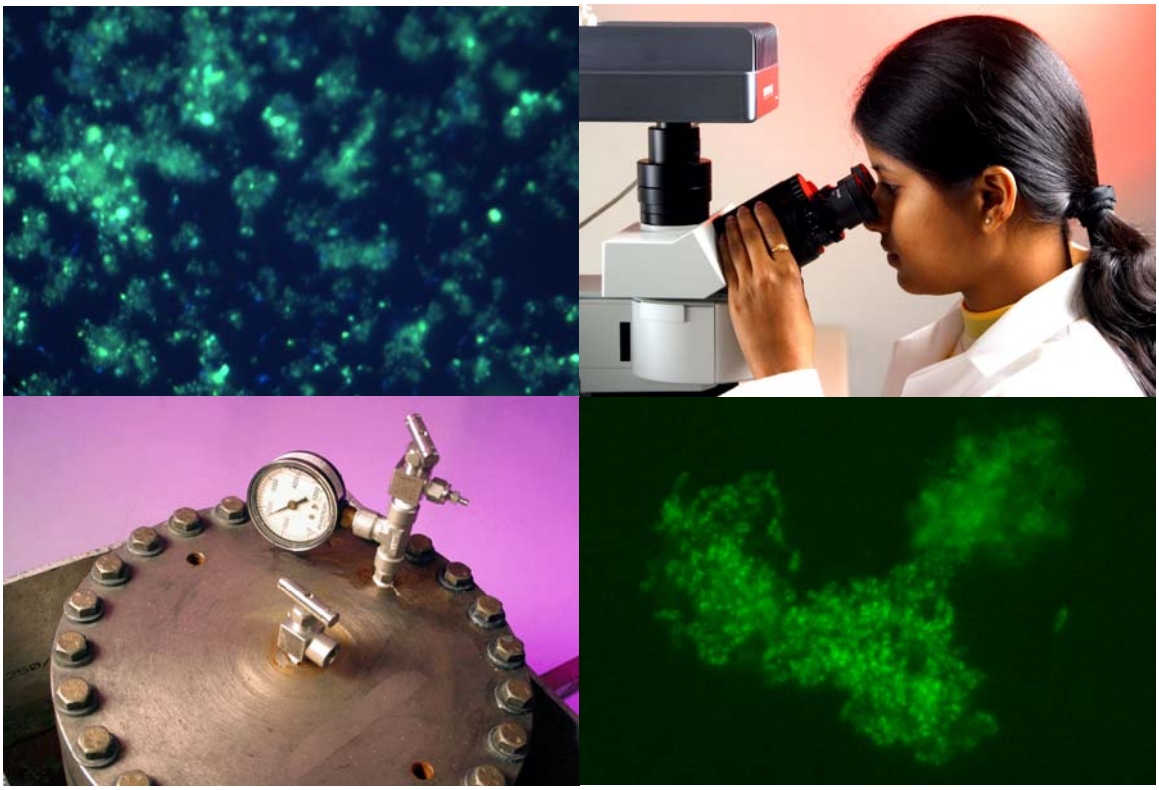
OCS Study
MMS 2006-028

Degradation of Synthetic-Based Drilling Mud Base Fluids by Gulf of Mexico Sediments

Final Report



MMS U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region

OCS Study
MMS 2006-028

Degradation of Synthetic-Based Drilling Mud Base Fluids by Gulf of Mexico Sediments

Final Report

Authors

Deborah J. Roberts
Alan H. Nguyen

Prepared under MMS Contract
1435-01-01-CA-31179
by
University of Houston
Department of Civil and
Environmental Engineering
N107 Engineering Bldg. 1
Houston, Texas 77204-4003

Published by

U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region

New Orleans
May 2006

DISCLAIMER

This report was prepared under contract between the Minerals Management Service (MMS) and the University of Houston. This report has been technically reviewed by the MMS, and it has been approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the MMS, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. It is, however, exempt from review and compliance with MMS editorial standards.

REPORT AVAILABILITY

Extra copies of this report may be obtained by the Public Information Office (Mail Stop 5034) at the following address:

U.S. Department of the Interior
Minerals Management Service
Gulf of Mexico OCS Region
Public Information Office (MS 5034)
1201 Elmwood Park Boulevard
New Orleans, Louisiana 70123-2394

Telephone: (504) 736-2519 or
1-800-200-GULF

CITATION

Suggested citation:

> Roberts, D.J. and A.H. Nguyen. 2006. Degradation of synthetic-based drilling mud base fluids by Gulf of Mexico sediments: Final report. U.S. Dept. of the Interior, Minerals Management Service. Gulf of Mexico OCS Region, New Orleans, LA. OCS Study MMS 2006-028. 122 pp.

ABOUT THE COVER

The cover art depicts a fluorescence photomicrograph of a culture of *Desulfovibrio salexigens* visualized using fluorescence in situ hybridization (top left), a student performing fluorescence microscopy on sediment samples (top right), the pressure chamber designed and constructed to allow the incubation of sediment samples at high hydrostatic pressures (bottom left), and a fluorescence photomicrograph of Gulf of Mexico sediment stained with DAPI (bottom right). The photograph of the pressure chamber and student at the microscope were taken by Jeff Shaw, the Cullen College of Engineering photographer.

ABSTRACT

An important consideration in the environmental assessment of deep-sea drilling operations in the Gulf of Mexico is the discharge of cuttings coated with synthetic-based fluids (SBF). Synthetic-based fluids are one type of nonaqueous drilling fluid and are used in drilling mud to lubricate the drill bit, control reservoir pressure, and bring rock chips, or cuttings, to the surface. Synthetic-based fluids, which can be composed of linear alpha olefins, internal olefins, esters, or paraffins, are released into the marine environment as a residue on the cuttings as they are discharged. This study addressed the fate of the synthetic base fluid portion of the drilling mud in Gulf of Mexico sediments by determining the potential of marine sediment microbes to degrade representative SBF under deep-sea conditions. A model to predict how fast the sediment will recover under realistic conditions was developed to form a scientific basis for evaluating impacts from the discharge of SBF in the deep-sea. This study also examined the effect of the discharge on the microbial ecology of the sediments.

Sediments collected from three locations in the Gulf of Mexico, 66 m to 1135 m depth, near field (NF) and far field (FF) locations (relative to drill sites) were used in a modified Closed Bottle Test (CBT) method to measure degradation rates of surrogate SBF tetradecene and ethyl oleate. The sediments were incubated at 4°C and under a hydrostatic pressure equal to that of the depth they were taken from, or at atmospheric pressure. The microbial population in the sediments was characterized by fluorescent in-situ hybridization (FISH) and most-probable number (MPN). A model that can be used to predict the fate of SBF deposited to the floor of the Gulf of Mexico was developed.

Analyses of the microbial ecology of the sediment were not as useful as had been hoped due to interference of natural sediment components with the methods used for analyses. The results did show an increase in the number of sulfate reducing bacteria (SRB) present in sediments that had been exposed to SBF (comparing impacted samples with unimpacted) or incubated in the presence of surrogate SBF tetradecene or ethyl oleate.

The study showed that the CBT could be adapted to reflect deep-sea pressure and demonstrated the activities of cold tolerant, anaerobic microorganisms from the Gulf of Mexico sediments for degrading surrogate SBF components tetradecene and ethyl oleate. The results showed that the degradation of ethyl oleate and tetradecene by sediment organisms was not dependant on incubation pressure. Sulfate was determined to be the major electron acceptor involved in the degradation process in contaminated sediments. The anaerobic incubations revealed that the removal of ethyl oleate from a contaminated site could be described using a first order k value of -0.22 ± 0.02 week^{-1}. The removal of ethyl oleate from an uncontaminated site would occur at a rate of -0.11 ± 0.02 week^{-1}. A lag time for ethyl oleate removal is predicted to be between 0 and 11 weeks. The average first order decay coefficient (k value) for the removal of tetradecene linked to sulfate reduction was -0.05 ± 0.01 week^{-1}. Tetradecene degradation typically required a much longer lag period (4-28 weeks).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS AND SYMBOLS

Abbreviations

AO	acridine orange
AODC	acridine orange direct counts
API	American Petroleum Institute
ARCH915	oligonucleotide probe for Archaebacteria
ATCC	American type culture collection
BAA	broad agency announcement
CBT	closed bottle test
CLOST I	oligonucleotide probe for Clostridia
Co-A	coenzyme A
DAPI	4'-6-diamino-2-phenylindole
DNA	deoxyribonucleic acid
DOE	U.S. Department of Energy
EDTA	ethylenediaminetetraacetic acid
EB963	Ewing Bank Block 963
EI346	Eugene Island Block 346
EPA	U.S. Environmental Protection Agency
Eub 338	oligonucleotide probe for eubacteria
EMOBF	enhanced mineral oil based fluids
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate fluorochrome
FF	far field (relative to drill site location)
GA	general anaerobes
GC112	Green Canyon Block 112
GC-FID	gas chromatography with flame ionization detector
GC-MS	gas chromatography with mass spectrometry
GOM	Gulf of Mexico
HP	Hewlett Packard
ID	inner diameter
IO	internal olefin
LA	lag time adjusted
LHS	left hand side of an equation
LAO	linear alpha olefin
MC496	Mississippi Canyon Block 496
MMS	Minerals Management Service
MPN	most probable number
MP 288	Main Pass Block 288
MP299	Main Pass Block 299
na	analysis not applicable
NABF	nonaqueous base fluid
NADF	nonaqueous-based drilling fluid
NF	near field (relative to drill site location)
NL	not linked

NOUR	natural oxygen uptake rate
NPDES	nonpoint discharge elimination system
OBF	oil-based fluid
OCS	Outer Continental Shelf
OS	original supernatant
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	negative log of the hydrogen ion concentration
RHS	right hand side of an equation
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RPD	redox potential discontinuity
SBF	synthetic base fluids
SBM	drilling mud made using synthetic base fluids
SC	stoichiometric coefficient
SD	standard deviation
SRB	sulfate reducing bacteria
SRB 385	oligonucleotide probe for sulfate reducing bacteria
T_H	hybridization temperature
TGY	tryptone, glucose yeast extract (microbial medium)
TRITC	Texas red fluorochrome
UH	University of Houston
U.S.	United States
UV	Ultraviolet
VBA	visual basic computer language
VK916	Viosca Knoll Block 916
WBF	water-based fluid

Symbols

Δx	depth increment (cm)
Δt	time increment (week, month)
ε	porosity
ρ_s	density of sediment (kg/L)
θ	turtuosity ($1/\varepsilon$)
a	ion specific diffusion coefficient ($cm^2\ s^{-1}\ °C^{-1}$)
C	general term for concentration of substrate in sediment or water (mg/kg or mg/L)
$coeff_{db}$	coefficient of exponential bioturbation decrease
D	general molecular diffusion coefficient
D^O	general molecular diffusion coefficient at 0 degrees
D^T	general molecular diffusion coefficient at temperature T
D_E	general effective diffusion coefficient
$D`_O$	diffusion coefficient of O_2 in sea water (cm^2/d)
$D`_{SO4}$	diffusion coefficient of SO_4 in sea water (cm^2/d)

D_O	diffusion coefficient of O_2 in sediment pore water (cm^2/d)
D_{SO4}	diffusion coefficient of SO_4 in sediment pore water (cm^2/d)
D_{b0}	bioturbation coefficient in bioburbated layer (cm^2/d)
F	SBF or surrogate compounds concentration (mg/kg dry sediment)
k	general first order decay coefficient ($time^{-1}$)
$k_{F,O2}$	first order decay coefficient of SBF in aerobic condition ($time^{-1}$)
$k_{F,SO4}$	first order decay coefficient of SBF in anaerobic condition ($time^{-1}$)
K_{ow}	octanol water partition coefficient (dimensionless concentration unit ratio)
K_s	general half saturation constant (mg/kg or mg/L)
$K_{s,1}$	half-saturation concentration of F for aerobic degradation (mg/kg)
$K_{s,2}$	half-saturation concentration of F for anoxic (SO_4) deg. (mg/kg)
$K_{s,3}$	half-saturation concentration of F for anoxic (CO_2) deg. (mg/kg)
Ks,o	O_2 limitation for oxygen consumption (mg/L)
$K_{S,SO4}$	SO_4 limitation for sulfate utilization (mg/L)
$K`_{O,SO4}$	O_2 inhibition of SO_4 reduction (mg/L)
$K`_{O,CO2}$	O_2 inhibition of methanogenesis (mg/L)
$K`_{SO4,CO2}$	SO_4 inhibition of methanogenesis (mg/L)
O	oxygen concentration (mg/L or mM pore water)
r^2	linear correlation coefficient
$r_{F,O}$	rate of base fluid degradation in the oxygen utilizing layer
$r_{F,SO4}$	rate of base fluid degradation in the sulfate utilizing layer
$r_{F,CO2}$	rate of base fluid degradation in the methanogenic layer
r_S	rate of substrate utilization
S	sulfate concentration (mg/L or Mm pore water)
SC	stoichiometric coefficient
T	temperature (°C typically)
T_H	hybridization temperature
t	time (week, month, year)
μ	general term for maximum specific cell growth rate
μ_1	maximum specific growth rate (O_2) (d^{-1})
μ_2	maximum specific growth rate (SO_4) (d^{-1})
μ_3	maximum specific growth rate (CO_2) (d^{-1})
x_b	thickness of constant bioturbation layer
X	general term for cell concentration
$X_{1,O}$	initial aerobic cell concentration (mg/kg) (extra subscripts $_{I, F}$ indicate initial and final concentrations)
$X_{2,O}$	initial anaerobic (SO_4) cell concentration (mg/kg) (extra subscripts $_{I, F}$ indicate initial and final concentrations)
$X_{3,O}$	initial anaerobic (CO_2) cell concentration (mg/kg) (extra subscripts $_{I, F}$ indicate initial and final concentrations)
$X_{1,F}$	final aerobic cell concentration (mg/kg) (extra subscripts $_{I, F}$ indicate initial and final concentrations)
$X_{2,F}$	final anaerobic (SO_4) cell concentration (mg/kg) (extra subscripts $_{I, F}$ indicate initial and final concentrations)

$X_{3,F}$	final anaerobic (CO_2) cell concentration (mg/kg) (extra subscripts $_{I, F}$ indicate initial and final concentrations.
Y	general term for microorganism yield (g cells/g substrate)
Y_1	yield (g aerobic cells/g hexadecane)
Y_2	yield (g SRB/g hexadecane)
Y_3	yield (g methanogens/g hexadecane)
Z	depth

CHAPTER 1 INTRODUCTION

Synthetic base fluids (SBF) are one component of the drilling mud system referred to as synthetic-based mud (SBM) that are important in difficult deep water drilling operations. SBF combine the technical advantages of oil-based fluids and the low toxicity of water-based fluids. When drilling mud and the cuttings it carries reach the surface they are passed through separation processes, the drilling mud is pumped back to the mud tank and used for the next drilling processes, and the cuttings that have SBF adherent to them are disposed of. The most economical disposal method is direct ocean discharge. The cuttings and adherent SBF settle to the sea floor. The concentration of SBF in the sea floor environment may decrease with time due to resuspension, bed transport, bioturbation, and biodegradation. Biodegradation is expected to be the most significant mechanism of SBF attenuation and thus environmental recovery.

Biodegradation of SBF is the use of the organic compounds as carbon and energy sources, and is expected to occur with different electron acceptors. Oxygen is the most energetically favorable electron acceptor, but in the floor of the Gulf the average oxygen concentration is 6.8 mg/L (0.21 mM) and the oxygen only naturally diffuses a few centimeters into the sediment. The deposition of SBF contaminated cuttings will cause the sediment to become anoxic and sulfate will then be used as the electron acceptor. The average sulfate concentration in the sea bottom is 2.77 g/L (28.9 mM) and the sulfate concentration in the sediment pore water will be almost the same as this concentration in first 30 cm. If the sulfate in the sediment is depleted, methanogens will then use carbon dioxide in the sediment and produce methane with the biodegradation of SBF. When the concentration of SBF in sediments is reduced to very low levels the oxygen and sulfate concentration will increase due to diffusion from the overlying water body and complete recovery of the sediment from the cuttings disposition will be possible. The key to predicting sediment recovery will be the kinetics of the biodegradation of the SBF.

