

the experimental conditions. Yet, the inherent microbial assimilative capacity of petroleum hydrocarbons in salt marshes ought to be fully utilized to provide cost-effective remedial options. However, the behaviour and fate of spilled oil are site specific making it difficult to generalize from one case to another.

5.2 Future Research

It is anticipated that, under real field conditions, there may be some variations from the laboratory results obtained in this work, primarily due to the influence of tidal mixing and sedimentation and other environmental factors. Therefore, field trials may be worth undertaking to confirm the laboratory results.

The full extent of the influence of flooding and spill recurrence on microbial diversity, community structure and their enzymatic activity in oil-contaminated coastal marshes has yet to be wholly revealed. Therefore, it may be interesting to explore further on this subject preferably using molecular techniques.

Tidal waters flooding salt marshes are normally turbid with the vegetation trapping the sediments, resulting in increase in marsh surface elevation with time (Adam, 1990). Sediment burial (accretion) may have a significant role on the fate of crude oil as it creates additional barrier to oxygen diffusion reaching the contaminated sediments, far from the overlaying water column. Therefore, it may be of interest to explore the effect of sediment burial on the biodegradation potential of crude oil.

LITERATURE CITED

- Adam, P. (1990). *Salt Marsh Ecology*. Cambridge University Press, Great Britain.
- Al-Hadhrami, M. N., Lappin-Scott, H. M. and Fisher, P. J. (1996). "Effects of the Addition of Organic Carbon Sources on Bacterial Respiration and *n*-Alkane Biodegradation of Oman Crude Oil". *Marine Pollution Bulletin*, 32(4): 351-357
- Atlas, R. M. (1981). "Microbial Degradation of Petroleum Hydrocarbons: An Environmental Perspective". *Microbiological Reviews*, 45: 180-209
- Atlas, R.M. (1988). "Biodegradation of Hydrocarbons in the Environment". In: *Environmental Biotechnology*, Plenum Press, pp. 211-222
- Baker, J. M. (1973). "Recovery of Salt Marsh Vegetation from Successive Oil Spillages". *Environmental Pollution*, 4:223-230
- Bauer, J.E., and Capone. D. G. (1985). "Degradation and Mineralization of the Polycyclic Aromatic Hydrocarbons Anthracene and Naphthalene in Intertidal Marine Sediments". *Applied and Environmental Microbiology*, 50: 81-90.
- Bekins, B.A., Godsy, E.M., and Warren E.(1999). "Distribution of Microbial Physiologic Types in an Aquifer Contaminated by Crude Oil". *Microbial Ecology*, 37:263-275
- Beller, H. R., Grbicgalic, D. and Reinhard, M. (1992). "Microbial Degradation of Toluene under Sulfate-Reducing Conditions and the Influence of Iron on the Process". *Applied and Environmental Microbiology*, 58(3): 786-796.
- Berry, D. F., Francis, A. J. and Bollag, J. M. (1987). "Microbial Metabolism of Homocyclic and Heterocyclic Aromatic Compounds under Anaerobic Conditions". *Microbiological Reviews*, 51(1): 43-59
- Bragg, J. R., Prince, R. C., Harner, E. J. and Atlas, R. M. (1994). "Effectiveness of Bioremediation for the *Exxon Valdez* Oil Spill". *Nature*, 368:413-418
- Caldwell, M. E., Gareth, R. M., Prince, R. C., and Suflita, J. M. (1998). "Anaerobic Biodegradation of Long-Chain Alkanes under Sulfate Reducing Conditions". *Environmental Science and Technology*, 32:2191-2195
- Carman, K. R., Fleeger, J. W., Gambrell, R. P. and Portier, R.J. (2000). "Interactive Effects of Metals and PAHs on Benthic Food Webs", Available online: <<http://www.biology.lsu.edu/webfac/kcarman/onr.htm>>

