

# **ANAEROBIC BIODEGRADATION OF CRUDE OIL IN SALT MARSHES**

John H. Pardue

Eun-Ju Lee

Julius Enock

Department of Civil and Environmental Engineering  
Louisiana State University

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## Anaerobic Biodegradation of Crude Oil in Salt Marshes

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### Abstract

The bioremediation of crude oil in marsh systems may represent one of the most promising remedial technologies for these environments. The relative contribution of anaerobic and aerobic processes is, to date, unknown. An experimental study was previously conducted to investigate biodegradation of crude oil components in a *Spartina alterniflora* salt marsh. Results from the field trial suggested that anaerobic biodegradation may be of potential significance. More detailed investigations were necessary to understand the specific role of anaerobic microorganisms in the salt marsh. Microcosm experiments were conducted under strictly anaerobic conditions using the Hungate Technique and inhibitors specific to the sulfate reducing bacteria. Rate and extent of biodegradation were measured referenced to the non-biodegradable marker hopane. Results indicated that alkanes and two PAHs, phenanthrene and pyrene, will biodegrade under anaerobic conditions apparently linked with sulfate reduction. Sulfate reduction rates were simultaneously measured to provide a further confirmation of sulfate reduction activity. Crude oil biodegradation was also monitored in greenhouse core studies using material from the same salt marsh. Three treatments were utilized: non-flooded, tidally (intermittently flooded), and continuously flooded. Although small differences were observed in the biodegradation rates depending on the extent of flooding, complete flooding of the cores did not terminate oil biodegradation. Environmentally relevant degradation rates occurred under anaerobic conditions linked to sulfate reduction. These results strongly suggest that complete natural recovery of crude oil in salt marshes is possible through a combination of aerobic and anaerobic microbial processes.

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### 1.0 Introduction

Bioremediation of crude oil was shown to be a promising cleanup technology following its application in the Alaska *Valdez* spill response. Significant effort has been expended over the past five years to further explore whether bioremediation is a sound

technique for removing crude oil from the marsh environment in Louisiana (Jackson *et al.* 1996; Jackson and Pardue 1997; 1999; Shin *et al.* 2000) and Texas (Bonner *et al.* 1999). As a result of these studies, the potential of crude oil biodegradation is better understood.

Coastal salt marshes are at considerable risk of crude oil contamination from a variety of sources, since major oil production, refining, and transport operations are located near these areas. The efficiency of nutrient stimulated bioremediation of crude oil spills has been studied in a variety of environments (Lee and Levy 1989, 1991; Prince *et al.* 1994). Salt marshes have an intrinsic potential to biodegrade oil spills (Jackson *et al.* 1996; Jackson and Pardue 1999), and enhanced degradation rates have been observed when nutrients are added to laboratory microcosms (Jackson and Pardue 1999).

Salt marshes are inherently anaerobic environments. Much of the mineralization of organic matter in coastal marine sediments (Jorgensen 1978; Jorgensen *et al.* 1990) and salt marshes (Howarth 1979; Howarth and Jorgensen 1984; Howarth and Giblin 1983; Howes *et al.* 1984) occurs through sulfate reduction, in the absence of oxygen. Dissimilatory reduction of sulfur compounds results in the formation of extracellular hydrogen sulfide. Sulfide oxidation by O<sub>2</sub> occurs at the interface between the reduced sediment and the oxidized zone (Jorgensen 1988). The oxidation of these reduced sulfides and the aerobic respiration of natural organic matter in the oxidized zone are the major oxygen sinks in the salt marsh (Howes *et al.* 1984; Jorgensen 1988). An oil spill in a salt marsh would represent an extra carbon source generating additional oxygen demand through aerobic respiration of crude oil components and potentially through the stimulation of sulfate reduction (oxidation of reduced sulfides) (Shin *et al.* 2000).

Biodegradation of crude oil components such as alkanes and PAHs linked to alternate electron acceptors such as sulfate has been recently documented (Rabus *et al.* 1993; Rueter *et al.* 1994; Lovley *et al.* 1995; Coates *et al.* 1996a,b; Zhang and Young 1997; Reinhard *et al.* 1997; Kazumi *et al.* 1997; Caldwell *et al.* 1998; So and Young 1999). In salt marshes, anaerobic sulfate reducing conditions predominate over other terminal electron accepting processes due to the abundance of dissolved sulfate and the higher energy available for microorganisms carrying out sulfate reduction (Shin *et al.* 2000). Recent evidence of anaerobic biodegradation of hydrocarbons was found in crude oil (Caldwell *et al.* 1998; Coates *et al.* 1996a,b; Phelps *et al.* 1996; Rueter *et al.* 1994). Despite these studies, the relative contribution of aerobic and anaerobic processes is unclear in the salt marsh environment.

Many crude oil components are biodegradable in the presence of oxygen; however, a full spectrum of crude oil components has not been examined under anaerobic conditions. Rueter *et al.* (1994) showed that crude oil components could be degraded directly by sulfate reducing bacteria growing under anaerobic conditions in marine sediments. Moderately thermophilic pure cultures selectively utilize *n*-alkanes in oil for sulfate reduction to sulfide. In addition, a mesophilic sulfate reducing bacteria enrichment culture was shown to oxidize alkylbenzenes in oil. The study concluded that sulfate

reducing bacteria using aliphatic and aromatic hydrocarbons as electron donors may represent a significant contribution to crude oil degradation.