The objective of this research is to model the fate of the synthetic base drilling fluids in the Gulf of Mexico. This will be accomplished by:

- developing a conceptual model;
- conducting a sensitivity analyses to determine which modeling parameters have the most important effect on the fate of SBF in Gulf of Mexico sediments;
- performing experiments to determine the specific values of the parameters deemed important; and
- using the experimentally determined kinetic data to finalize a mathematical model to predict the fate of SBF in deep Gulf sediments.

CHAPTER 2 LITERATURE REVIEW

2.1 OFFSHORE DRILLING

The main functions of drilling muds are to balance pressures preventing a blowout; to cool, lubricate, and support part of the weight of the bit and pipes; and to transport cuttings to the surface (Neff 1987, Darley and Gray 1988). During drilling, the drilling mud is pumped from the mud tanks down the hollow drill pipe and through nozzles in the drill bit. The flowing mud sweeps cuttings from beneath the bit and carries them back up the annular space between the drill pipe and the borehole or casing to the surface. The mud is then passed through solids control equipment to remove the drilling mud from the cuttings. The mud is then circulated back to the mud tanks where the cycle is repeated (Neff et al. 2000).

2.1.1 Drilling Mud

Drilling muds are composed of a base fluid to which various compounds such as barite ($BaSO_4$), clay, caustic soda, lignite, lignosulfonates, and/or polymeric materials are added. There are two general types of base fluids used in drilling mud: water and nonaqueous base fluids. Fresh or salt water is used as the continuous phase in water-based drilling fluids (WBF) and are used during shallow drilling operations and to drill the shallow phase of deeper wells. All muds may also contain low concentrations of specialty chemicals added to solve some particular problem that is affecting mud properties.

The continuous phase of nonaqueous-based drilling fluids (NADF) is a liquid hydrocarbon mixture or other insoluble organic chemical termed nonaqueous base fluid (NABF). NABF are more expensive than WBF but are used in difficult drilling situations where their technical advantages are required. There are three types of NADF, based on the chemical composition of the base fluid in the mud. These are oil-based fluids (OBF), enhanced mineral oil-based fluids (EMOBF), and synthetic based muds (SBM). The term SBM is used here to represent the whole drilling fluid or mud, and the base fluid portion is represented by the term SBF.

The OBF contain diesel fuel or conventional mineral oil as the continuous phase. They are the least expensive and were the only ones in use until the late 1980s (Dicks et al. 1986/87). EMOBF contain an enhanced mineral oil as the continuous phase. Enhanced mineral oils are conventional paraffinic mineral oils that have been or purified to remove all aromatic hydrocarbons. Although EMOBF are less toxic than OBF, the EPA does not permit the discharge of EMOBF coated cuttings to U.S. territorial waters.

The base fluid or continuous phase of an SBM is a water insoluble synthetic organic material. The EPA defines SBM as being different from other NADF because the SBF continuous phase is produced from a chemically defined feed stock. Since they are synthesized by the reaction of purified compounds, they should be free of polycyclic aromatic hydrocarbons (PAH) (U.S. EPA 1996). Thus SBF are designed to be less toxic and degrade faster than OBF, and they yield mud systems similar to OBF in drilling performance (Friedheim and Conn 1996). With the use of SBF the drilling times were reduced by 50 to 60 percent, and well costs were generally cut in

half for the SBF wells. Burke and Veil (1995) also point out that when SBF are used instead of WBF, the total quantity of waste discharged is greatly reduced. SBF provide good carrying capacity for cuttings, improving cuttings transport in long, cold, deep-water risers. Because of their low toxicity and persistence, SBF are thought to have environmental impacts intermediate between those of WBF and OBF. Linear alpha olefins (LAO) and internal olefins (IO) are very common synthetic base fluids used in drilling muds in the Gulf of Mexico.

The SBF can be classified into four categories: synthetic hydrocarbons, ethers, esters, and acetals. The synthetic hydrocarbons are typically polymerized olefins and linear paraffins. They are often used in blends that are designed to achieve balance among the physical properties important to the drilling operation (e.g. viscosity, pour point, flash point, etc). Ethers are only poorly degraded and acetals are not very stable so they have never been used in U.S. waters (Friedheim and Conn 1996). Esters and olefins are most commonly used in today's market due to their low toxicity, relatively rapid biodegradation rate, low environmental impacts, and cost. Olefins are less costly than esters, more stable at higher temperatures, less viscous at low temperature, and more adaptable to deep water drilling environments. However, esters are much more biodegradable than olefins (Neff et al. 2000).

2.1.1.1 Drilling Mud Additives

The types and amounts of chemical additives included in the mud formulation vary according to the required characteristics of the mud. The functions and types of the major chemical additives used in different drilling muds are summarized as follows.

- Weighting materials are added to increase the weight of the mud, so that adequate well control can be maintained. The weighting agent is normally a major component of the mud system. The most common weighting material is barite ($BaSO_4$).

- Viscosifiers promote viscosity through complex interactions with the emulsions. Bentonite clay is often used with most mud types.

- Fluid loss control agents are compounds added to reduce the loss of fluid from the mud into the drilled formation. Types of agents include bentonite clay, lignite, and polymers (e.g. carboxymethylcellulose, polyanioniccellulose, and modified starch).

- Emulsifiers are added to stabilize oil-in-water emulsions. A combination of two emulsifiers is often used to ensure a homogenous composition. Types of primary emulsifiers include fatty acids (and derivatives) and rosin acid (and derivatives). Types of secondary emulsifiers include amines, amides, sulfonic acids, lignosulfonates, alcohols, and related co-polymers. These are used to improve emulsion stability, especially at high temperatures, and to wet the drilled solids

- Brines are used 1) to balance the interactions of drilling fluid with clay and soluble salts in the formation, 2) because they are denser than the oil/synthetic oil

phase, thereby increasing the weight considerably, and 3) because the emulsion formed has a higher viscosity than either of the two phases alone.

- Alkaline chemicals are normally added to control the pH, which is beneficial to reduce corrosion and to activate some emulsifiers. Lime ($Ca(OH)_2$) is normally used with drilling muds made with SBF.

- Lost circulation materials are added to block pores, voids, or fractures (all mud types). Many types of materials are used, for example crushed nut shells, shredded vegetable fiber, mica flakes, graded sizes of calcium carbonate, shredded cellulose, and diatomaceous earth.

2.1.2 Cuttings Discharge

Drill cuttings are particles of crushed rock and range in size from clay-sized particles to coarse gravel. When SBM are used the EPA restricts the amount of base fluid remaining adherent to discharged cuttings. Prior to the implementation of current regulations, cuttings discharged offshore contained an average of 12 percent SBF. Current discharges are now in compliance with the limits in Table 2.1.

Table 2.1

EPA NPDES Allowances for SBF Adherent on Cuttings to be Discharged Overboard

Standard of Adherence	Level
C_{16}-C_{18} Internal Olefins	<6.9 g SBF/100 g discharged cuttings
C_{12}-C_{14} Ester	<9.4 g SBF/100 g discharged cuttings
C_8 Ester	<9.4 g SBF/100 g discharged cuttings
Discharged directly at sea floor	14 - 15%*

*Percentage of the base fluids retained on the cuttings. Source: *Federal Register* 2004.

When NADF are discharged to the ocean, they tend to clump together in large particles that settle rapidly to the sea floor (Delvigne 1996, Brandsma 1996). Ester based SBF cuttings are more easily dispersed than olefinic SBF cuttings. Water cannot easily penetrate the oleophyllic mass of cuttings, so they do not disperse efficiently (Neff et al. 2000). Therefore, most SBF cuttings settle rapidly and accumulate at the bottom near the platform discharge sites.

Gallaway et al. (1998) monitored SBF cuttings distribution in 565 m of water south of the Mississippi River in the northern Gulf of Mexico. Total cutting discharges from the rig included 7700 bbls of WBF cuttings, 5150 bbls of SBF cuttings, and an estimated 7695 bbls of Petrofree LE (a SBF base material containing 90 percent LAO and 10 percent ester). Maximum cuttings accumulations were observed to be 20 to 25 cm thick and maximum LAO concentration was 198,000 mg/kg in surficial sediment. The mean concentration of LAO in surficial sediments (all sampling stations combined) was 4,000 mg/kg in 1997 and 2,000 mg/kg in 1998. Gallaway`s

monitoring showed that SBF cuttings were distributed very heterogeneously in surface and subsurface sediment.

2.2 PHYSICAL PROCESSES AFFECTING THE FATE OF SBF IN SEDIMENTS

The concentration of SBF on the sea floor after deposition can be affected by various physical properties. The diffusion of SBF from the cuttings and sediment and the resuspension of sediments containing cuttings affect the SBF directly. Other properties, such as the diffusion of electron acceptors such as oxygen and sulfate effect the concentrations of SBF indirectly due to the stimulation (or lack of it) of the microbial population responsible for SBF metabolism.

2.2.1 Molecular Diffusion

SBF base chemicals are hydrophobic compounds with low solubility and they do not significantly disperse in the water column following discharge. Table 2.2 shows measured or estimated values for log octanol/water partition coefficient (log K_{ow}) of several SBF base chemicals. There is an inverse relationship between log K_{ow} and aqueous solubility. The esters would represent the most soluble of the SBF.

Since the SBF are so poorly soluble, the diffusion of the SBF off of the sediment or into the overlaying water body is minimal and can be ignored when predicting the fate of SBF in sediments.

Table 2.2

Measured or Estimated Log Octanol/Water Partition Coefficients of Several SBF

SBF Base Chemicals	Log K_{ow}	Reference
Ester	1.69	Growcock et al. 1994
Acetal	11.8	Vik et al. 1996a
Linear-α-Olefin	>6.43	McKee et al. 1995
Internal Olefin	8.57	Zevallos et al. 1996,
Poly-α-Olefin	11.2-13.7	Vik et al. 1996a
	15.4	Friedheim et al. 1996
	11.2	Zevallos et al. 1996

Neff et al. 2000

The diffusion of oxygen and sulfate is important because they are used as electron acceptors during biodegradation of SBF in sediment. The free solution diffusion coefficient (D^T) (units of $cm^2\ s^{-1}$) for sulfate at the ambient temperature T (°C) is calculated from the zero-degree coefficient D_0.

$$D^T = D^0 + at \qquad \textbf{Equation 2.1}$$

where a = an ion specific coefficient. For sulfate D^0 is 4.88 x 10^{-6} cm^2 s^{-1} and a is 0.232 x 10^{-6} cm^2 s^{-1} $°C^{-1}$ (Li and Gregory 1974). The ambient temperature of Gulf sediments is 5 °C.

Therefore,

$D^T = 4.88 \times 10^{-6} + 0.232 \times 10^{-6} * 5 = 6.04 \times 10^{-6}$ cm^2 s^{-1} (0.522 cm^2 d^{-1}) **Equation 2.2**

The following formula is used to calculate D^T for oxygen (Boudreau 1997)

$D^T = (0.2604 + 0.006383 * (t / \mu * 0.01)) * 10^{-5}$ **Equation 2.3**

where T is the absolute temperature (°K) and μ is the dynamic viscosity in units of poise (g cm^{-1} s^{-1}).

In the sediment pore water, because of the convoluted path molecules must follow to circumvent the sediment particles, the effective diffusion coefficient of a dissolved substance in the sediment (D_E) is lower than its free-solution diffusion coefficient in seawater (D^T). D_E is related to D^T by:

$D_E = D^T / \theta^2$ (Berner 1980) **Equation 2.4**

where θ is the turtuosity of the sediment. The turtuosity is related to sediment porosity (Ullman and Aller 1982). The effective diffusion coefficient of a dissolved substance in the sediment is then calculated as:

$D_E = D^T * \varepsilon^2$ **Equation 2.5**

where ε is the porosity of the sediment.

2.2.2 Advection and Compaction

Advection is the process of material transport due to deposition of new material at the sediment surface or to the mixing of the sediment through currents or geochemical forces (Berner 1980). Advection of sediment due to currents or other physical mixing forces is highly unpredictable. These actions would cause reaeration and dispersion of the cuttings over a larger area, which would increase the degradation rate of the SBF and thus the recovery rate of the sediment. Ignoring this type of mixing results in a worst case scenario for prediction of sediment recovery.

Bioturbation, the process of sediment mixing by the activity of organisms, is commonly modeled with Boudreau's random diffusion-like process. It is assumed that bioturbation is constant in the upper few cm and that it decreases exponentially with sediment depth. Equation 2.6 and Equation 2.7 can be used to calculate bioturbation.

If $z \leq z_b$ $Db_z = Db_{o,}$ **Equation 2.6**

If $z > z_b$ $Db_z = Db_o * e^{-(z-zb)/coeffDb}$ **Equation 2.7**

where Db_z the rate of bioturbation at depth z (cm^2 d^{-1}) and coeffDb is the coefficient for exponential bioturbation decrease.

Deposition of new material to the sediment will also cause compaction. It can be assumed that the recovery process is started after deposition and sedimentation are completed and that the natural deposition rate is very slow. Therefore, the effects of neglecting the advection and compaction to the outcome of any fate model would be minimal. In summary, assuming constant porosity, no sedimentation, and no compaction, Equation 2.8 can be used to describe the SBF mass balance:

$$\frac{\partial C}{\partial t} = \left(\frac{D^o}{\theta^2} + D_b\right)\left(\frac{\partial^2 C}{\partial z^2}\right) + \sum \text{Reactions} \qquad \textbf{Equation 2.8}$$

The left hand side (LHS) is the change of concentration of the compounds (base chemical, sulfate, or oxygen) with time. The right hand side (RHS) of the equation accounts for diffusion, bioturbation, and biodegradation.

2.3 BIODEGRADATION OF SBF

Biodegradation is the breakdown of an organic substance, by the action of living organisms (especially bacteria) and is a key factor in reducing the long-term environmental impacts of SBF. The SBF are used as carbon and energy sources for microorganisms. The presence of an electron acceptor is required for complete biodegradation to occur. When oxygen is present (aerobic conditions) it will be used as the electron acceptor. When oxygen is absent (anaerobic conditions), the major electron acceptors are nitrate, Fe (III), sulfate, or carbon dioxide. The order of preference would depend mainly on the available concentrations of the compounds in the environment but if all were present, the electron acceptors would be used in the order listed. For biodegradation to occur, several basic conditions must be satisfied (Alexander 1994). The most important of these are:

- the presence of organisms with the necessary enzymes to perform the reactions;
- the chemicals must be accessible to the organisms;
- the conditions in the environment must be favorable to the growth of the microorganisms (i.e. temperature, pH, salinity, and the presence of other essential nutrients).

There are many reasons why some chemicals are degraded rapidly and others are not. The structure and concentration of the chemical are both crucial. For some compounds, low concentrations may result in low biodegradation rates, and there is commonly a threshold concentration, below which biodegradation does not occur (Alexander 1994). Higher concentrations of the organic compounds may result in increased degradation, because the increased availability of carbon and energy sources may support a larger microbial population.

Alternatively, high concentrations of the organic compound may have toxic effects that reduce degradation rates (Cornelissen and Sijm 1996).

The presence of other compounds may also influence biodegradation. The presence of other more easily degradable organic compounds will result in a higher total microbial activity and thus may increase degradation rates of specific compounds. Alternatively, biodegradation of slowly degradable compounds could cease completely due to the availability of more preferentially degraded compounds (Cripps et al. 1999). In seawater, the reaction that yields the most energy is the aerobic oxidation of organic matter to CO_2. Whenever oxygen is present, aerobic oxidation will be dominant. When oxygen is consumed or limited, sulfate reduction is expected to dominate because of the high content of sulfate in seawater.