- Christian, R. R. and Wiebe, W. J. (1978). "Anaerobic Microbial Community Metabolism in *Spartina alterniflora* Soils", *Limnology and Oceanography*, 23(2): 328-336
- Coates, J. D., Anderson, R. T. and Lovley, D. R. (1996). "Oxidation of Polycyclic Aromatic Hydrocarbons under Sulfate Reducing Conditions". *Applied and Environmental Microbiology*, 62: 1099-1101
- Connell, D. W. and Miller, G. J. (1981). "Petroleum Hydrocarbons in Aquatic Ecosystems - Behaviour and Effects of Sublethal Concentrations: Part 1". *CRC Critical Reviews in Environmental Control*, 11(1): 37-104
- De P. K. and Bose, N. M. (1938). "A Study of the Microbiological Conditions Existing in Rice Soils". *Industrial Journal of Agriculture and Science*, 8: 487-498
- de Jonge, H., Freijer, J. I. , Verstraten, J. M., Westerveld, J. and Vanderwielen, F. W. M. (1997). "Relation Between Bioavailability and Fuel Oil Hydrocarbon Composition in Contaminated Soils". *Environmental Science and Technology*, 31(3): 771-775
- DeLaune, R. D., Patrick, Jr., W. H. and Buresh R. J. (1979). "Effect of Crude Oil on a Louisiana *Spartina Alterniflora* Salt Marsh", *Environmental Pollution*, 20 (1): 21-31
- Edwards, E. A. and Grbic-Galic, C. D. (1994). "Anaerobic Degradation of Toluene and *o*-xylene by a Methanogenic Consortium". *Applied and Environmental Microbiology*, 60(1): 313-322
- El-Tarabily, K. A. (2002). "Total Microbial Activity and Microbial Composition of a Mangrove Sediment are Reduced by Oil Pollution at a Site in the Arabian Gulf". *Canadian Journal of Microbiology*, 48(2): 176-182
- Fleury, B. E. (2000). "The Louisiana Environment: The Salt Marsh". Available online: <<http://www.tulane.edu/~bfleury/envirobio/saltmarsh.htm>>
- Freedman, B.(1995). *Environmental Ecology - The Ecological Effects of Pollution, Disturbance, and Other Stresses*. 2nd ed., Academic Press, New York.
- Gambrell, R. P. and Patrick, W. H., Jr. (1979). "Chemical and Microbiological Properties of Anaerobic Soils and Sediments". In Hook, D. D. and Crawford, R. M. M. (Eds). *Plant Life in Anaerobic Environments*. Ann-Arbor Science, Michigan (USA)
- Hambrick, G. A. (1979). "Effect of Sediment pH and Oxidation-Reduction Potential on Microbial Degradation of Crude Oil". *M. S. Thesis*, Louisiana State University, Baton Rouge (LA), USA

- Harayama, S., Kishira, H., Kasai, Y. and Shutsubo, K. (1999). "Petroleum Biodegradation in Marine Environment". *Journal of Molecular Microbiology and Biotechnology*, 1(1): 63-70
- Hayes, L. A., Nevin, K. P. and Lovley, D. R. (1999). "Role of Prior Exposure on Anaerobic Degradation of Naphthalene and Phenanthrene in Marine Harbor Sediments". *Organic Geochemistry*, 30 (8B): 937-945
- Herbert, B. E., Bertsh, P. M. and Novak, J. M. (1993). "Pyrene Sorption by Water-Soluble Organic Carbon". *Environmental Science and Technology*, 27(2): 398-403
- Hunter R. G. (2000). "Comparison of Biogeochemical Functions Between Restored and Natural Bottomland Hardwood Wetlands". *Ph. D. Dissertation*, Louisiana State University, Baton Rouge (LA), USA
- Jackson, W. A. (1996). "Hydrocarbon Degradation in Louisiana Salt and Fresh Water Marshes: Inherent Potential, Enhancement, and *In Situ* Monitoring Techniques". *Ph. D. Dissertation*, Louisiana State University, Baton Rouge (LA), USA
- Jackson, W. A. and Pardue J. H. (1999). "Potential for Intrinsic and Enhanced Crude Oil Biodegradation in Louisiana's Freshwater Marshes". *Wetlands*, 19 (1): 28-34
- Johnston, R. (1970). "The Decomposition of Crude Oil Residues in Sand Columns". *Journal of Marine Biology Association of UK*, 50: 925-937
- Leahy, J. G. and Colwell, R. R. (1990). "Microbial Degradation of Hydrocarbons in the Environment". *Microbiological Reviews*, 54(3): 305-315
- Lee, R. F. (1980). "Processes Affecting the Fate of Oils in the Sea" in Geyer, R. A (Ed). *Marine Environmental Pollution, Vol. 1-Hydrocarbons*. Elsevier Scientific Publishing Company, New York.
- Li, Y., Morris, J. T. and Yoch, D. C. (1990). "Chronic Low Level Hydrocarbon Amendments Stimulate Plant Growth and Microbial Activity in Salt Marsh Microcosms". *Journal of Applied Ecology*, 27(1): 159-171
- Long S. C., Aelion C. M., Dobbins D. C., Pfaender F. K. (1995). "A Comparison of Microbial Community Characteristics Among Petroleum-contaminated and Uncontaminated Subsurface Soil Samples". *Microbial Ecology*, 30 (3): 297-307
- Lovley, D.R., Coates, J. D., Woodward, J. C. and Phillips, E. J. P. (1995). "Benzene Oxidation Coupled to Sulfate Reduction". *Applied and Environmental Microbiology*, 61: 953-958.