Oxidation of [ $^{14}\text{C}$ ] naphthalene and [ $^{14}\text{C}$ ] phenanthrene to  $^{14}\text{CO}_2$  was observed without a detectable lag under strict anaerobic conditions in harbor sediments that were heavily contaminated with PAHs. The results indicate that the self-purification capacity of PAH contaminated sulfate reducing environments may be greater than previously recognized. The potential use of iron oxide to stimulate *in situ* hydrocarbon degradation in anaerobic petroleum contaminated harbor sediments was investigated (Coates *et al.* 1996b).

Successful benzene degradation was observed in enrichment cultures from salt marsh sediments using sulfate as the terminal electron acceptor (Phelps *et al.* 1996). Mineralization was confirmed by release of  $^{14}\text{CO}_2$  from radiolabeled benzene. The dependence of biodegradation on sulfate reduction was demonstrated by stoichiometric balances and the use of the specific inhibitor (molybdate). The result shows that anaerobic benzene degradation takes place when coupled with sulfate reduction.

Caldwell *et al.* (1998) showed the ability of anaerobic microcosms to degrade a wide variety of crude oil components using chronically hydrocarbon contaminated marine sediment. Complete removal of *n*-alkanes ( $\text{C}_{15}\text{-C}_{34}$ ) from weathered oil was observed under sulfate reducing conditions after 201 days of incubation using the Hungate Method (Hungate 1969) to achieve anaerobic conditions. Anaerobic degradation of crude oil components was confirmed by comparing sterile control microcosms and nonsterile microcosms. Nonsterile incubations amended with oil exhausted the available sulfate reserves within 125 days, but sterile controls showed no significant loss of sulfate during incubation. Inhibition of biodegradation by molybdate indicates that sulfate reduction is the major terminal electron acceptor process.

This study was conducted to: (1) evaluate *in situ* bioremediation strategies based on the results of our laboratory studies, and (2) to evaluate the overall potential of crude oil degradation in salt marshes. Different treatments (control, chemically, and biologically inhibited) were conducted to determine whether anaerobic degradation of crude oil naturally occurs in coastal salt marshes. In addition, biodegradation processes were studied by performing simultaneous measurements of crude oil components from greenhouse cores. Sulfate reduction rates were also measured.

## **2.0 Materials and Methods**

### **2.1 Sampling**

#### **2.1.1 Site Description**

The study site was located near Port Fourchon at the southwestern end of the Barataria Basin in Louisiana. The site is the location of large oil recovery, processing,

and shipping operations. This site is continuously flooded, experiences small tidal variation, and is dominated by *Spartina alterniflora*.

### **2.1.2 Petroleum Source**

The standard petroleum used in this study was a “Sweet” Louisiana Crude oil (SLCO). SLCO is a relatively non-toxic oil with high alkane, low polar, and moderate PAH concentration. Phenanthrene and pyrene were spiked (0.003g/ml) to obtain more accurate measurements of degradation rates.

### **2.1.3 Sample Collection**

Soil, sediment, and pore water samples were collected from the study site. The top 10 cm of soil were removed, and samples were collected from the newly uncovered soil. Soil samples were transferred to a bucket, the bucket was sealed tightly with a lid, and the samples were transferred to the laboratory. Pore water was collected from below the marsh surface and stored in a 10 liter Nalgene® bottle. Pore water samples were analyzed for initial sulfate concentrations. Sediment cores were collected using thin walled aluminum core tubes. Sediment columns approximately 20 cm long were taken between the culms of *Spartina alterniflora*. The columns excluded standing above-ground litter during low tide. The cores were transferred to 15 cm-id, 30 cm long, thick walled glass tubes. The cylinders were sealed tightly at the bottom with acrylic caps and transferred to the greenhouse.

## **2.2 Microcosm Study**

### **2.2.1 Microcosm Preparation**

Microcosm slurries 1:10 (soil: water, w/w %) were prepared using soil and water collected from the study site. Soil was homogenized (operators' hands were protected by latex-gloves) and mixed with pore water using a blender. Approximately 120 mL of slurry were placed in sterile 150 mL serum bottles and sealed with Teflon lined silicon septa (Bellco Glass Co.). Slurry construction and amendments were performed by purging the headspace of serum bottles with oxygen free nitrogen gas using the Hungate Technique (Hungate 1969) to maintain anaerobic conditions. The serum bottles were kept in an anaerobic glove box connected to a nitrogen gas tank. The microcosm studies were conducted using three microcosm treatments: (1) replicates of molybdate inhibited, (2) sterilized, and (3) control (no inhibition). The sterilized microcosms were prepared by autoclaving for 30 minutes at 15 psig and 121°C. Twenty mm of sodium molybdate were used to prepare molybdate inhibited microcosms. Molybdate is a specific inhibitor of sulfate reducing bacteria. All experiments were performed in the anaerobic glove box at room temperature. Next, 1.2 mL of crude oil pre-filtered through a bacteria free filter (Nalgene MF75™, Nalgene International Corporation, Rochester, NY) were added to the serum bottle under anaerobic conditions.