Another rule of thumb when estimating the biodegradability of organic compounds is that a compound that is structurally similar to a natural compound will more readily degrade than compounds that have completely foreign structures. When considering SBF compounds, the esters can be predicted to be the easiest to degrade since all organisms have ester bonds in the membranes and most organisms are capable of degrading membrane esters.

Biodegradation of ester compounds begins with hydrolysis of the ester to its component acid and alcohol. The esterase enzymes necessary to hydrolyze an ester bond are very common and can be found in most environmental samples. The fatty acid is then degraded via the β-oxidation pathway (Figure 2.1) where the molecule is degraded two carbons at a time through a series of oxidation then reduction reactions. Most organisms are capable of performing β-oxidation since it is the pathway used to degrade cellular fatty acids. The alcohol can be degraded by a number of mechanisms depending on the alcohol, in most cases being transformed to one of the central metabolic pathway intermediates such as acetate, pyruvate, fumarate, or succinate.

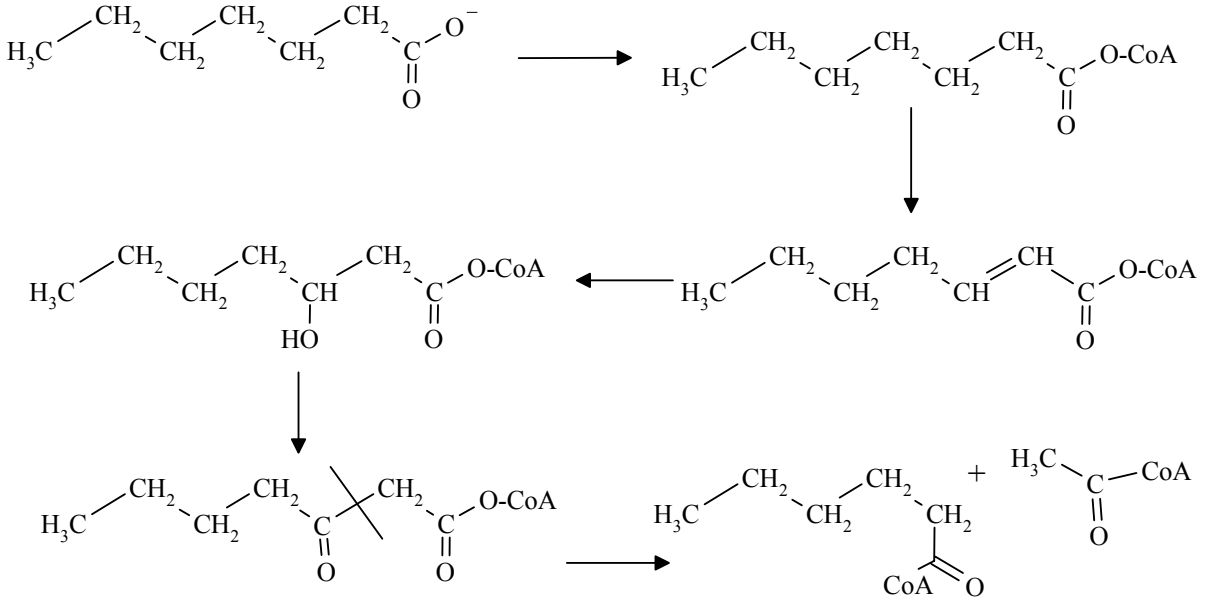


Figure 2.1. The β-oxidation pathway.

2.3.1 Aerobic Hydrocarbon Degradation

Aerobic degradation of hydrocarbons has been commonly accepted as the major mechanism of removal of hydrocarbons from many contaminated environments. Aerobic organisms like *Pseudomonas* (Tagger et al. 1990), *Flavobacterium* (Okpokwasili et al. 1984), *Moraxella* (Tagger et al. 1990), *Marinobacter* (Gauthier et al. 1992), and *Vibrio* (West et al. 1984) were found to degrade PAH under aerobic marine conditions. *Pseudomonas testosterone*, *P. putida*, and *P. stutzeri* biovars were isolated from Spanish Mediterranean sediments and were found to be capable of degrading naphthalene by Garcia-Valdes et al. (1988). *Cycloclasticus* was isolated from Puget Sound sediments (Dyksterhouse et al. 1995, Geiselbrecht et al. 1996) and from Gulf of Mexico sediments (Geiselbrecht et al. 1998). Shen et al. (1998) found that the genus *Pseudomonas* was the most prevalent among the aerobes degrading hydrocarbons. These organisms listed above are all classed as Eubacteria.

The biodegradation pathways of aromatic and saturated hydrocarbons have been reasonably well studied. Studies to determine the aerobic pathways for the degradation of both saturated and aromatic compounds have shown that monooxygenase or dioxygenase enzymes are required for initial activation of the hydrocarbon chain. Oxygenase enzymes incorporate one or both of the atoms of molecular oxygen into the hydrocarbon, ultimately forming an alcohol (or catechol for aromatic compounds). The saturated hydrocarbon alcohol is oxidized again to form a carboxylic acid (Figure 2.2), making a fatty acid which is then degraded by the cell using β-oxidation (Figure 2.1). The catechol formed during the degradation of an aromatic compound is then cleaved to form a dicarboxylic acid (Figure 2.3), which is then further degraded via β-oxidation (Figure 2.1).

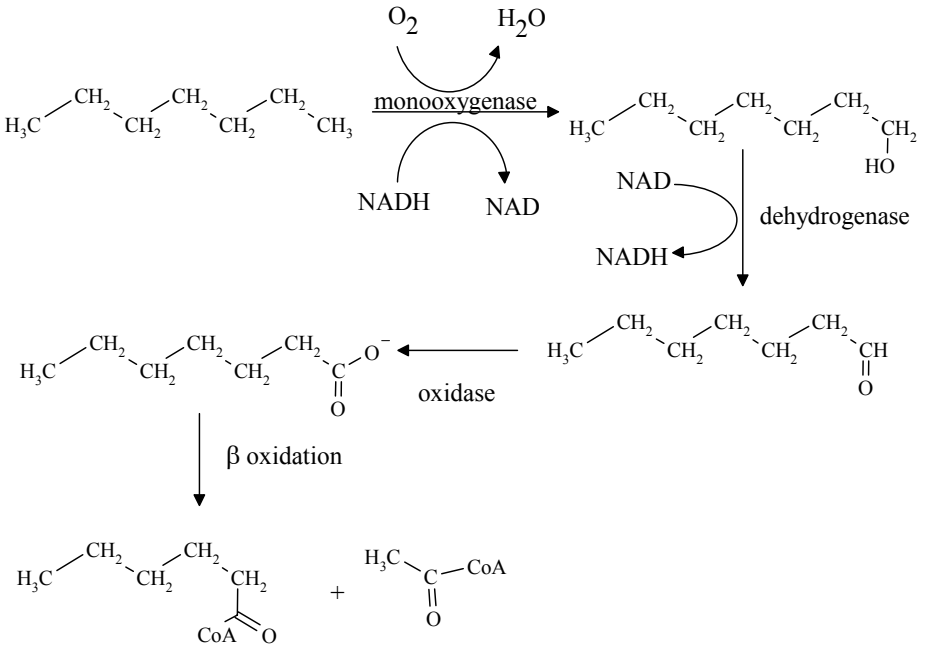


Figure 2.2. Monooxygenase pathway for activation of hydrocarbons.

Although there is limited information in the literature concerning the degradation of alkenes, it is proposed by Britton (1984) that the metabolism of alkenes may be initiated using four major routes:

- oxygenase attack upon a terminal methyl group to the corresponding alcohols and acids;
- subterminal oxygenase attack to the corresponding alcohols and acids;
- oxidation across the double bond to the corresponding epoxide;
- oxidation across the double bond to the corresponding diol.

All of the products formed are further metabolized by means of the β-oxidation pathway producing intermediates of the central metabolic pathway such as acetate.

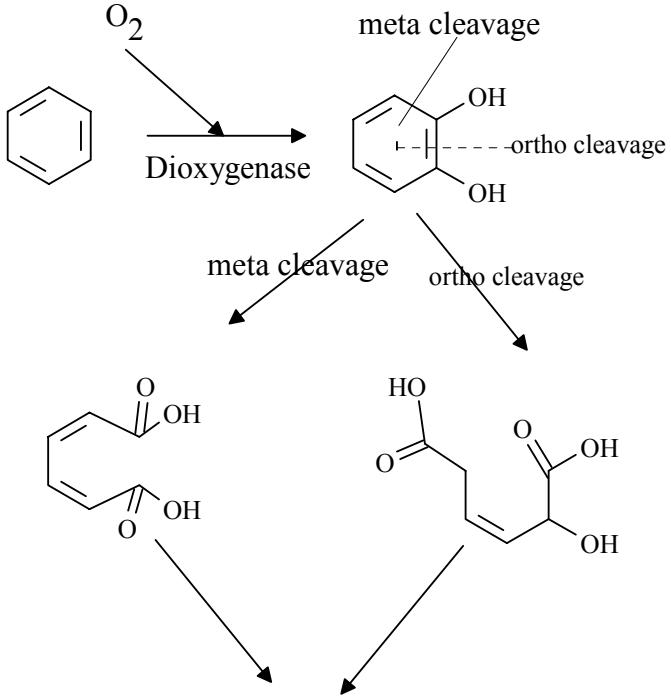


Figure 2.3. Summary example of aerobic pathways for the degradation of aromatic compounds.

2.3.2 Anaerobic Hydrocarbon Degradation

Only recently has reliable evidence demonstrating the degradation of aromatic hydrocarbons in anoxic environments been presented. The anaerobic degradation of alkanes is a rapidly developing field but the anaerobic degradation of alkenes is not very well studied.

The research concerning the anaerobic degradation of alkanes documents the removal of alkanes, linked to various anaerobic electron acceptors. The results suggest this is a very slow process. The information on types and numbers of organisms involved and the pathways that they use is limited. Aeckersberg et al. (1991; 1998) and Caldwell et al. (1998) found that sulfate-reducing bacteria (SRB) could metabolize straight chain saturated hydrocarbons. So *et al*. (1999a) reported the isolation and characterization of a novel sulfate-reducing bacterium (SRB) capable of degrading alkanes. Meckenstock et al. (2000) reported on a sulfate-reducing naphthalene-degrading freshwater culture enriched from a contaminated aquifer. So et al. (2001) enriched anaerobic alkane degrading microorganisms under four different reducing conditions (sulfate-reducing, denitrifying, iron-reducing, and methanogenic) using estuarine sediment. They found that sulfate was the major electron acceptor in the estuarine environment. Koizumi et al. (2002) established a toluene-degrading consortium and an ethylbenzene-degrading consortium under sulfate-reducing conditions. Kleikemper et al. (2002) studied the activity and diversity of SRB in petroleum hydrocarbon contaminated aquifers and showed that SRB from the environment were able to use a variety of organic carbon sources.

The major obstacle for the anaerobic biodegradation of hydrocarbons is the initial oxidation step. So and Young (1999b) proposed the subterminal oxidation of the hydrocarbon by an unknown mechanism, which was not a direct carboxylation. Studies that are more recent have shown that at least one mechanism for the anaerobic bacterial metabolism of alkanes is initiated by a fumarate addition reaction at the subterminal methyl group of the alkane (Kropp et al. 2000, Geig and Sulfita 2002) (Figure 2.4). Fumarate is an unsaturated 4-carbon dicarboxylic acid and a component of well established pathways for the oxidation of sugars. The addition of fumarate to a hydrocarbon results in the formation of a succinate derivative that has been identified using gas chromatograph-mass spectrometry (GC-MS) analysis (Geig and Sulfita 2002). The addition of an organic acid as an initial step in the oxidation of saturated hydrocarbons may represent a common theme for the anaerobic degradation of a broad range of hydrocarbon contaminants.

The anaerobic pathway for the biodegradation of olefins (unsaturated hydrocarbons), which are the dominant hydrocarbon present in SBF, has received little attention. Unsaturated hydrocarbons, such as those found in SBF, are known to biodegrade more quickly in anaerobic environments compared to saturated hydrocarbons of similar length. The presence of an unsaturated double bond appears to raise the reactivity of olefins. It is not known if the same organic acid addition reactions proposed for the biodegradation of saturated hydrocarbons would also be applied to unsaturated hydrocarbons, or if the mechanisms involving the incorporation of water across the double bond of the unsaturated compound forming an epoxide or an alcohol as seen in aerobic degradation of unsaturated hydrocarbons would occur.

Schink (1985) enriched methanogenic cultures on normal alkanes, branched alkanes, and alkenes. Only 1-hexadecene and squalene increased methanogenesis over that of controls. About 78-91 percent of the theoretical methane production was observed from hexadecene. Growth was much poorer and methanogenesis stopped after a short period when squalene was used as the substrate. It was proposed that hydration across the double bond to produce an alcohol was the most likely reaction. Later Schink (1989) noted that the initial reaction might be either a biological or an abiotic chemical reaction. There was no evidence for or against either of

these possibilities. It can be concluded that anaerobic degradation of alkenes appears to be feasible but the information concerning the types of alkenes that are degraded anaerobically and the mechanisms that organisms can use to degrade these compounds is limited.

It is evident that many different organisms have the capability to degrade hydrocarbons in different anaerobic conditions i.e., different reducing conditions. During anaerobic degradation nitrate, sulfate, and carbon dioxide serve as main terminal electron acceptors. Kleikemper et al. (2002) concluded that sulfate reduction was responsible for 70 percent of petroleum hydrocarbon attenuation at 38 petroleum hydrocarbon contaminated aquifers. SRB were found to grow on contaminants such as benzene, toluene, ethylbenzene, xylenes, naphthalene, phenanthrene, alkanes, and halogenated compounds. This information plus the fact that sulfate is present in high concentrations in seawater suggest that SRB will be major factors contributing to the anaerobic degradation of SBF in marine sediments.

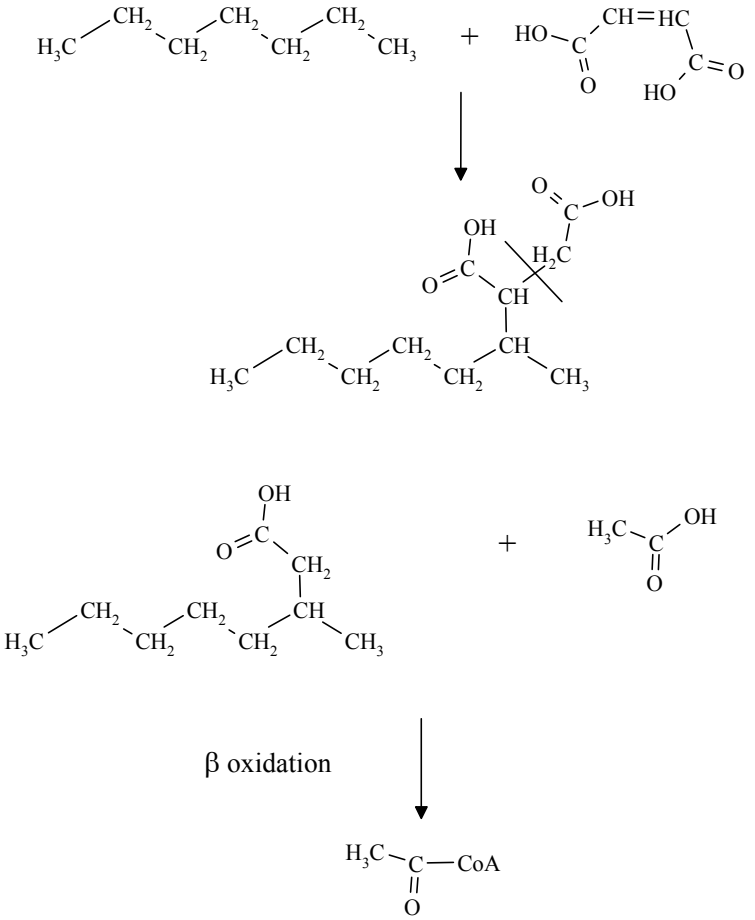


Figure 2.4. Incorporation of fumarate into saturated hydrocarbons during anaerobic degradation.