- Marschner, B. and Bredow, A. (2002). "Temperature Effects on Release and Ecologically Relevant Properties of Dissolved Organic Carbon in Sterilised and Biologically Active Soil Samples". *Soil Biology and Biochemistry*, 34:459-466
- Miller, P. A (1999). "Exxon Valdez Spill : Ten Years Later". *Technical Paper for Alaska Wilderness League*. Available online:
<<http://arcticcircle.uconn.edu/SEEJ/Alaska/miller2.htm>>
- Mitsch, W and Gosselink, J. G. (1993). *Wetlands*. Van Nostrand Reinhold, New York
- National Academy of Sciences (1975). "Petroleum in the Marine Environment : Inputs, Fates, and the Effects", NAS, Washington.
- Nelson-Smith, A. (1973). *Oil Pollution and Marine Ecology*. Plenum Press, New York.
- Nyman, J. A. (1999). "Effect of Crude Oil and Chemical Additives on Metabolic Activity of Mixed Microbial Populations in Fresh Marsh Soils". *Microbial Ecology*, 37:152-162
- Nyman, J. A. and DeLaune, R. D. (1991). "CO₂ Emission and Soil Eh Responses to Different Hydrological Conditions in Fresh, Brackish and Saline Marsh Soils" *Limnology and Oceanography*, 36(7): 1406-1414
- Pardue, J. H., Lee, E.-J., Shin, W. S. and Jackson, W. A. (2001). "Anaerobic Biodegradation of Crude Oil in Salt Marshes". *Proceedings of the Sixth International In Situ and On-Site Bioremediation Symposium*, Battelle Press, San Diego, California
- Penfound, W. T. and E. S. Hathaway (1938). "Plant Communities in the Marshlands of Southeastern Louisiana". *Ecological Monographs*, 8(1): 3-56
- Pfaender, F.K., and Buckley, E. N. (1984). "Effects of Petroleum on Microbial Communities". In: R. M. Atlas (ed). *Petroleum Microbiology*, MacMillan Publishing Co., New York, pp. 508-536
- Phelps, C. D. and Young, L. Y. (1999). "Anaerobic Biodegradation of BTEX and Gasoline in Various Aquatic Sediments". *Biodegradation*, 10(1):15-25
- Rockne, K. J., Chee-Sanford, J. C., Sanford, R.A., Helund, B. P., Staley, J. T. and Strand, S. E. (2000). "Anaerobic Naphthalene Degradation by Microbial Pure Cultures Under Nitrate-Reducing Conditions". *Applied and Environmental Microbiology*, 66 (4): 1595-1601
- Rozas L. P., Minello T.J., and Henry C. B.(2000). "An Assessment of Potential Oil Spill Damage to Salt Marsh Habitats and Fishery Resources in Galveston Bay, Texas". *Marine Pollution Bulletin*, 40 (12): 1148-1160

- Schnurer, J. and Rosswall, T. (1982). "Fluorescein Diacetate Hydrolysis as a Measure of Total Microbial Activity in Soil and Litter". *Applied and Environmental Microbiology*, 43(6): 1256-1261
- Shelton, T. B., and Hunter, J. B. (1975). "Anaerobic Decomposition of Oil in Bottom Sediments". *Journal of Water Pollution Control Federation*, 27(9): 2256-2269
- Shin, W. S. (1998). "Bioremediation of Petroleum Hydrocarbons in Louisiana's Salt Marshes: Sediment Oxygen Demand Model". *Ph. D. Dissertation*, Louisiana State University, Baton Rouge (LA), USA
- Shin W. S., Tate, P. T., Jackson W. A., and Pardue J. H. (1999). "Bioremediation of an Experimental Oil Spill in a Salt Marsh". In: Means J. L. and Hincsee, R.E. (Eds) *Proceedings of the International Conference on Wetlands and Remediation*. Batelle Press, Columbus (Ohio), USA
- Stringfellow, W. T. and Aitken, M. D. (1995). "Competitive Metabolism of Naphthalene, Methylnaphthalenes, and Fluorene by Phenanthrene-Degrading *Pseudomonads*". *Applied and Environmental Microbiology*, 61(1): 357-362
- Sugai, S. F., Lindstrom, J. E. and Braddock, J. F. (1997). "Environmental Influences on the Microbial Degradation of *Exxon Valdez* Oil on the Shorelines of Prince William Sound, Alaska". *Environmental Science and Technology*, 31: 1564-1572
- Taylor III, J. H (1995). "The Effects of Altered Inundation and Wrack Deposition on Nitrification, Denitrification, and the Standing Stocks of NO_3^- and NO_2^- ". *M. S. Thesis*. East Carolina University, USA
- Vroblesky, D. A., Bradley, P. M. and Chapelle, F. H. (1997). "Influence of Electron Donor on Minimum Sulfate Reduction in a Petroleum Hydrocarbon-Contaminated Aquifer". *Environmental Science and Technology*, 30:1377-1381
- Wright, A. L., Weaver, R. W. and Webb, J. W. (1997). "Oil Bioremediation in Salt Marsh Mesocosms as Influenced by N and P Fertilization, Flooding, and Season". *Water, Air and Soil Pollution*, 95:179-191