### **2.2.2 Anaerobic Condition**

Hungate's Technique and an anaerobic glove box were used to maintain strictly anaerobic conditions. The basis of this method was first developed in Europe in 1950 by Hungate, who used the technique to isolate and study strict anaerobic microorganisms in sewage sludge (Holland 1987). Fundamental to this technique is a supply of oxygen free nitrogen gas. Oxygen free nitrogen gas is prepared by passing the gas through a copper column (Acros Organics, Fisher Scientific, Pittsburgh, PA) held in a glass tube heated to 500°C by heating tape (Cole Parmer, Vernon Hills, IL) (Holland 1987). This process removes any traces of oxygen in the microcosm bottles.

Use of a glove box also helped maintain anaerobic conditions. After use of the Hungate Technique, all prepared microcosm bottles were incubated in an anaerobic glove box (Cole Parmer Instrument Company, Cheltenham, PA). The nitrogen source was connected by tubing and purged to remove residual air from the glove box.

## **2.3 Oil Analysis**

### **2.3.1 Oil Extraction**

Ten mL of slurry were subsampled from the 150 mL microcosm serum bottles and transferred into 50 mL Teflon centrifuge tubes containing 10 mL of hexane/acetone (50/50, v/v %). Each centrifuge tube was labeled and shaken for 48 hours to extract the oil from the slurry. After shaking, each tube was centrifuged for 15 minutes at 3000 rpm. The liquid was separated into aqueous and nonaqueous phases using a separatory funnel. The aqueous phase was then discarded. The nonaqueous phase was passed through sodium sulfate to remove any remaining water and placed in 20 mL scintillation vials. The collected nonaqueous phase of each vial was condensed to a volume of 5 mL under a clean stream of N<sub>2</sub> gas. One mL of this extract was transferred to amber GC/MS autosampler vials and refrigerated until analysis was performed. Ten µL of semivolatile internal standard (Supelco, Bellefonte, PA) were added. The extract was then analyzed on GC/MS using 17 (H), 21 (H)-hopane as a standard marker.

### **2.3.2 GC/MS Analysis**

Crude oil analyses were performed using a Hewlett Packard 5890 gas chromatograph coupled with a 5972A mass spectrometer (GC/MS). Crude oil components were quantified by the selected ion monitoring mode with a semivolatile internal standard. Semivolatile internal standards (2000 µg/mL in methylene chloride) contain the following components: 1,4-dichlorobenzene-d<sub>4</sub>, naphthalene-d<sub>8</sub>, acenaphthene-d<sub>10</sub>, phenanthrene-d<sub>10</sub>, chrysene-d<sub>12</sub>, and perylene-d<sub>12</sub>. Stock crude oil quantitative mix and TCLP aromatic hydrocarbons mix for calibration standards were obtained from Supelco (Bellefonte, PA). Stock pristane (2, 6, 10, 14-tetramethylpentadecane, with purity of 99.5% or greater) was obtained from Alltech Associates Inc. (Deerfield, IL). Rates of hydrocarbon degradation were measured using the hopane ratio technique (Prince *et al.* 1994). Hopanoids are components of oil, which volatilize and biodegrade very slowly as compared to other oil components. Hopane was used as a conservative marker to fingerprint the oil. Hopane has been successfully used as a standard marker (Prince *et al.*

1994), allowing confirmation of *in situ* biodegradation, since it allows a more accurate determination of relative rates and lag periods (Jackson *et al.* 1996).

## 2.4 Sulfate Reduction

### 2.4.1 Measurement of Sulfate Reduction Rate

In addition to monitoring oil disappearance, the rate of sulfate reduction was monitored using labeled sulfate. A two-step method (Fossing and Jorgensen 1989) was used for determination of sulfate reduction rates using  $^{35}\text{SO}_4$ . Sulfate reduction rates were measured in sediment using direct injection of tracer quantities of  $^{35}\text{SO}_4^{2-}$  (Jorgensen 1978). The radiosulfate was purchased from Amersham Corporation (Arlington Heights, IL) in the form of  $\text{Na}_2^{35}\text{SO}_4$ . Using a Hamilton micro-syringe, a volume of three  $\mu\text{L}$  of carrier-free  $^{35}\text{SO}_4^{2-}$  (1.75 Ci) was injected into the serum bottle through the Teflon lined silicon septa. Three replicates of molybdate inhibited, sterilized, and control samples (prepared as described above) were incubated in an anaerobic glove box for 72 hours at  $25^\circ\text{C}$ . Sulfate reduction was terminated by freezing at  $-65^\circ\text{C}$ .

### 2.4.2 Two-Step Method

The total reduced inorganic sulfur (TRIS) = acid volatile sulfur (AVS) + chromium reducible sulfur (CRS) was distilled simultaneously with HCl and  $\text{Cr}^{2+}$  and the amount of  $^{35}\text{SO}_4^{2-}$  reduced was calculated from the total  $\text{H}_2^{35}\text{S}$  released (Shin *et al.* 2000). Preserved samples at  $-65^\circ\text{C}$  were analyzed for  $^{35}\text{S}$  labeled acid volatile sulfides (AVS:  $\text{H}_2\text{S} + \text{FeS}$ ), pore water sulfate, and chromium reducible sulfides (CRS:  $\text{S}^0 + \text{FeS}_2$ ), using a two-step method (Fossing and Jorgensen 1989). Seven mL of 33% HCl were injected into each microcosm bottle through the rubber stopper. The volatilized  $\text{H}_2\text{S}$  was then stripped from the bottle using  $\text{N}_2$  as carrier gas. The  $\text{H}_2\text{S}$  was precipitated in two traps connected in series. The traps consisted of 10 mL of 5% zinc acetate and one drop of antifoam (Antifoam B, Sigma Chemical Co., St. Louis, MO). After purging with  $\text{N}_2$  gas for 15 minutes, the traps were disconnected from the microcosm bottles and refrigerated.