2.3.3 Modeling Microbial Activities

Microbial growth and degradation of compounds is typically modeled using Monod type kinetics as shown in equation (Equation 2.9). In cases where the substrate is not limiting ($K_s << C$, the Monod equation reduces to zero order, while in cases where $K_s >> C$ it reduces to a first order equation.

$$r_S = -\left(\frac{\mu XC}{Y\left(K_S + C\right)} \right) \qquad \textbf{Equation 2.9}$$

Where; r_S = rate of substrate removal (mg/L • h), μ = maximum specific growth rate (mg cell/mg cell · time), X = concentration of microorganisms in sediment (mg cells/L), C = concentration of substrate (mg/L), Y = yield (mg cell produced/mg substrate consumed) and K_s = half saturation coefficient (mg/L).

The Monod equation relates the rate of degradation of a substrate to the rate of microbial growth by the yield of cells from the degradation of the substrate. The microbial yield from any individual chemical can be calculated using thermodynamic principles (McCarty 1972) or it can be measured in laboratory studies. In many cases, it is easier to measure the rate of removal of a compound and then convert this to a growth rate using a yield than it is to try and directly measure a yield in environmental samples.

There is very little literature concerning rates of microbial growth on hydrocarbons and the growth yields from this. Table 2.1 presents some information gathered for hexadecane, which is a 16-carbon paraffin rather than an olefin, but it is the best example available in the literature. There are large differences between the calculated yields and the experimental yields; each of the experimental yields is about 22 percent of the expected yields.

Table 2.3

Calculated* and Experimental Yields for Organisms Grown on Hexadecane

Electron Acceptor	Calculated Yield (g cell/g hexadecane)	Yield Found in Literature (g cells/g hexadecane)
Oxygen	1.3965	0.3 (Chakravarty et al. 1975)
Sulfate	0.1446	0.0327 (So and Young 1999b)
CO_2	0.0637	No Data

*The calculations were performed using the thermodynamic model first described by McCarty (1972).

The other factor that it is important to model to determine the fate of SBF in sediment is the lag phase or acclimation period. When cells are exposed to a new carbon source, they typically are not able to metabolize it immediately. There is typically a period (from hours to years) in which the organisms learn to be able to grow on and degrade a substrate. Although there are many theories to explain a lag phase, there are no widely accepted models to account for a lag phase. The theories of why microorganisms experience a lag phase can be summarized as follows.

Several theories have been proposed to explain the acclimation of microorganisms to new compounds. These theories include:

- Proliferation of small populations. This theory suggests that the microorganisms capable of degrading the new substrate are present in the natural population, but in very small numbers. The acclimation period, then, would reflect the induction of the appropriate enzymes and the increase in numbers of the organisms capable of growing on the new substrate. This would be reflected in low numbers and slow rates of degradation initially, then an increase in numbers of organisms and a corresponding rate increase.

- Presence of toxins. This theory suggests that the presence of toxic chemicals suppresses biodegradation until some organism is capable of degrading or are not inhibited by the toxin develop.

- Predation by protozoa. It has been observed that when protozoa (which normally feed on bacteria) are inhibited the lag times seen for acclimation to new materials are decreased dramatically. It is presumed that the grazing of the protozoa on the bacteria keeps the population of the acclimating bacteria so low that noticeable degradation does not occur.

- Appearance of new genotypes. Natural mutations occur about once every million cell divisions in a bacterial culture. If the mutation results in a new genotype with altered enzymes that are capable of degrading the new substrate it will give the culture a selective advantage.

- Diauxie. This theory suggests that some substrates are degraded preferentially over other substrates. For example if glucose is present in a media, many organisms will degrade only it until it is gone then they will begin to degrade the other substrates. The lag phase would be due to the organisms degrading the other substrate first.

- Enzyme induction. This theory presumes that the acclimation period is due to enzyme induction in a population that is already present. The enzymes are only produced when the substrate is present. When organisms capable of degrading a substrate, but have been degrading something else, are then exposed to the substrate, it usually takes them a while to recognize the new substrate and turn on the appropriate enzyme systems.

In most cases, several of the theories will apply to the overall explanation of the acclimation or lag period.

2.3.4 Methods for Enumerating Microorganisms in Sediment

To model the microbial degradation of SBF in deep Gulf sediments it is necessary to study the population of organisms present in the sediment, especially those that respond to the presence of SBF. The study of environmental microbial ecology has bloomed in the last few years due to the advent of newer methods based on genetic profiling of organisms and analysis of specific cellular components such as phospholipid fatty acids. Two types of methods were selected for this research: growth-based methods and molecular-based methods.

2.3.4.1 Growth-Based Techniques

Growth based techniques for environmental microbiology rely on the ability to induce the organisms in the environmental sample to reproduce and give some sort of positive response to their presence. The response may be the formation of a visible colony on an agar plate or causing a color change in a liquid medium. The use of solid medium to culture organisms is the foundation behind the majority of medical microbiological studies, but is not recommended for growing environmental organisms. The Most Probable Number (MPN) technique is a liquid based technique. An MPN enumeration is based on the observation of whether or not growth occurs in a series of culture containers of progressively increasing dilution. Determination of the dilution separating growth from no-growth can be used with a statistical technique to infer the concentration of organisms in the original sample. Turbidity is often used as an indicator of growth. The indicator could also be the measurement of a substrate consumed or end product produced. The tubes that show growth are scored and the distribution of positive tubes among the dilutions is statistically related to the number of organisms present in the original sample. This is done using the MPN tables that are based on the mathematical approaches of Halvorson and Ziegler (1933). Cochran (1950) later contributed procedures that estimate error and calculate confidence limits. Woomer (1994) also gave attention to the reliability of experimental results, including the criteria for discarding data that do not comply with the principle assumptions that underlie the use of this technique. The MPN data can also be interpreted by using the Thomas equation (Thomas 1942).

2.3.4.2 Molecular Based Techniques

The discovery of DNA and the development of tools for purification isolation and rapid sequencing of DNA have all contributed to the development of molecular methods for use in environmental microbiology. A variety of molecular based techniques has been described over the last two decades, which give direct counts of microorganisms in environmental samples. This involves direct microscopic observations, which yields high numbers, has less bias and counts both live and dead cells (if necessary). Staining procedures are developed to allow direct counts of living bacteria. As all the active cells are expected to have DNA or RNA, the most common staining procedures involve the DNA or RNA probes. The initial probes developed were used for determining the total counts of bacteria. Later, rRNA based probes were developed to identify the phylogenetic groups of bacteria.

Total counts: Several dyes have been developed to enumerate total bacterial content of samples. In 1978, Rublee and Dornseif determined the number of bacteria in the sediments of North Carolina salt marsh by direct count epifluorescent illumination and acridine orange (AO) stain. Acridine orange direct counting (AODC) is based on the RNA/DNA ratio in a cell. Yu et al. (1995) stated that at relatively low AO concentration, the active bacteria (cells with high amounts of RNA) could be counted as fluorescent red-orange cells because of the predominance of RNA while inactive bacteria have mostly DNA and will fluoresce green. In natural and undefined bacterial communities, the RNA/DNA ratio in a cell and thus the AO color reaction could be affected by growth media. AO has also been noted to react nonspecifically with

organic material in soils and sediments thus making AODC a poor method for assessment of the activity of cells in environmental samples.

Porter and Fieg (1980) reported the use of 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI) for identifying and counting aquatic microflora. The DAPI stain binds to DNA due to intercalation between base pairs or by associating itself with the adenosine thymine clusters in the minor groove of the DNA double helix. DAPI can also bind to the RNA by somewhat different mechanism that is expected to involve adenosine uracil selective intercalation. Fluorescent enhancement is observed when DAPI binds to the DNA due to the displacement of water molecules from both DAPI and the minor groove (Kubista et al. 1987; Tanious et al. 1992; Pineda de Castro and Zacharias 2000; Molecular probes 2001). DAPI was chosen as a dye for total cell counts over acridine orange for this research, in the hopes of minimizing interference from natural organic matter in the sediment.

Phylogenetic identification: Many methods have been developed to analyze DNA and RNA of cells. The focus has been on the use of the polymerase chain reaction (PCR) in the detection and analysis of the DNA. This method can determine whether a specific organism is present in the sample, but most PCR methods are not quantitative in nature. Real time or quantitative PCR where the detection of a signal based on how many rounds of thermal cycling of PCR reaction has taken place has been developed in the last two to three years, but is not widely proven in environmental samples. The major improvement in detection and enumeration of specific environmental organisms is the sequencing and comparative analysis of RNA molecules. The sequencing of ribosomal RNA (rRNA) has provided the first consistent taxonomic description of microorganisms. Stahl (1986) and Amann et al. (1995) have described in detail the various DNA and RNA based methods.

The 5S, the 16S (and 16S-like), the 23S (and the 23S-like) rRNA, are common types of rRNA present in the ribosomes of prokaryotes and eukaryotes. The rRNA is composed of structural domains. With increase in phylogenetic distance, sequence variations occur in these structural domains. While the most variable regions in these domains help distinguish between species and genera, the regions that vary to small extents allow inference of relationships between members of three main domains: Eubacteria, Eukaryotes, and Archaebacteria. These differences in sequence conservation are the basis for designing nucleic acid probes. The newly designed oligonucleotide probes can be used to determine environmental diversity, as there are group- and species-specific probes. To date over 5,000 16S-like rRNA sequences have been developed and are available in the GenBank database, which have been used to explore natural microbial diversity and phylogeny.

In order to detect that an oligonucleotide probe has bound to its complementary DNA or RNA in a cell the probes are usually labeled with single fluorescent dye molecule attached to the 5’ end via a linker molecule. There are several fluorescent dyes available typically fluorescing green or red. The dyes chosen for this study were Fluorescein, which was one of the first dyes used and fluoresces green and Texas red which fluoresces red and has the longest excitation and emission wavelengths, preventing cross talk between the green emission and red excitation. Table 2.4 presents the oligonucleotide sequences of the phylogenetic probes chosen from literature for

enumerating different groups. The Texas red fluorescent dye was used for the probe directed at eubacteria (Eub 338). The other probes are linked to fluorescein dye.

Table 2.4

Molecular Probes for Microbial Enumeration

Probe	Specificity	Probe Sequence (5' to 3')	Reference
Eub 338	Eubacteria	GCTGCCTCCCGTAGGAGT	Amann et al. 1990a
Arch 915	Archaea	GTGCTCCCCCGCCAATTCCT	Amann et al. 1990b
Clost I	Clostridia	TTCTTCCTAATCTCTACGCA	Kusel et al. 1999
SRB 385	Sulfate-reducing bacteria	CGGCGTCGCTGCGTCAGG	Amann et al. 1995

Two methods have been developed for quantitative microbial analysis using phylogenetic probes. Quantitative dot blot hybridization involves hybridization of the probe to total rRNA, extracted from the environment. Fluorescent whole-cell or in situ hybridization (FISH) involves hybridization of the probes to the DNA or RNA of whole cells for quantitative enumeration by direct microscopic visualization. FISH was chosen as a molecular tool for enumerating different phylogenetic groupings of organisms in the sediment samples.

FISH is a technique where rRNA is specifically detected within intact cells and can be used to directly identify and enumerate microorganisms belonging to a particular phylogenetic group (Amann et al. 1995). This method provides more information than dot blot hybridization as it involves analysis at the single-cell level. This method not only helps in determining the morphology and abundance of uncultured microorganisms but also in analyzing their spatial distributions in situ and estimating the in situ growth rates.

Though the FISH technique has become a powerful tool for quantitative and phylogenetic analysis of microorganisms in various environments, it still has problems that need to be resolved. It is a direct microscopy method so sediment and detritus will interfere during hybridization or by autofluorescence during microscopy. Because of its potential promise of direct cellular quantification the FISH method was attempted in this study.

An important step is the ability to visualize the cells from the samples using microscopy. Several studies have investigated the quantitative extraction of cells from sediments. Methods have typically involved ultrasonication, vigorous homogenization, and/ or the addition of detergents and dispersants. Several of the following methods were attempted in this study. Scheraga et al. (1979) reported that the best method for the recovery of bacteria from sediments would involve addition of 0.0001 percent cetyltrimethylammonium bromide to the initial dilution blank followed by shaking. However, they did not include the comparison of the techniques such as sonication and grinding. Ellery and Schleyer (1984) in their studies on sandy sediments found that ultrasonication (performed using an ultrasonication bath with a frequency range of 40 - 50 kHz) was more effective than homogenization at 23000 rpm. They also stated that ultrasonication destroyed many bacteria in the sediment they used.

Velji and Albright (1985) examined in detail the dispersal effect of pyrophosphate and ultrasound on bacterial cells in marine surface sediments. They reported that samples treated with pyrophosphate and sonication in combination showed a decline in variation within sub samples. Schallenberg et al. (1989) reported that the majority of published abundance data underestimate the bacterial numbers to various extents due to the difficulties in removing the organisms from the sediments. Epstein and Rossel (1995) examined three different ways to dislodge bacteria: homogenization in a blender at 16000 rpm, sonication in a sonic bath, and sonication by sonic probe (disintegrator with 5 mm tapered microtip). They concluded that the use of a sonic probe for 3 times at 60 sec each with an 109 μm amplitude to be the most effective device for bacterial dislodgement. Tso and Taghon (1997) used glutaraldehyde to strengthen the bacterial cells before sonication. Their studies indicated that dispersant tetra sodium pyrophosphate did not allow the cells to reattach to the sediment particles or clump together after sonication. This helped in distributing the cells evenly for any further analysis. Sonication for 1 minute was reported to have resulted in maximal abundances of bacteria and to be effective in dislodging the cells from the detritus or sediment particles. Frischer et al. (2000) evaluated a modified protocol for extracting cells from salt marsh sediments that involved the use of homogenization, detergents, and dispersants, but did not require sonication. They reported that their technique (which involved addition of 0.01M sodium pyrophosphate and 0.09 percent Tween 80, rigorous vortexing, slow centrifugation, supernatant removal, and then cell collection by fast centrifugation of supernatant) allowed the rapid and quantitative extraction of cells from sediments that had been stored in formalin, while avoiding possible cell lysis by sonication. The resulting cell extract were substantially reduced in sediment and detritus content and were therefore amenable to hybridization protocols. The last two papers contradict each other and exemplify the troubles associated with separating organisms from sediments. It is important to note that the majority of the research reported to date used relatively sandy sediments so separation of organisms from the sediment is possible. In all of the research, once the organisms were dislodged from the sediment the larger sediment particles were allowed to settle and the organisms are assumed to be in the aqueous phase. The separation of organisms and solids in sediment with relatively higher organic matter and clay particles is much more difficult than for sandy sediments, researchers have avoided these types of sediments.

CHAPTER 3 METHODOLOGY

The sediments were collected by Continental Shelf Associates during the seabed survey sponsored by the MMS, DOE, and the Synthetic Based Muds Research Group. The VK916 samples were collected during the MMS Deepwater Study (2000-2002). The sediments were collected from seven geographic locations (Figure 3.1). Two samples were collected from each geographic location, one within 100 m of a drilling site where synthetic based mud cuttings were discharged (near field (NF)) and the other at a location near that site that was not expected to be impacted by drilling activity to serve as a control (far field (FF)). The sediments were shipped immediately on ice (arriving still cold) and have been kept and worked with at 4°C at all times. In order to use the four jars sent as one sample, they were homogenized into one sample and then subdivided again into two sub samples. The two were considered equal sub samples, representative of the entire sample set from each site.