APPENDIX A CALIBRATION CURVES

A1: Microbial Activity (FDA Hydrolysis) Analysis

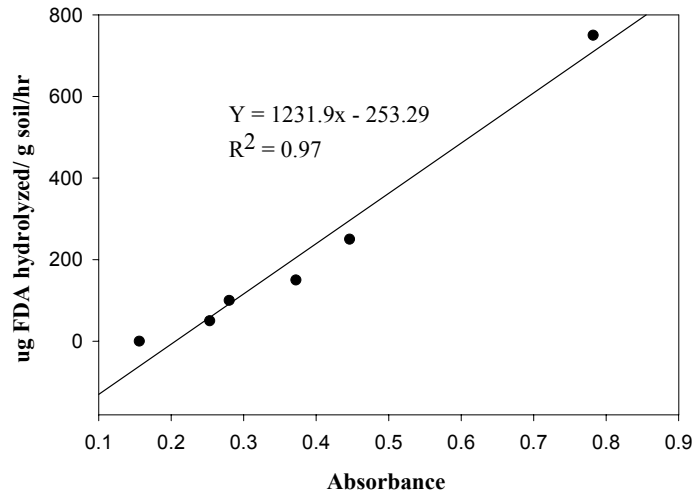


Figure A1: Calibration curve for FDA hydrolysis analysis using the spectrophotometer