The contents of each microcosm bottle were transferred to 30 mL centrifuge bottles in the anaerobic glove box. The slurry samples were then centrifuged at 3000 rpm for 15 minutes, and the supernatants were used to measure  $^{35}\text{SO}_4^{2-}$  radioactivity and the concentration of  $\text{SO}_4^{2-}$ .  $^{35}\text{SO}_4^{2-}$  radioactivity of 1 mL supernatant was determined by mixing with 10 mL of Eco-Lite liquid scintillation cocktail (ICN Pharmaceuticals Inc., Aurora, OH). The radioactivity of  $^{35}\text{S}$  was assayed with a liquid scintillation counter (Beckman LS 6000SC). Pore water samples were collected by centrifuging the slurries from three to four parallel microcosms. The concentration of unlabelled  $\text{SO}_4^{2-}$  was determined using a Hach DR 2010 spectrophotometer (Hach DR 2010, Hach Co., Loveland, CO).

The water content of the slurry was determined by weight loss after drying to a constant weight of  $105^\circ\text{C}$ . The water content was used to convert concentration dimensions so that all results were represented as per unit weight of soil. The reduced  $^{35}\text{S}$

in the sediment pellet was mixed and transferred into a round bottom boiling flask, then mixed with 8 mL of distilled water and 8 ml of ethanol. The refrigerated traps were connected. The traps were prepared as for AVS, containing 10 mL of 5% zinc acetate and one drop of antifoam. The reaction flask was degassed with N<sub>2</sub> gas for 15 minutes; 8 mL of 12 N HCl and 16 mL of 1 M Cr<sup>2+</sup> in 1.0 N HCl were then added. The mixture was boiled and stirred for 45 minutes. H<sub>2</sub>S distilled from CRS was collected in the zinc acetate traps. The total reduced inorganic sulfur (TRIS = AVS + CRS) was distilled as H<sub>2</sub><sup>35</sup>S and precipitated as ZnS in the zinc acetate (ZnAc) traps. The ZnS was then suspended using a vortex mixer, 5 mL of subsample were withdrawn, mixed with 10 mL of Eco-Lite liquid scintillation cocktail (ICN Pharmaceuticals Inc., Aurora, OH), and the radioactivity of the <sup>35</sup>S was assayed with a liquid scintillation counter (Beckman LS 6000SC). The Sulfate Reduction Rate (SRR) was calculated in each time step using the following equation (Fossing and Jorgensen 1989):

where  $a_{ZnS}$  is the radioactivity of sulfide (ZnS) (cpm),  $A_{SO_4^{2-}}$  is the total radioactivity of sulfate (<sup>35</sup>SO<sub>4</sub><sup>2-</sup>) after incubation (cpm),  $C_{SO_4^{2-}}$  is the sulfate concentration (mg/L),  $t$  is the incubation time in hours, and 1.06 is the correction factor for the expected isotope fractionation.

### 2.4.3 Chromium Reduction

The Cr<sup>2+</sup> solution was prepared by reduction of 1 M CrCl<sub>3</sub>·6H<sub>2</sub>O in 1 N HCl by amalgamated zinc. Amalgamated zinc was prepared by stirring 50 g of 20 mesh granular zinc (Sigma Chemical Co., St. Louis, MO) in 20 mL of 3N HCl for 30 seconds, then adding 20 mL of 0.013 M HgCl<sub>2</sub> and stirring for three minutes. The amalgamated zinc was rinsed with distilled water and dried. In a glass bottle, 50 to 100 mL of 1M CrCl<sub>3</sub>·6H<sub>2</sub>O in 1 N HCl were added to the amalgamated zinc to produce the Cr<sup>2+</sup>. While continuously flushing with N<sub>2</sub>, the solution changed color from dark green (Cr<sup>3+</sup>) to bright sky blue (Cr<sup>2+</sup>). Complete reduction of chromium was indicated by color change to sky blue. The used amalgamated zinc can be reused by rinsing with 1N HCl.

## 2.5 Core Study

### 2.5.1 Core Preparation

In parallel to the microcosm study, a core study was conducted to investigate the effect of the tidal cycle on the biodegradation of crude oil in coastal salt marshes. Collected sediment cores were transferred to the greenhouse. Sediment columns were approximately 20 cm long and fully saturated with pore water samples collected from the study site. Sediment cores, taken between the culms of *Spartina alterniflora*, were collected using thin walled aluminum core tubes to minimize compaction. The cores were

transferred into 15 cm id, 30cm long, thick walled glass tubes before being transported to the greenhouse. The air temperature of the greenhouse was  $22 \pm 4^{\circ}\text{C}$ .