Sub samples of the sediments were extracted analyzed to determine SBF exposure using gas chromatographic analysis (Section 3.5.3). Table 3.1 presents the results and other pertinent data for the sediments. This data was used to decide which sediments should be used for further study. MP299 was chosen because it was from the shallowest depth. GC112 was chosen because it was from a medium depth and the near field sample showed some contamination with SBF. VK916 was chosen because it was from the deepest site and the near field sample was contaminated with SBF. Samples of these sediments were then sent to the Texas A&M soils laboratory for analysis of important sediment properties. The results are presented in Table 3.2. These sediments all contain low percentage of sand and high amounts of silt and clay.

3.1 MPN ENUMERATION OF SPECIFIC NUTRITIONAL GROUPS

The MPN viable count procedure was used to determine the numbers of specific nutritional groups i.e. sulfate-reducing bacteria (SRB), methanogens (H_2 and acetate utilizing methanogens), general anaerobes, and hydrocarbon degrading aerobes. Each enumeration was performed using selective media in a five tube dilution series. One set of dilution blanks was prepared for each sample and used to inoculate the appropriate dilutions of each specific medium.

3.1.1 Sample Preparation

Approximately 10 g of wet sediment was weighed and added to 90 ml phosphate buffered saline adjusted to the salinity of seawater (1.236 g Na_2HPO_4, 0.18 g $NaH_2PO_4 \bullet H_2O$, 26.3 g NaCl and 1 ml resazurin (1 g/L stock solution) per liter type 1 water) which had been sterilized using an autoclave. This represented an initial 10^{-1} dilution. This initial dilution was shaken vigorously on a shaker for 10 min, then put in an ice bath and subjected to sonic disruption with a sonic probe at 40 percent power for three, 2 min bursts. The headspace of the dilution bottle was then flushed with N_2 gas. The initial and remaining dilution blanks were reduced with 10 ml of 10 mM thioglycolate stock solution and the dilutions were carried out mixing well by shaking each transfer.

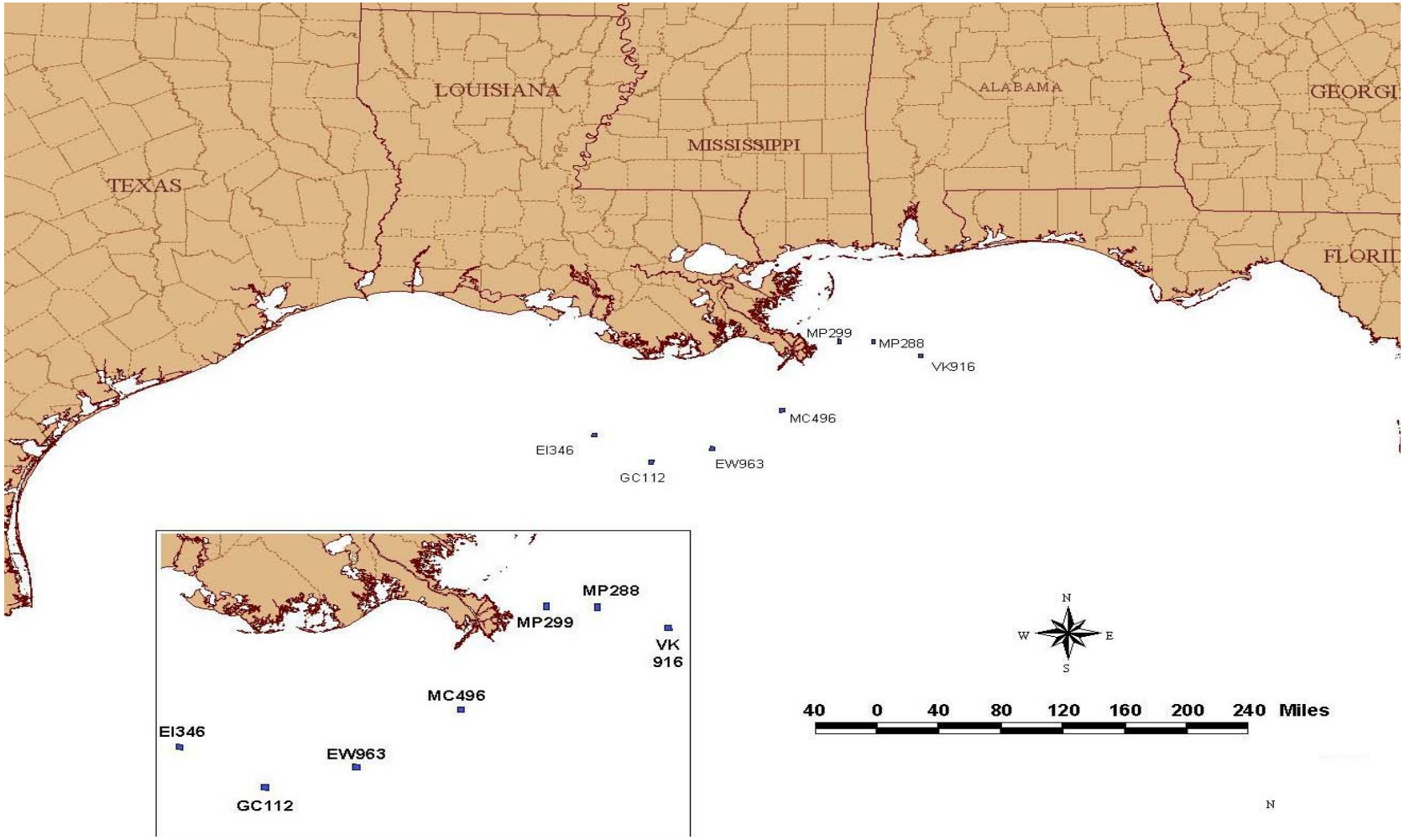


Figure 3.1. Geographic locations of the offshore sediments collected.

Table 3.1

Sediment Samples Received for Study

Sediment	Location	Depth (m)	Pressure at Depth (psi)	Presence of SBF (mg/kg dry sediment)	History**
MP299	NF	66	97	Not detected	Drilling activity 1962-2000; 966 bbl of IO/LAO cuttings mid 1990s-2000.
	FF	65	96	Not detected	
EI346	NF	93	137	Trace amounts	Drilling activity 1977-2000; 10,328 bbl of IO cuttings mid 1990s-2000.
	FF	95	140	Not detected	
EB963	NF	535	787	Trace amounts	598 bbl of IO cuttings, last discharge 1997.
	FF	440	647	Not detected	
GC112	NF	535	787	1068 $\pm$ 300	5,470 bbl IO cuttings last discharge 1997.
	FF	454	667	Not detected	
VK916	NF	1135	1668	11717 $\pm$ 636	2,510 bbl IO cuttings from Nov to Dec 2001.
	FF	1114	1638	Not detected	
MC496	NF	550	809	Not detected	1,674 bbl IO cuttings last discharge 1998.
	FF	440	647	Not detected	
MP288	NF	122	179	Not detected	Drilling activity 1968-1997; 98 bbl of IO cuttings mid 1990s-1997.
	FF	151	222	Not detected	

*The SBF in the sediments were extracted and then analyzed with GC/FID as in Section 3.4.3.
** The history data were taken from Continental Shelf Associates (2004). All samples were collected during the spring and summer, 2002.

Table 3.2

Chemical Properties of Selected Sediments

Sediment	% Sand	% Silt	% Clay	pH	Nitrate-N[1] (mg/L)	Phosphorus[1] (mg/L)	% Organic
MP299 NF	18	8	74	8.3	3	120	2.94
MP299 FF	28	40	32	8.1	2	114	3.24
GC112 NF	20	58	22	8.4	3	98	3.98
GC112 FF	18	54	28	8.5	2	96	4.13
VK916 NF	24	64	14	8.4	2	103	5.76
VK916 FF	30	54	16	8.2	2	104	5.36

[1] Available form

3.1.2 Media Preparation

Biosea Marine Mix was mixed with type 1 reagent grade water and was used as the liquid base during the preparation of media for SRB, general anaerobes, and hydrocarbon degraders. The SRB medium was prepared using modified Butlin and Postgate's medium B (3.5 g sodium acetate, 1.0 g yeast Extract, 2.0 g $MgSO_4.7H_2O$, 1.0 g NH_4Cl, 0.06 g $CaCl_2.2H_2O$, 0.5 g KH_2PO_4, 0.005 g $FeSO_4.7H_2O$, 0.1 g ascorbic acid, 1.0 ml resazurin stock (1 g/L) per liter of type 1 water. The medium was boiled and cooled under nitrogen, before 9 mL was transferred to Hungate screw cap tubes using strict anaerobic technique. Nails were added to pre-reduce the medium and reveal SRB activity. The nails were washed in dichloromethane (twice) to remove rust prevention oils and dried before adding one nail to each tube. The tubes were sealed with Hungate screw caps and septa and autoclaved before use.

The medium for general anaerobes was 1/4 strength TGY (Atlas, 1997), which contains 1.25 g Tryptone (pancreatic digest of casein), 1.25 g yeast extract, 0.25 g glucose (dextrose), 0.25 g K_2HPO_4, and 1 mL resazurin stock (1 g/L) per 100 ml of the Biosea Marine Mix.

The medium for hydrocarbon degraders was prepared by adding 1 mL of a solution of 425 mg KH_2PO_4, 575 mg K_2HPO_4, and 500 mg NH_4NO_3, per 125 ml distilled water which had been adjusted to pH 7 and filter sterilized (using a syringe filter) to 8 mL of Biosea Marine Mix water that had been pre-sterilized in test tubes. This was the only medium not made up or incubated under anaerobic conditions.

The medium for methanogens was modeled after Fedorak and Hrudey (1986) with added salt to produce marine conditions. The components are as follows: 10 mL macronutrient solution, 1.0 mL micronutrient solution, 10 mL Phosphate solution (50 g/L KH_2PO_4), 26.3 g NaCl, 1 mL resazurin (1 g/L) and 5.7 g sodium bicarbonate (added after the medium has been boiled) per liter of type 1 water. The macronutrient solution consisted of 50 g NaCl, 50 g NH_4Cl, 10 g $MgCl_2.6H_2O$, and 10 g $CaCl_2.2H_2O$ per liter of type 1 water. The micronutrient solution contained 10 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1 g $ZnSO_4.7H_2O$, 0.3 g H_3BO_3, 1.5 g $FeCl_2.4H_2O$, 10 g

$CoCl_2.6H_2O$, 0.03 g $MnCl_2.4H_2O$, 0.03 g $NiCl_2.4H_2O$, and 0.1 g $AlK(SO_4)_2$.$12H_2O$, per liter of type 1 water.

3.1.3 Inoculation, Incubation, and Results Interpretation

All inoculations and incubations were performed at 4°C. The incubations were carried out until indicators of growth were observed. Different indicators of growth were used for different classes or organisms. The detection of methane (using GC-FID), any peak at all above background levels, was used as an indicator of growth for methanogens. The dispersion (reduction of sheen from the hydrocarbon layer of the medium) after incubation at a 45° angle on a tube rotator was used as an indicator for hydrocarbon degraders. A blackening of the nails in the medium was used as an indicator of growth of SRB. Turbidity was used as an indicator of the growth of general anaerobes.

The positives or negatives per dilution set were scored and the Thomas Equation or the MPN table was then used to determine the MPN per g wet sediment. The value was then converted to MPN per g dry sediment by multiplying it with the wet to dry ratio (determined by drying sediment in the oven at 104^oC overnight). The same sediment, which was used for the inoculation, was used to determine the wet to dry ratio.

3.2 Enumeration Using Molecular Techniques

The molecular techniques used in this study included DAPI staining for enumeration of total cells and FISH for determination of the numbers of organisms in different phylogenetic groups. This study was the first application of these techniques in our lab. As such, a number of method validation and development techniques were carried out. The probe selection and protocols for application (including the hybridization temperature (T_H) and the concentration of formamide) were performed using pure cultures. Hybridization of the oligonucleotide probe to the sample only happens when the DNA is opened (unwound, melted) and the base pairs are then free to bind the probe. The hybridization temperature and the formamide concentration control the state of opening of the DNA. Each piece of DNA opens at a different T_H or formamide concentration. The objective of this initial method development was to see if all of the probes could be used at the same temperature, thus allowing multiple sample processing. Adjusting the formamide concentration is another way to allow this.

The protocol for separation of cells from sediment and probing these cells was worked up using sediments obtained from Galveston Bay. Pure cultures of known organisms were spiked into Galveston bay sediment samples for use as validation standards.

3.2.1 Cultures Used in Validation Activities

The pure cultures used for the method validation were purchased from American type culture collection (ATCC) or donated by the biology laboratory in the University of Houston (UH). The organisms used were *Clostridium alginoliticum* (ATCC# 17916), *Clostridium sordelli* (ATCC# 9714), *Desulfococcus multivorans* (ATCC# 33890), *Desulfovibrio salexigens* (ATCC# 14822),

Escherichia coli (biology lab, UH), *Bacillus subtilis* (biology lab, UH), *Halobacterium salinarium* (ATCC# 33170), *Micrococcus roseus* (biology lab, UH), *Methanosarcina mazei* (ATCC# BAA-159), *Pseudomonas aeruginosa* (biology lab, UH), *and Staphylococcus aureus* (biology lab, UH). The cultures purchased from ATCC were grown in the media specified by ATCC for each culture. The others were grown in Nutrient broth (Difco).

3.2.2 Fixing the Pure Cultures

The pure cultures were fixed by adding 270 ml of the fixative (29.73 ml of 37 percent formaldehyde to 275 mL sterile filtered Biosea Marine Mix, i.e 4 percent formaldehyde solution) to 90 ml of mid-log grown cells. The fixative was allowed to react with the cells on ice for at least 2 hrs. The fixed cells were then divided into 40 ml allotments in 50 ml centrifuge tubes and washed twice by sequential centrifugation and resuspension with filtered sea water to remove the formaldehyde. The centrifugation was done at maximum speed (1000 rpm) for 5 min using a MSE GT-2 table centrifuge. The final resuspension was in a 1:1 solution of phosphate buffered saline (PBS):ethanol. The cells were then transferred into 1.7 ml centrifuge tubes (in 1 ml aliquots) and stored at 4^{o}C ready for probing.

3.2.3 Fixing the Sediments

Samples of 100 g of sediment were mixed with 300 ml of fixative cooled to 4°C. The slurry was shaken well on a rotary shaker to suspend sediment in fixative and was allowed to sit for 2 - 3 hrs at 4^{o}C. The sediment was then poured into 50 ml centrifuge tubes. The sediment suspension was washed twice by sequential centrifugation and resuspension with filtered PBS to remove the formaldehyde. The final resuspension was in a 1:1 solution of PBS:ethanol. The tubes were stored at –20^{o}C.

3.2.4 Preparing the Sediment for FISH

The pure cultures were ready for the application of the FISH method once fixed, but the sediments required further treatment to separate the cells from the solids in the sediment. Many methods were tried to separate the cells from the various particles in the sediment but none of them worked effectively (Section 5.1.1). The final procedure was as follows: The fixed sediment (about 15 ml was required, if not present in one tube then two tubes were combined together) was taken from the freezer and was allowed to warm up. The supernatant (ethanol + PBS) was removed by decanting after centrifugation. Five grams of the sediment pellet was weighed into an aluminum dish and the wet to dry ratio was then determined by drying it overnight at 104^{o}C. A second 5 g of sediment was added to a bottle containing 50 mg sodium pyrophosphate in 95 ml PBS. The contents were homogenized by placing the bottle horizontally on a rotary shaker for about 15 min. The final volume of the sediment slurry in the bottle was noted. This sediment slurry was then used for FISH analysis.

3.2.5 FISH Examination of the Sediment

The molecular probes used for the FISH analyses of the sediment were Arch 915 (*Archaea*), Clost I (*Clostridium*), SRB 385 (*sulfate-reducing bacteria*) and Eub 338 (*Eubacteria*) (Table 2.4)

made up in 0.1 ml aliquots of (0.2 μg/mL) in TE (10 mM Tris and 1mM EDTA (pH 7.5-8)). DAPI was included for total cell counts and was prepared as a 100 μg/mL solution.