A2: SOC Concentration Analysis

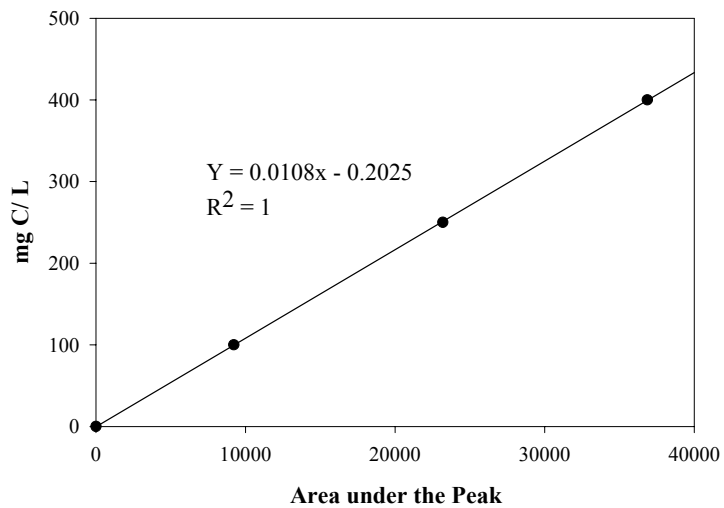


Figure A2: Calibration curve for SOC analysis using the TOC Analyzer

APPENDIX B RESIDUAL HYDROCARBON DEGRADATION DATA

B1: Effect of Flooding on Phenanthrene and Pyrene Degradation

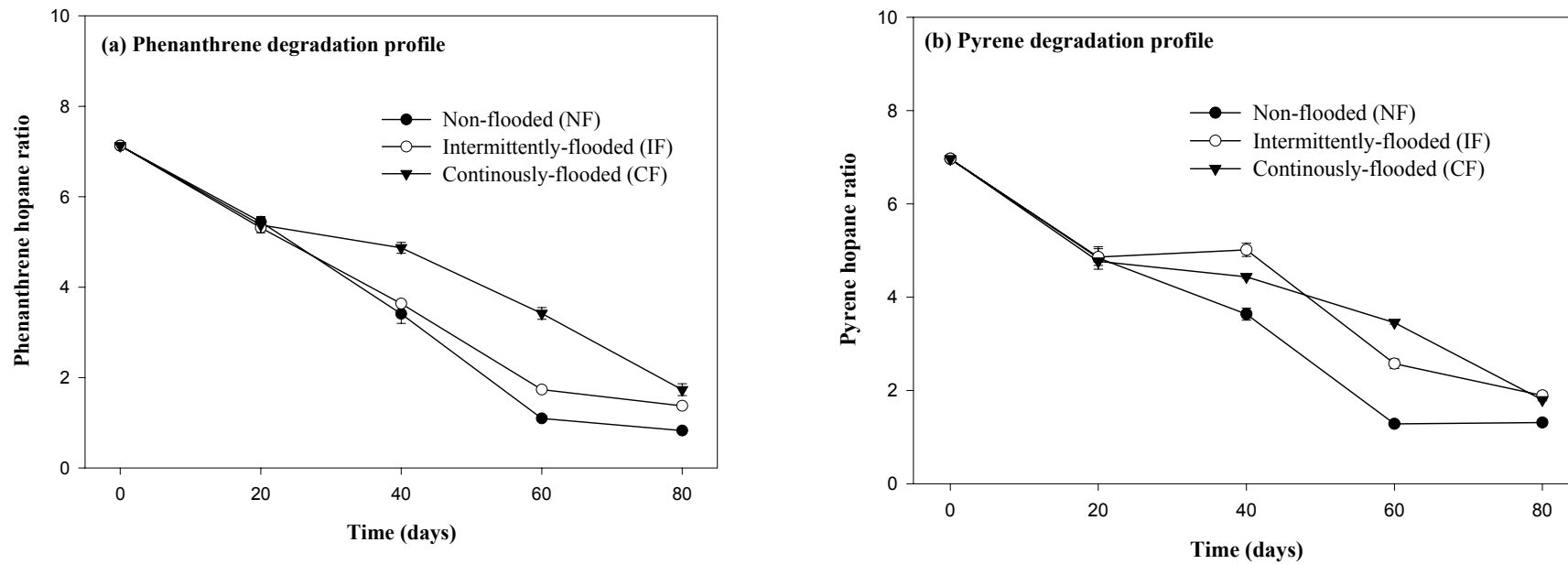


Figure B1: The effect of flooding on degradation of phenanthrene and pyrene

B2: Effect of Oil Spill Recurrence on Phenanthrene and Pyrene Degradation

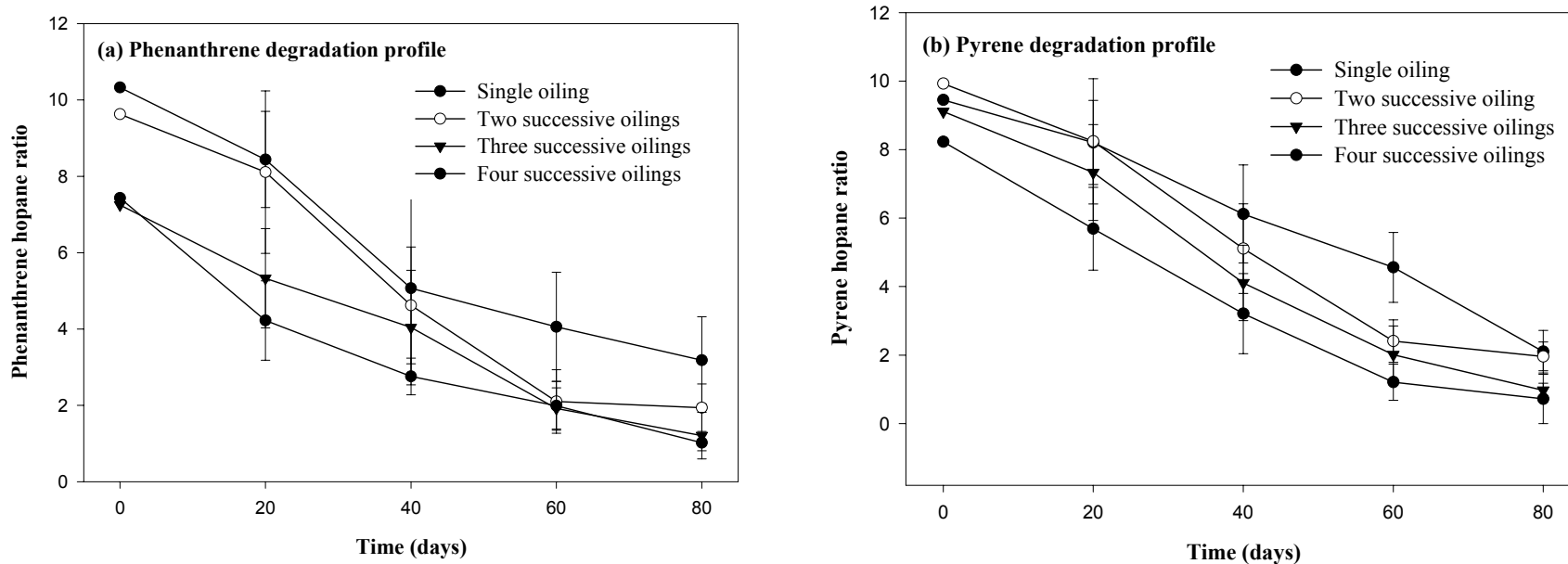


Figure B2: The effect of oil spill recurrence on degradation of phenanthrene and pyrene