Nine sediment columns were set up; each was spiked with 35mL of weathered crude oil and left for seven days for pre-equilibration. The column sides were wrapped with aluminum foil to repel light and minimize algae growth. The core studies were conducted using three treatments conducted in triplicate: (1) non-flooded (NF), (2) intermittently flooded (IF), and (3) continuously flooded (CF) regimes. The CF cores were flooded with approximately 5 cm of sea water for the duration of the experiments. The IF cores were intermediately flooded with sea water for two days and drained for two days alternately. The water was drained from the cores by siphoning with a small diameter tube. Water in the NF cores was flush with the sediment surface, with only enough liquid to wet the cores. To compensate for water evaporation from the cores, artificial sea water was added daily, depending on the respective flooding treatment. Samples were taken every 20 days.

Sediment samples were taken from the intact cores by scooping with a knife and removing a section approximately 2 cm square and 5 cm deep. The sampled cavities in the cores were then refilled with sand and a marker was placed to label each sampled spot. Care was taken not to take repeated samples from the same plots of soil.

### **2.5.2 Oil Analysis**

Subsamples were taken to extract remaining oil components using liquid extraction with hexane: acetone (1:1, v/v%). The extracts were passed through  $\text{NaSO}_4$  to remove water and concentrated under a stream of dry nitrogen. The oil extracts were analyzed by GC/MS using 17 (H), 21 (H)-hopane as a normalizing compound (Prince *et al.* 1994), a method which allowed calculation of loss due to biodegradation only. Oil extraction and GC/MS analysis were described in Section 2.3.

## **3.0 Results and Discussion**

### **3.1 Microcosm Study**

Microcosm studies were conducted using replicates for each treatment to determine degradation of PAHs and alkanes under sulfate reducing conditions. The microcosm studies demonstrated slow but measurable degradation of alkanes in the control treatment (no inhibition), while crude oil alkane components in molybdate inhibited and sterilized treatments were not degraded as measured by the total hopane ratio of the alkanes (Figure 3.1). As previously described for alkanes, biodegradation of PAHs was observed over the time frame of the study in control samples (Figure 3.2).

The rate of alkane and PAH degradation differed between treatments. The control treatment degraded with an observed first-order rate constant of  $0.006 \text{ day}^{-1}$ . Reductions in alkane hopane ratios from ~95 to ~55 were observed over 80 days. Reductions in PAH hopane ratios from ~22 to ~11 were observed over 80 days. Between the initiation of the experiment and 80 days, approximately 60% and 50% of the alkanes and PAHs

biodegraded respectively (as referenced to the non-biodegradable hopane). No biodegradation was detected in samples amended with molybdate or in the killed controls. The inhibition of degradation after addition of molybdate strongly suggests that sulfate reducers are responsible for degradation, since their activity is inhibited by molybdate. In the field, sulfate is regularly replenished by tidal action and flooding of sulfate rich waters. These data confirm the findings of other microbial studies that linked sulfate reduction to degradation of crude oil. The experimental results further confirm that the sulfate reduction process is important to the natural recovery of salt marshes following an oil spill.

**Figure 3.1** *Anaerobic biodegradation of alkane in salt marsh microcosms.*

**Figure 3.2** *Anaerobic biodegradation of PAH in salt marsh microcosms.*

These data suggest that anaerobic microbes indigenous to salt marsh soils can degrade both alkanes and PAHs under anaerobic conditions. Similar results were reported by Jackson *et al.* (1996), who found that salt marsh soils were capable of hydrocarbon degradation and that fertilizers affected degradation rates of some compounds. Jackson *et al.* found a slow decrease in alkanes in the unfertilized treatment.

Caldwell *et al.* (1998) reported that crude oil components were degraded anaerobically within 201 days of incubation when sulfate reduction was the predominant electron accepting process. Complete removal of *n*-alkanes (C<sub>15</sub>-C<sub>34</sub>) was observed by gas chromatography analysis. Caldwell *et al.* confirmed anaerobic degradation of crude oil components by comparing sterile control microcosms and nonsterile microcosms.

The rate of phenanthrene and pyrene degradation also differed between treatments. The control treatment degraded with an observed first-order rate constant of 0.006 day<sup>-1</sup>. Reductions in phenanthrene hopane ratios from ~7 to ~2 were observed over 80 days. Reductions in pyrene hopane ratios from ~7 to ~2 were observed over 80 days. Between the initiation of the experiment and 80 days, approximately 70% of the phenanthrene and pyrene biodegraded. No biodegradation was observed in molybdate inhibited and

sterilized treatments. The phenanthrene hopane ratio and pyrene hopane ratio decrease is presented in Figures 3.3 and 3.4.