The probe efficiency and specificity were tested using the pure cultures described above. Further method validation was performed by adding 1 ml of fixed cells of pure cultures (positive controls for the respective probes) to make sure that the probes were working as expected in the sediments.

The procedure followed for probing the pure cultures and sediments was as follows: A sample of 1 mL of the fixed cell solutions or 0.1 mL of the sediment slurry was added to 5 ml prewarmed (to 53.5°C) hybridization buffer containing 200 ng of each probe combination. The hybridization occurred for 9 hrs (overnight) in a heating block at 53.5°C. The cells were then centrifuged, the supernatant poured off and the pellet resuspended in 5 ml prewarmed hybridization buffer with no probes and incubated for 1 hr at 53.5°C. The cells were then centrifuged again, the supernatant poured off and the pellet resuspended in 5 ml prewarmed hybridization buffer before 350 μl DAPI was added and allowed to incubate for 10 min at 53.5°C. The excess DAPI was washed off by a third and fourth centrifugation and replacement of the buffer with clean hybridization buffer and incubation for 10 min at 53.5°C. The sample (100 μl) was then filtered onto a 0.2 μm black polycarbonate filter (Millipore Isopore) allowed to dry and placed on a microscope slide.

Counting was done using fluorescence microscopy (Olympus BX51). The fluorescence microscope was equipped with specific filters to expose the specimen to the specific excitation and pass through the emission wavelengths as required by the fluorescent tag on the probes or the DAPI stain. The specificity, emission color of the probes, and the name of filter used to visualize the fluorescence are given in Table 3.3. The DAPI filter allowed light in the UV range to pass through to the sample as the excitation wavelength and the blue fluorescent light from DAPI-stained DNA to pass from the specimen to the observer (camera optics or optic lenses). The FITC filter allowed light from the blue spectrum to pass through to the specimen and green emission light from the fluorescein stained specimen to pass to the observer. The TRITC filter allowed excitation light in the green wavelengths to pass to the specimen and the red fluorescence from the Texas Red fluorochrome to pass to the observer. The fluorescing cells were counted in approximately 20 microscopic fields for each fluor. The average number of cells was then converted into the number of cells per g dry sediment originally used in the preparation of the sediment sample taking into account the area of the microscopic field, the geometry of the filter, the volume of probed sediment filtered and the original weight of sediment added to the buffer.

Table 3.3

Expected Fluorescence of Molecular Probes

Probe	Specificity	Fluorescing Color on Filter (if positively probed)	
		Color	Filter
Eub 338	All Eubacteria	Red	TRITC
Arch 915	*Archaea*	Green	FITC
Clost I	*Clostridia*	Green	FITC
SRB 385	Sulfate-reducing bacteria	Green	FITC

3.3 ANAEROBIC BIODEGRADATION TESTING

The basis for the anaerobic SBF degradation studies is the closed bottle test (CBT) approved by the EPA for testing SBF biodegradability (Herman and Roberts 2005) modified to allow high pressure incubations. The traditional incubation vessels for anaerobic testing (serum bottles) were changed to flexible walled heavy-duty polyethylene heat-seal bags that would allow the transfer of pressure from pressure chambers into the cultures. Three pressure chambers (Figure 3.2) were constructed, one capable of holding up to 800 psi (corresponding to 503 m in water depth), one holding up to 1200 psi (corresponding to 750 m in depth) and one holding up to 1700 psi (corresponding to 1000 m in depth). The pressure chambers were made from 1-in thick stainless steel tubes that were 10 in tall and had 10 in internal diameters. Plates made of 1-in stainless steel were made to fit over the top and bottom of the tubes. The end plates have an extended inner core, which holds two O-rings used to form an airtight seal with the inner wall of the stainless steel tube. The end plates are secured by 18 or 24 steel bolts, depending on the vessel. Once the end plates have been secured, water is pumped into the pressure vessel to raise the hydrostatic pressure to mimic conditions at the depth at which the sediment had been collected. The pressure vessels are incubated in a walk-in room at 4^{o}C.

To prove that the plastic bags would be suitable vessels for the tests, near shore sediment was placed in plastic sample bags, pressurized to 800 psi and left for 72 hours. The bags did not leak. Further proof that the bags would be suitable incubation vessels was obtained by inoculating near shore sediment into both the plastic bags and serum bottles. The bags were incubated in the pressure vessel (but were not pressurized since these were near shore sediments). The serum bottles were incubated alongside the pressure vessel. The results showed the change of incubation vessel did not affect the removal of ethyl oleate or tetradecene.

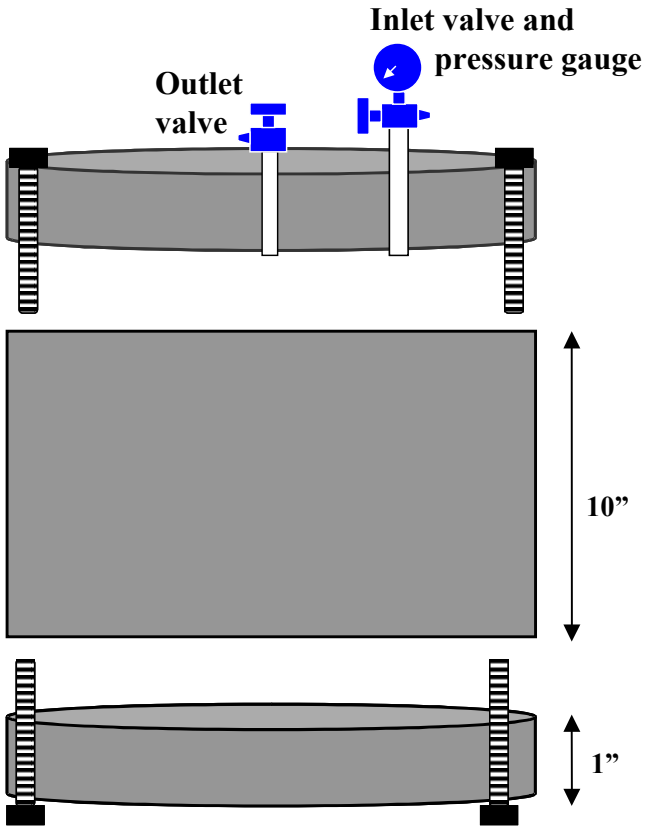


Figure 3.2. Pressure vessel.

3.3.1 General Test Procedures

Ethyl oleate (a surrogate for an ester based SBF and the positive control used in the EPA CBT) and tetradecene (a surrogate for olefin-based SBF) were spiked into the test sediments to a final concentration of 2000 mg carbon/kg dry sediment. The sediments were then mixed with synthetic marine water (Forty Fathoms Crystal Sea Marine Mix) and a volume of sediment/marine water slurry equivalent to 30 g dry sediment was transferred into the incubation vessel. Controls consisted of sediment mixed with artificial seawater but not spiked with test substrate.

When high-pressure incubations were required, the sediment seawater slurry was placed into 150 mL heavy-duty polyethylene heat-seal bags. The bags were heat sealed with a small straw in one side of the top. Any air in the bag was pushed up through the small hole left by the straw and then the bag was heat-sealed again. The bags were placed inside a water-filled pressure vessel. Hydrostatic pressure inside the vessels was applied by using a pump to force excess water into a sealed pressure vessel. Sediments were pressurized to a level equal to the pressure encountered at the depth of sampling and incubated at 4°C.

When atmospheric pressure incubations were performed synthetic seawater (Crystal Sea/Forty Fathoms Marine mix, Marine Enterprises International, Baltimore, MD) was added to make a slurry, and 75 mL of this slurry, containing 30 g/dry wgt sediment, was added to 125 mL serum bottles. A drop of resazurin solution (0.5 g/L) was added to indicate the redox conditions inside the bottles. The headspace of each vial was then flushed with a stream of nitrogen gas for 1 to 2 minutes to remove oxygen from the headspace, and the bottles were sealed with 20 mm diameter rubber stoppers (Bellco Glass, Vineland NJ) and crimp cap seals (Fisher Scientific). The headspace within the bottles was vented to establish an internal pressure equivalent to atmospheric pressure. The cultures were incubated at 4°C.

At timed intervals, the pressure vessels were opened and triplicate bags from each treatment were removed. Triplicate bottles were also sacrificed if set up. Microbial activity in the bags or bottles was monitored by 1) measuring the production of methane gas, 2) monitoring the utilization of sulfate by SRB, and, 3) determining the concentration of SBF surrogate by extraction and gas chromatography with a flame ionization detector (GC-FID) analysis.

3.3.2 First Pressure Vessel Experiment

The first experiment using the Gulf of Mexico sediment had the dual objectives of 1) investigating the anaerobic biodegradation of SBF under simulated deep-sea conditions, and 2) determining the necessity of placing sediment under hydrostatic pressure (matching the sampling depth). Sediment from two NF sites (MP299 and GC112) was prepared as described above for both high-pressure and atmospheric pressure incubations. The MP299 sediment was collected from 66 meters depth so it was incubated in bags at 97 psi. The GC112 sediment was collected from 535 meters so it was incubated in bags at 787 psi. Samples of both sediments were also incubated at atmospheric pressure in serum bottles.

3.3.3 Other Anaerobic Incubations

Since the first pressure vessel experiment showed that for MP299 and GC112, the pressure of incubation did not cause a significant difference in the degradation of ethyl oleate or tetradecene, samples from the far field sites were incubated only at atmospheric pressure.

Sediment from VK916 NF site was incubated at both atmospheric pressure and 1700 psi. The substrates were removed faster than expected so the experiment was started again. There were indications that the removal of the substrates was faster at higher pressure so the incubations were only performed at high pressure (1700 psi) when the incubations were restarted. The VK916 FF sediment was also incubated at 1700 psi.

3.4 AEROBIC BIODEGRADATION TEST

The natural oxygen uptake rate and aerobic degradation of SBF was measured using a BI-1000 electrolytic respirometer (Biosciences Inc.). Sediment from MP299 FF was divided into two sub-sets and prepared at 4^{o}C for the aerobic study. Triplicate samples of the test sub-set (200 g of dry sediment each, spiked with 466 mg of tetradecene) and identical triplicate samples of the

control sub-set (200 g of dry sediment each, not spiked) were placed in reactors and incubated at 4°C. Oxygen consumption data were collected at two hour intervals for 1174 hours (49 days).

The natural oxygen uptake rate was determined from the average oxygen consumption in the control sub-set. The oxygen uptake rate due to the biodegradation of tetradecene was determined from the net difference in oxygen consumption between the spiked sub-set and the control sub-set.

3.5 ANALYTICAL METHODS

3.5.1 Methane Analysis

An aliquot of 0.1 cc was taken from the headspace of the MPN tubes or cultures and injected into the GC. The GC-FID analysis (HP 6890 Series GC system) was performed with a 30 m HP-5 (0.32 mm ID, 0.25 micron film) column. Helium was the carrier gas. The oven, injector and detector temperatures were set at 50°C, 50°C, and 250°C respectively. Any peak at all above background levels, was taken as a positive for methane production.

3.5.2 Sulfate Analysis

Sulfate analysis was performed using a DX-100 Ion Chromatograph (Dionex, Sunnyvale CA). Eluent (2.7 mM Na_2CO_3 + 0.3 mM $NaHCO_3$) was pumped at a flow rate of 1.5 ml/min through AG12 and AS12 columns. The concentration of sulfate was determined using an external standard curve.

3.5.3 SBF in Sediment

An aliquot (20 g wet weight) of sediment from each vial was transferred into a 400 ml Pyrex beaker and spiked with 1 ml of an internal standard solution. Two internal standards were used, hexamethylbenzene and heneicosane (C21), which were prepared to 500 mg/L in dichloromethane. The internal standard solution was mixed into the sediment sample using a glass rod, and the sediment was then dried by the addition of anhydrous sodium sulfate. The dried sediment was extracted with approximately 100 ml of dichloromethane, and sonicated in a water bath (Fisher Scientific) for 20 min. The solvent was decanted through a sodium sulfate filter into round bottom flasks. Three dichloromethane washes of the dried sediment were performed. Rotary evaporation was used to reduce the solvent volume to 1.8 ml, which was transferred into an auto-sampler vial. GC-FID analysis (HP 6890 Series GC system) was performed with a 30 m HP-5 (0.32 mm ID, 0.25 micron film) column. Flow rate of helium was 1 ml/min, and the injector and detector temperatures were set at 275°C. A ramped temperature regime was used, which had an initial temperature of 100°C for 1 min, then was increased by 2°C per min to a final temperature of 225°C, which was held for 2 min. Total run time was 66 min.

3.6 STATISTICAL ANALYSIS

Confidence intervals (95 percent) for MPN values were calculated as described in Standard Methods for the Examination of Water and Wastewater (Greenberg et al. 1992). Cochran's analyses (Cochran 1950) was used for comparison of MPN data. Confidence intervals (95 percent) for the FISH values were calculated using Microsoft Excel or SigmaStat software. Student's t-tests were performed using Microsoft Excel or SigmaStat software.

CHAPTER 4 INITIAL MODEL DEVELOPMENT

The model developed starts after discharge is completed and cuttings are uniformly settled on the sea floor. The main mechanism of removal of SBF is through microbial degradation. The biodegradation of SBF base materials and other hydrocarbons is much more rapid in the presence of oxygen (aerobic) than its absence (anaerobic) (Scherrer and Mille 1989). But the amount of oxygen in the sea bottom is very limited and in the first centimeters or even millimeters all oxygen will be used by aerobes. This depletion of oxygen in the sediment will render the sediments anaerobic. Sulfate is abundant in seawater (~29 mM) therefore it is dominant terminal electron acceptor for microbial oxidation of SBF base chemicals in anoxic marine sediments (Getliff et al. 1997). Methanogenesis occurs only when most of the available sulfate is consumed (Friedheim 1997). Because of the availability of electron acceptors in the system, the model is divided into three zones; oxygen consumption zone, sulfate consumption zone and CO_2 consumption zone. In addition to biodegradation, the concentrations of SBF and electron acceptors is affected by bioturbation, sedimentation, compaction, and diffusion.

4.1 SBF REMOVAL

4.1.1 Oxygen Consumption Zone.

In this zone, the factors affecting the degradation of SBF in the sediment are diffusion of oxygen to the sediment pore water from sea water, bioturbation, and biodegradation. Sediment mixing by organisms (bioturbation) and resuspension by bottom currents are two processes, which dilute and disperse contaminants that initially settle on the seafloor. Bothner et al. (1992) introduced contaminants at the sediment surface and they penetrated to a depth of 5 cm below the seafloor because of rapid vertical mixing by organisms living in the sediments and resuspension by bottom currents. Soetaert et al. (1996) also used the value of 5 cm for the constant mixed layer, after 5 cm they found an exponential decrease in bioturbation and bioturbation approached zero at about 10 cm depth.

Assumptions of the model:

1. The contaminant is SBF base chemical and it is non diffusible.
2. The oxygen consumption layer is well mixed via organisms for the first 5 cm, then mixing is neglected.
3. The initial concentration of SBF is uniform throughout the region.
4. There is no compaction in sediments.
5. The sediment has a constant porosity.
6. Only single component (hexadecane) mass transfer is described.