B3: The Effect of Flooding on Biodegradation of Crude oil Fractions

Table B1: Summary data for the degradation of alkanes ($n\text{-C}_{10}$ - $n\text{-C}_{36}$) in the flooding study

Time (days)	CONTINUOUSLY-FLOODED (CF)		INTERMITTENTLY-FLOODED (IF)		NON-FLOODED (NF)	
	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation
0	95.64	0.00	95.64	0.00	95.64	0.00
20	62.14	38.60	51.07	5.03	34.00	13.60
40	40.17	8.82	25.80	19.48	12.40	19.00
60	9.52	2.95	7.96	5.07	3.30	1.53
80	8.67	2.87	7.30	2.11	4.20	0.00

Table B2: Summary data for the degradation of PAHs in the flooding study

Time (days)	CONTINUOUSLY-FLOODED (CF)		INTERMITTENTLY-FLOODED (IF)		NON-FLOODED (NF)	
	Total PAH hopane ratio	Standard deviation	Total PAH hopane ratio	Standard deviation	Total PAH hopane ratio	Standard deviation
0	21.70	0.00	21.70	0.00	21.70	0.00
20	15.50	0.22	15.70	0.76	10.80	0.26
40	14.20	0.62	12.30	5.33	8.50	0.00
60	10.70	7.25	6.99	0.90	3.55	0.38
80	5.30	0.52	4.65	0.67	2.65	0.25

B4: The Effect of Oil Spill Recurrence on Biodegradation of Crude oil Fractions

Table B3: Summary data for the degradation of alkanes ($n\text{-C}_{10} - n\text{-C}_{36}$) in the oil spill recurrence study

Time (days)	SINGLE OILING		TWO OILINGS		THREE OILINGS		FOUR OILINGS	
	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation
0	78.64	0.00	74.46	0.00	67.53	0.00	65.61	0.00
20	38.32	13.11	33.84	11.34	23.61	13.33	20.21	7.12
40	11.45	6.20	10.51	2.33	7.62	3.21	5.11	1.50
60	9.64	2.20	9.23	3.34	5.83	2.17	4.34	1.32
80	9.12	5.60	9.11	2.21	3.49	1.12	2.96	1.11

Table B4: Summary data for the degradation of PAHs in the oil spill recurrence study

Time (days)	SINGLE OILING		TWO OILINGS		THREE OILINGS		FOUR OILINGS	
	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation	Total alkane hopane ratio	Standard deviation
0	18.72	0.00	16.13	0.00	12.15	0.00	10.62	0.00
20	16.52	7.62	14.25	11.64	8.36	14.13	7.28	14.25
40	13.11	9.85	12.58	5.43	6.73	9.72	3.25	10.22
60	11.92	8.29	9.83	9.25	3.16	5.76	2.82	5.46
80	7.43	5.73	6.82	8.72	2.93	5.26	2.57	5.72

APPENDIX C
MICROBIAL ACTIVITY (FDA HYDROLYSIS) ANALYSIS DATA

C1: Microbial Activity (FDA Hydrolysis) Data for the Flooding Experiments

Table C1: Summary data of microbial activity (FDA hydrolysis) for the flooding study

Time (days)	CONTROL			NON-FLOODED (NF)			INTERMITTENTLY_FLOODED (IF)			CONTINUOUSLY-FLOODED (CF)		
	μg FDA /g-soil/hr	Std Dev	Moisture content (%)	μg FDA /g-soil/hr	Std Dev	Moisture content (%)	μg FDA /g-soil/hr	Std Dev	Moisture content (%)	μg FDA /g-soil/hr	Std Dev	Moisture content
0	178.91	18.95	19.29	183.3	19.17	19.55	163.89	18.19	24.29	215.72	19.79	22.70
20	171.20	18.56	21.29	242.8	12.14	21.51	318.58	12.92	22.14	191.32	18.56	24.10
40	192.03	19.60	23.29	383.2	19.16	18.99	284.30	16.22	19.26	234.91	14.75	19.63
60	183.41	19.17	21.25	302.5	15.13	22.71	302.20	15.11	22.73	259.41	10.97	18.82
80	221.14	11.06	23.70	405.2	20.26	18.92	321.70	13.09	22.21	243.21	19.16	24.56