### 3.2 Degradation Rate Constants

Rate constants for the degradation of alkane and PAH compounds were determined using non-linear regression. The decrease in alkane concentrations was fitted using non-linear regression of a first-order exponential decay equation. The coefficient of determination for the fit was determined. The coefficient of determination,  $r^2$ , describes the fraction of the variability explained by the model. The degradation rate constant ( $k$ ) was calculated using the following equation:

where  $C$  is the substrate's hopane ratio,  $C_0$  is the initial hopane ratio,  $k$  is the degradation rate constant,  $\text{day}^{-1}$ , and  $t$  is the time, days. Degradation rate constants were calculated as  $0.006 \text{ day}^{-1}$  ( $r^2 = 0.99$ ) for both the total alkane hopane ratio and PAH hopane ratio. An  $r^2$  of 0.99 means that approximately 99 % of the variation in total alkane and PAH concentration can be attributed to first order degradation with the given rate constants. In salt marshes, summed alkanes and PAHs degraded slowly over 80 days at a degradation rate constant of  $0.006 \text{ day}^{-1}$ . This rate constant is similar to oil degradation rate constants observed in other microcosm experiments. Pardue *et al.* (1998) investigated the mineralization of hydrocarbons in Louisiana salt marsh soils using microcosms. Hydrocarbon degradation kinetics using  $^{14}\text{C}$ -labelled hexadecane and phenanthrene were determined. Degradation of alkane ( $\text{C}_{11}$ - $\text{C}_{14}$ ), pristane, and parent  $\text{C}_1$ - and  $\text{C}_2$ -naphthalene and phenanthrene was monitored. Degradation curves were analyzed by non-linear regression using first order kinetics. The curve analysis showed that salt marsh soils had slower kinetics for higher chain alkanes ( $> \text{C}_{17}$ ) in crude oil. Shorter chain alkanes ( $\text{C}_{11}$ - $\text{C}_{17}$ ) degraded rapidly in control microcosms. Rate constants ( $0.0032 \text{ day}^{-1}$ ) are similar to this study for certain alkane component degradation.

**Figure 3.3** *Anaerobic biodegradation of phenanthrene in salt marsh microcosms.*

**Figure 3.4** *Anaerobic biodegradation of pyrene in salt marsh microcosms.*

Tate (1998) demonstrated that the biodegradation of the *n*-alkane, pristane, and phytane fraction of a sweet Louisiana crude oil was modeled as first order decay with a rate of  $0.0054 \text{ day}^{-1}$ . These results are very similar to those found in this study, not only for the total alkane degradation rate but also for degradation rates for individual alkane components.

### **3.3 Sulfate Reduction Study**

The initial concentration of sulfate was 12.5 mM (~1500 ppm). The sulfate concentration decreased in the control treatment, while no significant decreases were observed in molybdate inhibited and sterilized treatments. In the control treatment, sulfate concentration significantly decreased from 12.5 mM to 4.8 mM over the time course of the study. This decrease is due to consumption of sulfate as an electron acceptor by sulfate reducing anaerobic microorganisms.

Sulfate reduction rate experiments were conducted to determine whether sulfate reduction occurred in the microcosms and to determine sulfate reduction rates during the anaerobic crude oil degradation study. The results of these experiments showed that

sulfate reduction was occurring during the crude oil degradation study. The sulfate reduction rates over time in the salt marsh microcosms are presented in Figure 3.5. The total reduced inorganic sulfur (TRIS,  $\mu\text{mol/g-soil/d}$ ) increased over time. The TRIS generated over time was approximately equal to the sulfate reduced over time on a molar basis.

Measured sulfate reduction rates increased from 0.1 to  $\sim 1.75$   $\mu\text{moles/g}$  of soil/day over the incubation time. Sulfate reduction rates increased continuously over time. Sulfate reduction rates were less than 0.1  $\mu\text{moles/g}$  of soil/day in molybdate inhibited and sterilized experiments.) Inhibition of metabolism by molybdate (20 mM) implicated sulfate reduction as the terminal electron acceptor process in the incubation. Sulfate consumed during the incubation period was 8.7 mM over 80 days.

Most research regarding sulfate reduction rates has been conducted in marine and brackish marsh environments where sulfate concentration and reduction rates are higher than in fresh water environments. Fossing and Jorgensen (1989) observed sulfate reduction rates of  $60.6 \pm 1.1$  ( $\text{nmol/cm}^3/\text{d}$ ) in fresh water (Odder stream) sediment in Denmark using a single-step chromium reduction method. This value is lower than rates found in this study since sulfate concentration and reduction rates in fresh water are lower than in salt marshes. Most research has focused on determining which terminal electron accepting processes (TEAPs) are dominant during contaminant degradation (Lovely *et al.* 1995; Coates *et al.* 1996a; Coates *et al.* 1996b). Kazumi *et al.* (1997) measured consumption of sulfate with benzene metabolism simultaneously and concluded that sulfate was the electron acceptor for benzene oxidation. Caldwell *et al.* (1998) demonstrated that sulfate reduction occurred with *n*-alkane degradation under anaerobic conditions.

**Figure 3.5** *Sulfate reduced over time in salt marsh microcosms.*

### **3.4 Molybdate Inhibition**

Molybdate inhibited treatments were conducted each time crude oil microcosms were constructed to explore the roles of sulfate reducing bacteria in mineralization. Twenty mM of molybdate was added as described in Edward *et al.* (1992b) and Phelps *et al.* (1996) to inhibit sulfate reduction. Molybdate is a specific inhibitor of sulfate reduction activity,  $\text{MoO}_4^{2-}$  (Odom and Singleton 1993). Molybdate ( $\text{MoO}_4^{2-}$ ) has a competitive interaction with sulfate ( $\text{SO}_4^{2-}$ ), which inhibits sulfate reducers (Odom and Singleton 1993). Crude oil degradation was consistently inhibited by addition of molybdate. These results indicate that sulfate was the predominant terminal electron accepting process (TEAP) for anaerobic crude oil degradation in salt marsh microcosms.