The biodegradation rate of hydrocarbons in the oxygen consumption zone is modeled with Monod type kinetics with a factor that allows the concentration of oxygen to be used as a rate-limiting factor. The rate of metabolic activity depends not only on the degradability of the hydrocarbons, but also on the availability of the oxygen utilized. This oxygen limitation is represented by a hyperbolic function with a half –saturation constant $K_{s,O}$. When the concentration of oxygen increases, the limitation becomes weaker and rate of hydrocarbon

consumption will increase, on the other hand as oxygen is removed from the system the activity predicted for aerobes slows down and stops (Equation 4.1).

$$r_{F,O} = -\left(\frac{\mu_1 X_1 F}{Y_1\left(K_{s,1} + F\right)}\right)\left(\frac{O}{K_{s,O} + O}\right) \qquad \textbf{Equation 4.1}$$

Where $r_{F,O}$ = the rate of SBF removal due to oxygen consumption, (mg SBF/kg dry sediment • time), X_1 = aerobe concentration in sediment (mg cells/kg dry sediment), F = SBF base chemical concentration (mg base chemical/kg dry sediment), μ_1 = maximum specific growth rate for aerobes (mg cell/mg cell · time), $K_{s,1}$ = half velocity coefficient in oxygen consumption zone (mg base chemical/kg dry sediment), Y_1 = yield for aerobes (mg cell produced/mg hydrocarbon consumed), O = oxygen concentration (mg oxygen/L pore water), and $\underline{K_{s,o}}$= half saturation constant for oxygen limitation (mg oxygen /L pore water).

4.1.2 Sulfate Consumption Zone

In the absence of oxygen or under limited oxygen concentration, organisms use sulfate as the electron acceptor. Because of the very low aqueous solubility of SBF base chemical, it will be biodegraded before it can diffuse to other layers. Since sulfate is an electron acceptor, sulfate availability will be a limiting factor in hydrocarbon biodegradation. This can be represented by the Monod equation with half-saturation constant $K_{s,\ SO4}$. In the sulfate consumption zone oxygen is inhibitory for sulfate reducing organisms. This inhibition is added to the rate equation with a function suggested by Van Capellan et al. (1993). When the concentration of oxygen increases the usage of sulfate in biodegradation of hydrocarbon will decrease. Limiting and inhibitory functions in the model will allow the use of a single equation for each electron acceptor zone. The equation describing the SBF concentration in the sulfate consumption zone is presented as Equation 4.2.

$$r_{F,SO_4} = -\left(\frac{\mu_2 X_2 F}{Y_2\left(K_{s,2} + F\right)}\right)\left(\frac{S}{K_{s,SO_4} + S}\right)\left(\frac{K'_{O,SO_4}}{K'_{O,SO_4} + O}\right) \qquad \textbf{Equation 4.2}$$

Where $r_{F,SO4}$ = the rate of SBF degradation in the sulfate utilizing region (mg SBF/kg dry sediment • time), X_2 = SRB concentration in sediment (mg cell/kg dry sediment), F = SBF base chemical concentration (mg base chemical/kg dry sediment), μ_2= maximum specific growth rate for SRB (mg cells/mg cells · time), $K_{s,2}$ = half velocity coefficient in sulfate consumption zone (mg base chemical /kg dry sediment), Y_2 = yield for SRB (mg cells produced/mg base chemical consumed), S = sulfate concentration (mg sulfate/L pore water), $K_{s,SO4}$= half saturation constant for sulfate limitation (mg sulfate/L pore water), O = oxygen concentration (mg oxygen/L pore water), and $K`_{O,SO4}$ =half saturation constant for oxygen inhibition against sulfate usage (mg oxygen/L pore water).

4.1.3 CO_2 Consumption Zone

In the absence of sulfate or under limited sulfate concentrations, methanogens will use CO_2 as an electron acceptor and degrade hydrocarbons. O_2 and SO_4 are inhibitory for methanogens and

inhibition functions must be included on the equation. The mass balance equation becomes Equation 4.3. The concentration of CO_2 is expected to be non-limiting since it is produced by the other organisms in the O_2 and sulfate consumption zones and it is produced in the methanogenic zone, so there is no component of the methanogenic equation limiting SBF degradation based on CO_2 concentration.

$$r_{F,CO2} = \left(\frac{\mu_3 X_3 F}{Y_3 \left(K_{s,3} + F \right)} \right) \left(\frac{K'_{SO_4,CO_2}}{K'_{SO_4,CO_2} + S} \right) \left(\frac{K'_{O,CO_2}}{K'_{O,CO_2} + O} \right) \qquad \textbf{Equation 4.3}$$

Where $r_{F,CO2}$ = the rate of SBF degradation in the methanogenic region (mg SBF/kg dry sediment • time), X_3 = methanogen concentration in sediment (mg cell/kg dry sediment), F = SBF base chemical concentration (mg base chemical/kg dry sediment), μ_3 = maximum specific growth rate for methanogens (mg cells/mg cells • time), $K_{s,3}$ = half velocity coefficient in carbon dioxide consumption zone (mg base chemical/kg dry sediment), Y_3 = yield for methanogens (mg cells produced/mg base chemical consumed), S = sulfate concentration (mg sulfate/L pore water), $K`_{SO4,CO2}$= half saturation constant for sulfate inhibition against $CO_2$ usage (mg sulfate/L pore water), O = oxygen concentration (mg oxygen/L pore water), and $K`_{O,CO2}$ = half saturation constant for oxygen inhibition against CO_2 usage (mg oxygen/L pore water).

4.1.4 General Formula for All Zones

When the above three equations are combined the result is Equation 4.4 which has biodegradation and mixing by bioturbation as the two effects that control changes in concentrations of SBF base chemical in the sediment.

$$\frac{dF}{dt} = \begin{pmatrix} -\left(\frac{\mu_1 X_1 F}{Y_1\left(K_{s,1}+F\right)}\right)\left(\frac{O}{K_{s,O}+O}\right) \\ -\left(\frac{\mu_2 X_2 F}{Y_2\left(K_{s,2}+F\right)}\right)\left(\frac{S}{K_{s,SO_4}+S}\right)\left(\frac{K'_{O,SO_4}}{K'_{O,SO_4}+O}\right) \\ -\left(\frac{\mu_3 X_3 F}{Y_3\left(K_{s,3}+F\right)}\right)\left(\frac{K'_{SO_4,CO_2}}{K'_{SO_4,CO_2}+S}\right)\left(\frac{K'_{O,CO_2}}{K'_{O,CO_2}+O}\right) \\ +D_b\left(\frac{\partial^2 F}{\partial z^2}\right) \end{pmatrix}. \qquad \textbf{Equation 4.4}$$

4.2 Fate of Pore Water Species (Oxygen and Sulfate)

The concentrations of oxygen and sulfate in the pore waters are both used in the SBF equations as controls on SBF degradation, equations that can predict their concentration in the pore water are necessary. The concentrations of these species in pore water are influenced by bioturbation, molecular diffusion, and biodegradation. In the initial model, the biodegradation of each pore water species is modeled using the theoretical requirement for each species based on hexadecane degradation.

The summary reactions when hexadecane is the electron donor, oxygen is electron acceptor, and NH_4^+ is the nitrogen source is:

$C_{16}H_{34}+12.25\ O_2+2.45\ HCO_3^- +2.45\ NH_4^+ \rightarrow 2.45\ C_5H_7O_2N + 6.2\ CO_2 +14.55\ H_2O$

This suggests that for each mole of hexadecane utilized 12.25 moles of oxygen will be required, or 1.7 g O_2 will be required per g of hexadecane consumed. The overall mass balance for oxygen in the sediment pore water then becomes Equation 4.5.

$$\frac{\partial O}{\partial t} = \left(\frac{D'_O}{\theta^2} + D_b\right)\left(\frac{\partial^2 O}{\partial z^2}\right) - 1.7 * \left(\frac{\mu_1 X_1 F}{Y_1\left(K_{S,1}+F\right)}\right)\left(\frac{O}{K_{s,O}+O}\right) \qquad \textbf{Equation 4.5}$$

When hexadecane is the electron donor, sulfate is the electron acceptor, and NH_4^+ is the nitrogen source the overall growth equation is:

$C_{16}H_{34}+ 6.125\ SO_4^{2-}+ 2.45\ HCO_3^- + 2.45\ NH_4^+ +6.2\ H^+ \rightarrow 2.45\ C_5H_7O_2N + 6.125\ HS^- +14.55\ H_2O +6.2\ CO_2.$

This suggests that 6.125 moles of sulfate will be required for each mole of hexadecane consumed (or 2.6 g/g). Equation 4.6 presents the overall mass balance for sulfate in the pore water.

$$\frac{\partial S}{\partial t} = (\frac{D_{SO4}^{'}}{\theta^2} + D_b)\left(\frac{\partial^2 S}{\partial z^2}\right) - 2.6 * \left(\frac{\mu_2 X_2 F}{K_{s,2} + F}\right)\left(\frac{S}{K_{s,so4} + S}\right)\left(\frac{K_{O,SO4}^{'}}{K_{O,SO4}^{'} + O}\right) \qquad \textbf{Equation 4.6}$$

As stated earlier, it is assumed that CO_2 will always be in excess, so there is no need to model its concentration in sediment pore waters.

4.3 SOLUTION FOR EQUATIONS

To predict the fate of SBF in the sediment it is important to determine concentrations with respect to both time and depth. Due to diffusion limitations the different electron acceptor processes will be occurring at different sediment depths. To derive a solution for this the depth dimension z must be added to the equations. For the oxygen consumption zone the equation then becomes Equation 4.7.

$$\frac{\partial O(z,t)}{\partial t} = \begin{pmatrix} (\frac{D_o'}{\theta^2} + D_b)\left(\frac{\partial^2 O(z,t)}{\partial z^2}\right) \\ -1.7 * \left(\frac{\mu_1 X F(z,t)}{Y_1\left(K_{s,1} + F(z,t)\right)}\right)\left(\frac{O(z,t)}{K_{s,O} + O(z,t)}\right) \end{pmatrix} \qquad \textbf{Equation 4.7}$$

With explicit differencing Equation 4.8 and Equation 4.9 are found.

$$\frac{O_i^{k+1} - O_i^k}{\Delta t} = \begin{pmatrix} (\frac{D_o'}{\theta^2} + D_b)\left(\frac{O_{i+1}^k - 2O_i^k + O_{i-1}^k}{\Delta z^2}\right) \\ -1.7 * \frac{\mu_1 X F_i^k}{Y_1\left(K_{s,1} + F_i^k\right)}\left(\frac{O_i^k}{K_{s,O} + O_i^k}\right) \end{pmatrix} \qquad \textbf{Equation 4.8}$$

$$O_i^{k+1} = O_i^k + \Delta t \left(\begin{array}{l} (\frac{D_o^{'}}{\theta^2} + D_b)\left(\frac{O_{i+1}^k - 2O_i^k + O_{i-1}^k}{\Delta z^2} \right) \\ -1.7 * \frac{\mu_1 XF_i^k}{Y_1(K_{s,1} + F_i^k)} \left(\frac{O_i^k}{K_{s,O} + O_i^k} \right) \end{array} \right)$$

Equation 4.9

Similarly Equation 4.10 and Equation 4.11 are found for the concentrations of sulfate in the pore water and the concentration of SBF in the bulk sediment.

$$S_i^{k+1} = S_i^k + \Delta t \left(\begin{array}{l} (\frac{D_{SO4}}{\theta^2} + D_b)\left(\frac{S_{i+1}^k - 2S_i^k + S_{i-1}^k}{\Delta z^2} \right) \\ -\left(2.6 * \frac{\mu_2 XF_i^k}{Y_2(K_{s,2} + F_i^k)} \left(\frac{S_i^k}{K_{s,S} + S_i^k} \right) \left(\frac{K_{O,SO4}^{'}}{K_{O,SO_4}^{'} + O_i^k} \right) \right) \end{array} \right)$$

Equation 4.10

$$F_i^{k+1} = F_i^k + \Delta t \left(\begin{array}{l} -\left(\frac{\mu_1 XF_i^k}{Y_1\left(K_{s,1} + F_i^k\right)} \right) \left(\frac{O_i^k}{K_{s,O} + O_i^k} \right) \\ -\left(\frac{\mu_2 XF_i^k}{Y_2\left(K_{s,2} + F\right)} \right) \left(\frac{S_i^k}{K_{s,SO_4} + S_i^k} \right) \left(\frac{K_{O,SO_4}^{'}}{K_{O,SO_4}^{'} + O_i^k} \right) \cdots\cdots \\ -\left(\frac{\mu_3 XF_i^k}{Y_3\left(K_{s,3} + F_i^k\right)} \right) \left(\frac{K_{SO_4,CO_2}^{'}}{K_{SO_4,CO_2}^{'} + S_i^k} \right) \left(\frac{K_{O,CO_2}^{'}}{K_{O,CO_2}^{'} + O_i^k} \right) \\ -D_b \left(\frac{F_{i+1}^k - 2F_i^k + F_{i-1}^k}{\partial z^2} \right) \end{array} \right)$$

Equation 4.11

Where: Δt is the time step, C_i^k is the concentration at the grid point $x=x_i$ at time t_k (time at the k^{th} time step).

4.4 SPREADSHEET SOLUTION TO THE MODEL

4.4.1 Layout

A solution to the initial model was developed using two Excel (™) spreadsheets. In the first sheet there was one input matrix and eight solution matrices with 365 rows and 40 columns each. In the second sheet there was one solution matrix with 365 rows and 40 columns. In the first sheet each solution matrix calculated one aspect of the overall model i.e. substrate concentration due to oxygen consumption, number of substrate consuming aerobes, oxygen concentration, etc. The new concentrations in each row or column were derived from previous rows or columns by applying the appropriate equations.

4.4.2 Input Parameters

The input parameters were obtained from the literature wherever possible, estimated from preliminary lab data, or obtained using Excel solver. These included the physical properties of the sediment, initial concentration of electron donor and electron acceptors, initial organism concentrations, time and depth increments, oxygen and sulfate diffusion coefficients, kinetics parameters for different electron acceptors consumption zones and bioturbation effect with changing depth. These initial parameters used in initial modeling are detailed in Table 4.1.

4.5 BOUNDARY CONDITIONS

Initial data (time=0) for sulfate and oxygen are taken from Prof. John H. Trefry (Florida State University) personal communications. Sulfate concentration was almost constant at 2688 mg/L in uncontaminated sites. This was used as the initial average sulfate concentration, at all depths. Oxygen was never found deeper than 6 cm. Therefore oxygen diffusion after 6 cm was neglected. The initial hexadecane concentration was assumed constant in all depths and 2000 mg hexadecane per kg dry sediment.

4.6 SENSITIVITY ANALYSIS

The initial model was developed based on first principles with the intent on using it to determine the parameters that would have the most influence on the fate of SBF base chemicals in marine sediments. This would then allow the focus on the experimental portion of the research to determine the most sensitive factors. The sensitivity of the model to the various parameters was investigated by varying one input parameter and generating curves for SBF (hexadecane), oxygen and sulfate concentrations in the sediment after two years at different depths, returning the changed parameter back to the initial setting (Table 4.1) and then changing the next parameter, etc.