Notes: The soil samples weighed about 10 g each

C2: Microbial Activity (FDA Hydrolysis) Data for the Oil Spill Recurrence Experiments

Table C2: Summary data of microbial activity (FDA hydrolysis) for the oil spill recurrence study

Time (days)	CONTROL			SINGLE OILING			TWO OILINGS			FOUR OILINGS		
	µg FDA /g-soil/hr	Std Dev	Moisture content (%)	µg FDA /g-soil/hr	Std Dev	Moisture content (%)	µg FDA /g-soil/hr	Std Dev	Moisture content (%)	µg FDA /g-soil/hr	Std Dev	Moisture content
0	178.91	18.95	19.29	186.30	18.34	24.29	302.51	18.13	24.22	352.21	13.61	25.18
20	171.20	18.56	21.29	152.40	16.62	21.45	367.90	15.39	18.29	431.50	19.58	22.18
40	192.03	19.60	23.29	163.61	17.18	18.56	356.72	18.84	23.71	482.42	21.12	18.37
60	183.41	19.17	21.25	136.10	18.81	25.30	393.20	19.66	21.14	421.40	17.07	19.29
80	221.14	15.06	23.70	144.32	15.22	18.37	425.33	17.27	19.33	444.31	19.22	21.41

Notes: The soil samples weighed about 10 g each

APPENDIX D SOLUBLE ORGANIC CARBON (SOC) DATA

D1: SOC Data for the Flooding Experiments

Table D1: Summary data of SOC analysis for the flooding study

Time (days)	CONTROL			NON-FLOODED (NF)			INTERMITTENTLY_FLOODED (IF)			CONTINUOUSLY-FLOODED (CF)		
	SOC (ppm)	Std Dev	Moisture content (%)	SOC (ppm)	Std Dev	Moisture content (%)	SOC (ppm)	Std Dev	Moisture content (%)	SOC (ppm)	Std Dev	Moisture content
0	11.2	1.82	19.29	20.34	2.29	19.55	11.94	1.83	24.29	17.84	1.31	22.70
20	12.52	3.22	21.29	15.25	3.22	21.51	15.46	2.93	22.14	22.45	1.82	24.10
40	12.83	2.13	23.29	38.92	2.17	18.99	13.89	0.01	19.26	18.26	2.85	19.63
60	10.33	1.67	21.25	16.68	3.24	22.71	10.37	1.92	22.73	32.45	1.99	18.82
80	7.83	3.81	23.70	17.56	2.11	18.92	15.46	2.85	22.21	18.26	2.14	24.56

Notes: The soil samples weighed about 10 g

D2: SOC Data for the Spill Recurrence Experiments

Table D2: Summary data of SOC analysis for the oil spill recurrence study

Time (days)	CONTROL			SINGLE OILING			TWO OILINGS			FOUR OILINGS		
	SOC (ppm)	Std Dev	Moisture content (%)	SOC (ppm)	Std Dev	Moisture content (%)	SOC (ppm)	Std Dev	Moisture content (%)	SOC (ppm)	Std Dev	Moisture content (%)
0	11.2	1.82	19.29	22.46	2.64	24.29	28.97	1.28	24.22	17.63	0.13	25.18
20	12.52	3.22	21.29	15.07	1.22	21.45	24.97	6.93	18.29	22.49	7.95	22.18
40	12.83	2.13	23.29	16.06	3.56	18.56	18.38	5.35	23.71	21.33	2.67	18.37
60	10.33	1.67	21.25	17.22	2.63	25.30	21.42	4.40	21.14	20.42	2.98	19.29
80	7.83	3.81	23.70	15.21	1.53	18.37	18.52	4.25	19.33	18.69	1.93	21.41

Notes: The soil samples weighed about 10 g

APPENDIX E

SAMPLE STEPWISE REGRESSION OUTPUT

E1: Brief Description

The stepwise linear regression is used when

- Predicting a trend in the data, or predict the value of one variable from the values of one or more other variables, by fitting a line or plane (or hyperplane) through the data
- Finding the model with suitable independent variables by adding or removing independent variables from the equation

E2: Sample Output from Stepwise Regression

The data used for this sample output were those for alkanes under the CF regime.

The output from the stepwise regression follows below.