### **3.5 Stoichiometry**

The purpose of this study was to determine whether anaerobic crude oil degradation is linked to sulfate reduction in coastal salt marshes. The results of these experiments showed that sulfate reduction occurred during the crude oil degradation study. The rate of alkane degradation differed significantly between treatments. The lack of degradation in

the molybdate inhibited treatments is evidence that sulfate reducing bacteria are responsible.

Stoichiometry was also used to determine whether the microbial oil degradation was linked to sulfate reduction. The amount of sulfate reduced during alkane degradation was compared to the amount predicted from the theoretical stoichiometric equations in Table 3.1.

**Table 3.1** Theoretical stoichiometric equations of individual alkane compounds

Compound	Theoretical stoichiometric equation	Sulfate consumed (mM)
Decane	$C_{10}H_{22} + 7.75 SO_4^{2-} + 15.5 H^+$ -> $10 CO_2 + 7.75 H_2S + 11 H_2O$	0.20
Tridecane	$C_{13}H_{28} + 10.00 SO_4^{2-} + 20.0 H^+$ -> $13 CO_2 + 10.00 H_2S + 14 H_2O$	0.26
Hexadecane	$C_{16}H_{34} + 12.25 SO_4^{2-} + 24.5 H^+$ -> $16 CO_2 + 12.25 H_2S + 17 H_2O$	0.61
Heptadecane	$C_{17}H_{36} + 13.00 SO_4^{2-} + 26.0 H^+$ -> $17 CO_2 + 13.00 H_2S + 18 H_2O$	0.61
Octadecane	$C_{18}H_{38} + 13.75 SO_4^{2-} + 27.5 H^+$ -> $18 CO_2 + 13.75 H_2S + 19 H_2O$	0.89
<i>n</i> -eicosane	$C_{20}H_{42} + 15.25 SO_4^{2-} + 30.5 H^+$ -> $20 CO_2 + 15.25 H_2S + 21 H_2O$	0.72
<i>n</i> -octacosane	$C_{28}H_{58} + 21.25 SO_4^{2-} + 42.5 H^+$ -> $28 CO_2 + 21.25 H_2S + 29 H_2O$	0.49
Total Alkanes	-	8.20

Sulfate consumed during the incubation period was 8.7 mM over 80 days. Based on stoichiometry, theoretical sulfate consumed during the total alkane degradation was 8.2 mM. For instance, 12.25 moles of sulfate are required to metabolize one mole of hexadecane.

Stoichiometry also indicated that alkane degradation occurred when sulfate was the terminal electron acceptor. Concentrations of alkanes were determined by GC/MS. Sulfate loss was then measured using the spectrophotometer. Sulfate loss occurred due to metabolism of carbon compounds present in crude oil. Results suggest that alkane mineralized to carbon dioxide, and that sulfate reduction is the terminal electron accepting process involved.

### 3.6 Core Study

The intent of this study was to evaluate the effect of flooding on crude oil degradation in salt marshes. The residual alkane and PAH concentrations were expressed relative to hopane concentration. After 100 days, the alkanes decreased 96.9%, 92.4%, and 90.9% in the NF, IF, and CF core soils, respectively. PAHs decreased by 96.9%, 78.6%, and 75.6% in the NF, IF, and CF core soils, respectively. This suggests that some biodegradation of the crude oil was occurring, with the effect of flooding being more pronounced for the PAHs than for the alkanes. The degradation profiles for both alkanes and PAHs (Figure 3.6 and 3.7), indicate that the non-flooded (NF) soil had a high inherent potential to degrade crude oil as compared to continuously flooded (CF) and intermittently flooded (IF) soils. The PAHs show higher degradation potential than the n-alkanes for both flooding regimes (Table 2). Unexpectedly, higher PAH degradation potential was observed in CF and IF than in NF cores.

In addition, the oil degradation rates under tidal and continuously flooding regimes were not found to be statistically different at  $\alpha = 0.05$  level of significance for both alkanes and PAHs. However, there was a statistically significant difference between CF and NF as well as IF and NF. Replicate cores for the same flooding regime did not differ in their biodegradation rates. This reflects a satisfactory reproducibility within individual treatments.

**Table 3.2** Summary results for first-order degradation rate constants for alkanes and PAH

		<u>Alkanes</u>			<u>PAH</u>	
<u>Treatment</u>	<u>k</u>	<u>Prob &gt;F</u>	<u>R<sup>2</sup></u>	<u>k</u>	<u>Prob &gt;F</u>	<u>R<sup>2</sup></u>
Non-flooded (NF)	0.028	0.007	0.90	0.045	0.005	0.87
Intermittently-flooded (IF)	0.021	0.003	0.79	0.110	0.021	0.88
Continuously-flooded (CF)	0.019	0.002	0.96	0.111	0.002	0.94

**Figure 3.6** *Alkane biodegradation in cores.*

**Figure 3.7** *PAH biodegradation in cores.*

### **3.7 Effect of Intact Core Flooding**

The residual petroleum hydrocarbon profiles signify the effect of flooding on the biodegradation of crude oil in salt marshes. The non-flooded (NF) cores had inherently higher crude oil degradation potential compared to intermittently flooded (IF) and continuously flooded (CF) cores, in that order. It is known that flooding may prevent oxygen from diffusing to the soil, increase nutrient inputs, dilute salinities, and may well affect the soil pH (Taylor III 1995).