Forward Stepwise Regression:

Dependent Variable: Col 1(Total alkane hopane ratio)

F-to-Enter: 4.0000 P = 0.1161

F-to-Remove: 3.9000 P = 0.1195

Step 0:

Standard Error of Estimate = 36.9

Analysis of Variance:

Group	F	DF	SS	MS
Residual		4	5444.5	1361.1
Group	F	P		
Residual				
Variables in Model				
Group	Coef.	Std. Coeff.	Std. Error	
Constant	43.23		16.499	

	Group	F-to-Remove	P	
	Constant			
Variables not in Model				
	Group		F-to-Enter	P
	Col 2 (FDA hydrolyzed)	4.089	0.1132	
	Col 3(SOC concentration)	0.801	0.4214	

Step 1: Col 2 (FDA hydrolyzed) Entered
R = 0.7595 Rsqr = 0.5768 Adj Rsqr = 0.4357
Standard Error of Estimate = 27.7

Analysis of Variance:

Group	DF	SS	MS
Regression	1	3140.4	3140.4
Residual3	2304.1	768.0	

Group	F	P
Regression	4.09	0.1364
Residual		

Variables in Model

Group	Coef.	Std. Coeff.	Std. Error
Constant	287.51		121.441
Col 2 (FDA hydrolyzed)	-1.07	0.759	0.528

Group	F-to-Remove	P
Constant		
Col 2 (FDA hydrolyzed)	4.09	0.1364

Variables not in Model

Group	F-to-Enter	P
Col 3 (SOC concentration)	0.107	0.7650

Summary Table

Step #	Vars. Entered	Vars. Removed	R
1	Col 2 (FDA hydrolyzed)		0.759

Step #	RSqr	Delta RSqr	Vars in Model
1	0.577	0.577	1

The dependent variable Col 1 (alkane hopane ratio) can be predicted from a linear combination of the independent variables:

	P
Col 2 (FDA hydrolyzed)	0.1364

The following variables did not significantly add to the ability of the equation to predict Col 1 (Alkane hopane ratio) and were not included in the final equation:

Col 3 (SOC concentration)

Normality Test: Passed (P = 0.3762)

Homoscedasticity Test: Passed (P = 0.0500)

Power of performed test with alpha = 0.0500: 0.2902

The power of the performed test (0.2902) is below the desired power of 0.8000. You should interpret the negative findings cautiously.

APPENDIX F PEARSON CORRELATION RESULTS

F1: Brief Description

Pearson correlation method is used to measure the strength of association between pairs of variables without regard to which variable is dependent or independent; and tests whether relationship, if any, between the variables is a straight line.

The P-value refers to the probability of being wrong in concluding that there is a true association between the variables. In our case, if $P > 0.05$, this reflects that there are no significant relationships between the pair of variables in the correlation table.

F2: Pearson Correlation for Intermittently-flooded (IF) Regime

Table F1: The P-values for Pearson correlation for the alkanes under the IF regime

	Total alkane hopane ratio	FDA Hydrolyzed	SOC concentration
Total alkane hopane ratio	-	0.0727	0.8640
FDA Hydrolyzed	0.7270	-	0.436
SOC concentration	0.8640	0.4360	-

Table F2: The P-values for Pearson correlation of PAHs under the IF regime

	Total PAH hopane ratio	FDA Hydrolyzed	SOC concentration
Total PAH hopane ratio	-	0.120	0.867
FDA Hydrolyzed	0.120	-	0.436
SOC concentration	0.867	0.436	-

F3: Pearson Correlation for the Four Oiling Treatment

Table F3: The P-values for Pearson correlation of alkanes in the four oiling treatment

	Total alkane hopane ratio	FDA Hydrolyzed	SOC concentration
Total alkane hopane ratio	-	0.0598	0.3550
FDA Hydrolyzed	0.0598	-	0.2480
SOC concentration	0.3550	0.2480	-

Table F4: The P-values for Pearson correlation of PAHs in the four oiling treatment

	Total PAH hopane ratio	FDA Hydrolyzed	SOC concentration
Total PAH hopane ratio	-	0.1060	0.6440
FDA Hydrolyzed	0.1060	-	0.2480
SOC concentration	0.6440	0.2480	-

VITA

Julius Enock was born on July 14, 1973, in Dar-es-salaam, Tanzania. He graduated from the University of Dar-es-salaam, Tanzania, in November, 1998, with a Bachelor of Science degree in chemical and process engineering.

Following graduation, he worked temporarily with the Institute of Production Innovation (IPI), University of Dar-es-salaam, Tanzania, as a research assistant for about a year. Then, in March, 2000, he joined the Division of Environment in the Vice President's Office (Tanzania), as an Environmental Engineer.

In August, 2000 he was awarded ATLAS (African Training for Leadership and Skills) scholarship to pursue a master's degree in civil engineering, majoring in environmental engineering, at Louisiana State University. He shall graduate in August, 2002.