The lack of oxygen to support aerobic metabolism is probably the most important factor influencing microbial activity in crude oil contaminated marine sediments (Hambrick 1979; Shin 1999). The NF cores have higher oxygen availability (redox potential) because the soil is more exposed, allowing more oxygen to reach the soil. The IF and CF cores, by contrast, were characterized by a water column over the soil surface. The highest redox potentials in wetland soils are found in the top 1 to 2 cm layer (Taylor III 1995; Shin 1999). Therefore, oil biodegradation can be expected to occur most quickly in this top layer, keeping in mind that less oxygen is available during flooding. It is estimated that with water as the oxygen carrier from the air to the soil, air will supply

about 8 mg O<sub>2</sub>/L water, and that about 400,000 kg of water would be required to degrade 1 kg of hydrocarbon (Riser-Roberts 1998). This indicates that oxygen may have been limited during flooding of the intact cores, with anaerobic conditions prevailing.

In addition, other factors may have contributed to the observed patterns. Based on the microcosm result, anaerobic degradation linked to sulfate reduction can explain much of the biodegradation observed in the cores. The kinetic rate constants are similar. Results are consistent with aerobic and anaerobic conditions occurring in the NF cores and strictly anaerobic conditions in the CF cores.

The higher biodegradation rate in NF cores may have been supported by attachment of the petroleum hydrocarbons onto the soil sediments, which facilitated direct contact with microbes (de Jorge *et al.* 1997; Carlsson 1998). This did not happen to the same extent in the CF and IF cores, in which a relatively greater portion of the petroleum hydrocarbons was suspended in the water column. The adsorption of crude oil components onto the salt marsh sediments may be of significance given that soil attached bacteria can generally be two to three times more metabolically active than 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, thus forming a biofilm.

In addition, the observed crude oil degradation trend may be related to organic matter decomposition, which is known to be affected by flooding. The decomposition rate of organic matter in flooded soil tends to be only about half the rate in non-flooded soil (Riser-Roberts 1998). This is due to the anaerobic conditions present during flooding, which result in lower microbial growth yield (mass). This, in turn, reduces the microbial population available to decompose organic matter and, by extension, petroleum hydrocarbons. Furthermore, the high concentrations of ethanol and hydrogen sulfide commonly produced in waterlogged soils can be toxic to some microbial species. These conditions can also slow microbial activity.

### **3.8 Implication for an Oil Spill**

In the field, fertilizer addition had a negligible impact on the observed biodegradation of the crude oil components monitored. Simultaneous measurements of areal oxygen uptake and sulfate reduction rates during the field trial suggest that both aerobic and anaerobic mechanisms contributed to natural recovery in salt marshes. The sulfate reduction rate increased in the serum bottle study. Alkane and PAH components were both degraded. Relative rates of PAH/alkane degradation are of interest since PAHs are more toxic than alkanes. PAH/alkane ratios (Table 3.3) did indicate increasing toxicity in the continuously flooded cores, but this was largely mitigated as time progressed.

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**Table 3.3** Relative changes in PAH content in cores.

Time (Days)	PAH/Alkane, Flooded	PAH/Alkane, Non-flooded
0	0.22	0.22
20	0.26	0.36
40	0.36	0.58
60	1.2	0.38
80	0.60	0.25

#### 4.0 Conclusion

The objectives of this study were to determine: (1) which oil components can be degraded anaerobically in salt marshes, (2) the associated degradation rates, and (3) the sulfate reduction rates at which degradation occurs to support the anaerobic oil degradation.

Based on experimental results concerning the anaerobic degradation of hydrocarbons under sulfate reducing conditions, crude oil, both alkanes, and PAHs were microbially degraded in the control treatment microcosms under anaerobic conditions at a first order rate constant of  $0.006 \text{ day}^{-1}$ . Crude oil was not degraded in molybdate inhibited and sterilized treatment microcosms. In terms of anaerobic alkane degradation, there were significant statistical differences in the control treatment compared with molybdate inhibited and sterilized treatments.

Sulfate concentrations decreased significantly in the control treatment, but were stable in molybdate inhibited and sterilized treatments. Sulfate reduction rates increased up to  $1.75 \mu\text{moles/g of soil/day}$  throughout the study as determined by  $^{35}\text{S}$  techniques. Alkane and PAH degradation were linked to sulfate reduction under strictly anaerobic conditions. Sulfate was used as the terminal electron acceptor. Evidence includes molybdate inhibition of the processes and independent measurements of sulfate reduction.

Natural recovery was faster under non-flooded and intermittently flooded (tidal) conditions than under continuously flooded conditions. Even when the system was continuously flooded under anaerobic conditions, biodegradation of alkanes and PAHs proceeded. The salt marshes investigated showed higher biodegradation potential for PAHs than *n*-alkanes in NF, IF, and CF cores. However, the effect of flooding was more pronounced on the biodegradation of PAHs than of alkanes. The results from this study reflect that the pattern and frequency of flooding may influence the biodegradation of crude oil in tidal salt marshes. Field trials are needed to widen our ability to predict the effect of flooding on the fate of crude oil in coastal marshes.

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