Table 4.1

Parameter Values Used in Initial Modeling

Param.	Value	Units	Description	Ref
ρ_s	2.65	kg/L	Density of sediment	1
ε	0.924		Porosity	2
θ	1.082		Turtuosity (1/ ε)	3
F	2000	mg/kg	Hexadecane concentration in dry sediment	A
O	6.8	mg/L	Oxygen concentration	1
S	2688	mM	Sulfate concentration	1
$X_{1.0}$	2.347	mg/kg	Initial aerobic cell concentration	4
$X_{2,0}$	5035	mg/kg	Initial anaerobic (SO_4) cell concentration	C.E
$X_{3,0}$	0.589	mg/kg	Initial anaerobic (CO_2) cell concentration	C.E
$X_{1.f}$	33.56	mg/kg	Average final aerobic cell concentration	A
$X_{2,f}$	50350	mg/kg	Average final anaerobic (SO_4) cell concentration	A
$X_{3,f}$	589	mg/kg	Average final anaerobic (CO_2) cell concentration.	A
μ_1	0.017	d^{-1}	Maximum specific growth rate (O_2)	M
μ_2	$7.5x10^{-6}$	d^{-1}	Maximum specific growth rate (SO_4)	M
μ_3	0.001	d^{-1}	Maximum specific growth rate (CO_2)	M
$K_{s,1}$	1.19	mg/kg	Half-saturation conc. F for aerobic degradation	5
$K_{s,2}$	$1.17x10^{-6}$	mg/kg	Half-saturation conc. F for anoxic (SO_4) deg.	A
$K_{s,3}$	$1.17x10^{-6}$	mg/kg	Half-saturation conc. F for anoxic (CO_2) deg.	A
Y_1	0.30	g/g	Yield (g aerobic matrices/g hexadecane)	5
Y_2	0.0654	g/g	Yield (g anaerobic (SO_4) matrices/g hexadecane)	6
Y_3	0.0637	g/g	Yield (g anaerobic (CO_2) matrices/g hexadecane)	C
Ks,o	0.0192	mg/L	Half saturation concentration for O_2 limitation for aerobes	5
$K_{S,SO4}$	156	mg/L	Half saturation concentration for SO_4 limitation SRB	7
$K`_{O,SO4}$	0.256	mg/L	Half saturation concentration for O_2 inhibition for SRB	8
$K`_{O,CO2}$	0.256	mg/L	Half saturation concentration for O_2 inhibition for methanogens	8
$K`_{SO4,CO2}$	98	mg/L	Half saturation concentration for SO_4 inhibition for methanogens	8
Δz	2	cm	Depth increment	M
Δt	2	day	Time increment	M
$D`_O$	0.955	cm^2/d	Diffusion coefficient of O_2 in sea water	9
$D`_{SO4}$	0.522	cm^2/d	Diffusion coefficient of SO_4 in sea water	10
D_O	0.815	cm^2/d	Diffusion coefficient of O_2 in sediment pore water	C
D_{SO4}	0.446	cm^2/d	Diffusion coefficient of SO_4 in sediment pore water	C
D_{b0}	$3.3x10^{-2}$	cm^2/d	Bioturbation coefficient	11
x_b	5	cm	Thickness of constant bioturbation layer	11
$coeff_{Db}$	1	cm	Coefficient of exponential bioturbation decrease	11

1)John H. Trefry, Florida State University, Personal Communication (2) Murray et al. 1978, (3) Ullman and Aller, 1982, (4) Berte-Corti and Bruns 1999, (5) Chakravarty 1975, (6) So and Young 1999b, (7) Boudreau and Westrich 1984, (8) Van Cappellen and Wang 1995, (9) Soetaert 1996, (10) Li and Gregory 1974, (11) Wijsman et al. 1997**
A) Assumed parameters, C.E) Calculated from experimental results, C) Calculated, M) parameter derived by model calibration. ** original data adapted to appropriate unit.

4.6.1 Physical Properties

4.6.1.1 Porosity and Tortuosity of the Sediment

The initial porosity assumption was based on work by Murray et al. (1978). The sediments in the Gulf of Mexico may have different porosities than those studied by Murray and the addition of cuttings may affect the porosity of the sediment. Sediment porosity could vary from as low as 0.4 (high clay soil) to 0.98 (sandy sediment). When the porosity assumption used in the model was 0.924 (Figure 4.1 a), the hexadecane was removed from the first 4 cm (due to oxygen consuming reactions) and decreased from the initial 2,000 mg/kg to about 1200 mg/kg in the rest of the sediment during the first two years. Oxygen was completely consumed in the first four cm. The concentration of sulfate decreased slightly but it never became limiting, so methanogenesis would not be expected to be important. When the porosity was reduced to 0.7 (Figure 4.1 b) the model predicted the concentration of hexadecane left in the sediment would increase slightly in the first four cm because of oxygen diffusion limitations but remained relatively the same in the rest of the sediment because sulfate was never depleted from the sediment, even though the concentration of sulfate decreased greatly due to diffusion limitations. When the porosity was decreased to 0.4 (Figure 4.1-c), the sulfate was depleted below 7 cm in the two year period and the hexadecane concentration remaining in the sediment increased to approximately 1600 mg/kg.

These results suggest that the model is sensitive to the porosity parameters, but it would take a significantly low porosity to cause a complete depletion of sulfate in the sediment.

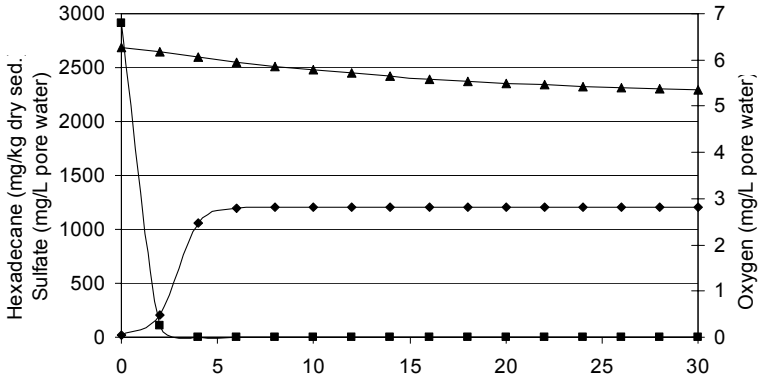
4.6.1.2 Oxygen Diffusion Coefficient

In this sensitivity analysis the initial assumption of the oxygen concentration with depth was not changed. The initial oxygen profile taken from non-contaminated Gulf sediment is used and in any point it is assumed that oxygen concentration cannot be higher than this initial condition. The diffusion coefficient can change with temperature and dynamic viscosity. The diffusion coefficient for oxygen was calculated at 0^oC, 5^oC, 20^oC to be 0.938, 0.955, and 1.01 cm^2/day respectively. In this temperature range changes in diffusion coefficient do not significantly change the results of model (Figure 4.2).

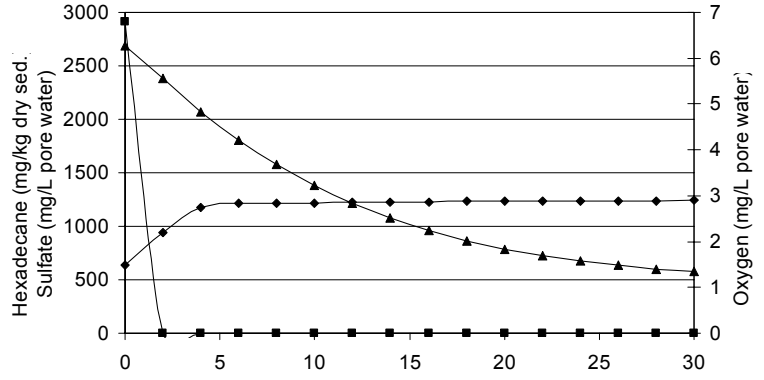
4.6.1.3 Sulfate Diffusion Coefficient.

Similarly to the oxygen diffusion coefficient the sulfate diffusion coefficient would be different at different water temperatures. The diffusion coefficient for sulfate was calculated for temperatures of 0^oC, 5^oC, 20^oC as 0.422, 0.522, and 0.823 cm^2/day respectively. In this temperature range changes in the sulfate diffusion coefficient do not change the results of model (Figure 4.3).

a) Porosity = 0.924



b) Porosity = 0.7



c) Porosity = 0.4

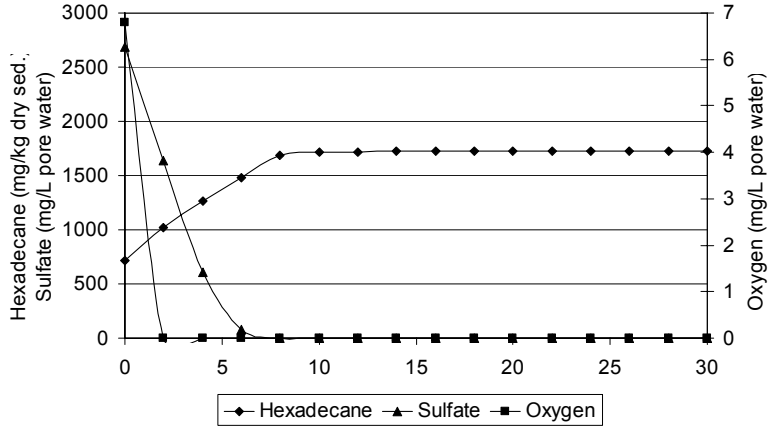
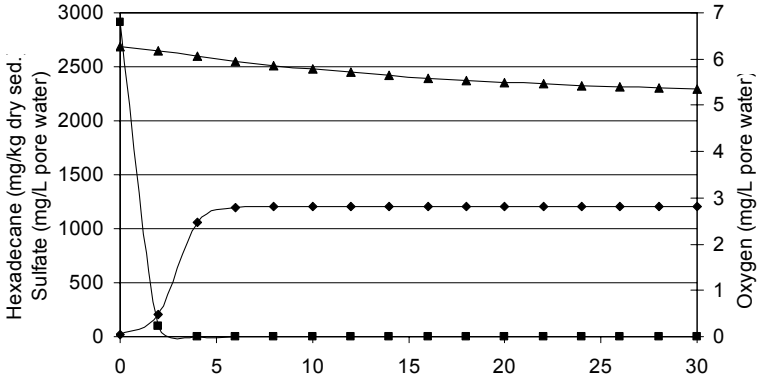
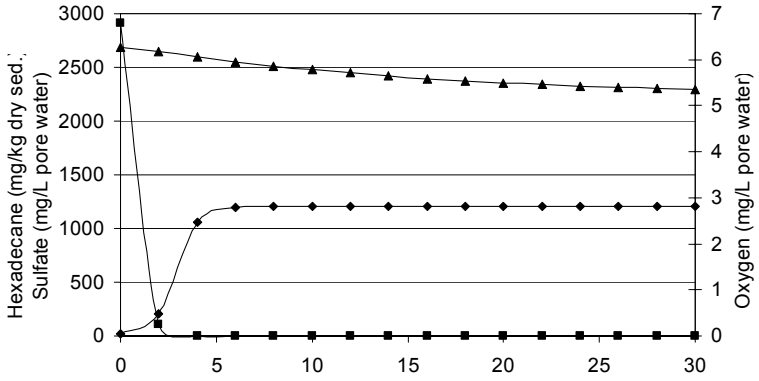


Figure 4.1. Effect of porosity on O_2, SO_4, and hexadecane concentrations in the first 30 cm after two years.

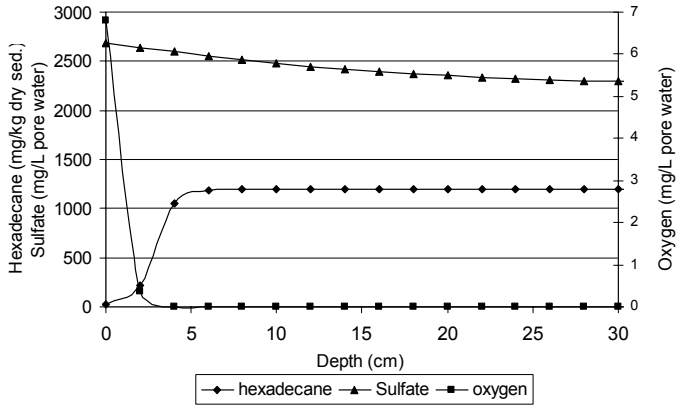
a) $D_O = 0.938\ cm^2/\ day$



b) $D_O = 0.955\ cm^2/\ day$



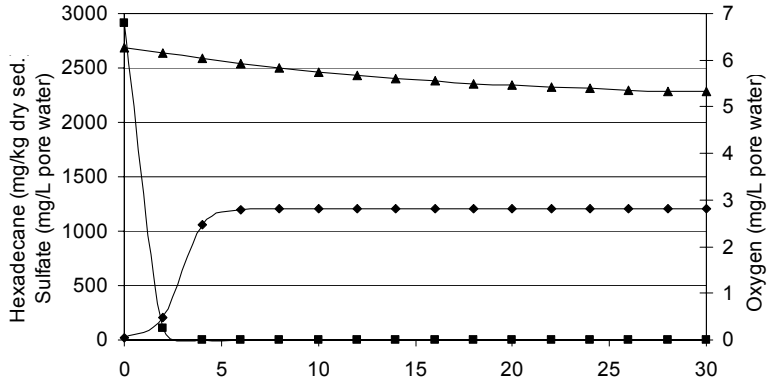
c) $D_O = 1.01\ cm^2/\ day$



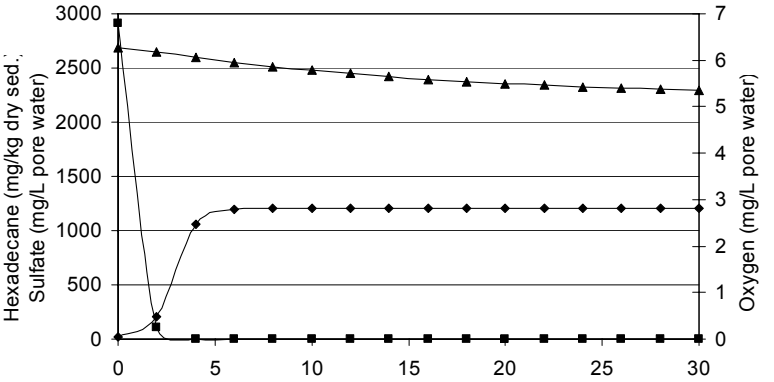
Depth (cm)

Figure 4.2. Effect of the oxygen diffusion coefficient on O_2, SO_4, and hexadecane concentrations in the first 30 cm after two years.

a) $D_{SO4} = 0.422\ cm^2/\ day$



b) $D_{SO4} = 0.522\ cm^2/\ day$



c) $D_{SO4} = 0.823\ cm^2/\ day$

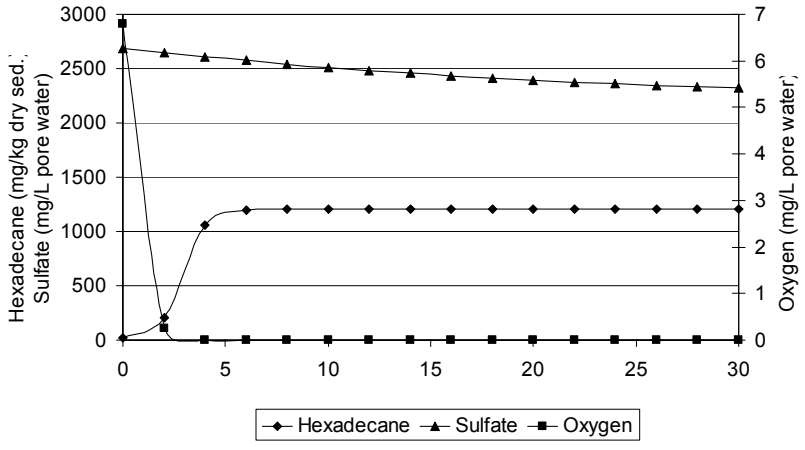


Figure 4.3. Effect of sulfate diffusion coefficient on O_2, SO_4, and hexadecane concentrations in the first 30 cm after two years.

4.6.1.4 Initial Concentration of Hexadecane

In the initial parameters it is assumed that the initial hexadecane amount in all sediment is homogenous and 2000 mg/kg dry sediment. In reality, the hexadecane concentration in the sediment will be different depending on how the cuttings are processed before disposal and how the cuttings become dispersed on the sediment. In this sensitivity analysis, the initial hexadecane concentration was varied from 400 to 4000 mg/kg sediment. As would be expected a lower initial hexadecane concentration results in more complete biodegradation of hexadecane in the two-year period (Figure 4.4). The model is sensitive to different initial hexadecane concentrations.

4.6.2 Biological Parameters

Although the theory for modeling biological systems is quite well developed, there are very few studies reported in the literature that can be used as sources of the coefficients for modeling biological processes in the environment. The sensitivity analysis was generally conducted by varying the biological coefficient 10-fold higher and lower than the value used as the initial assumption.

4.6.2.1 Bioturbation Coefficient

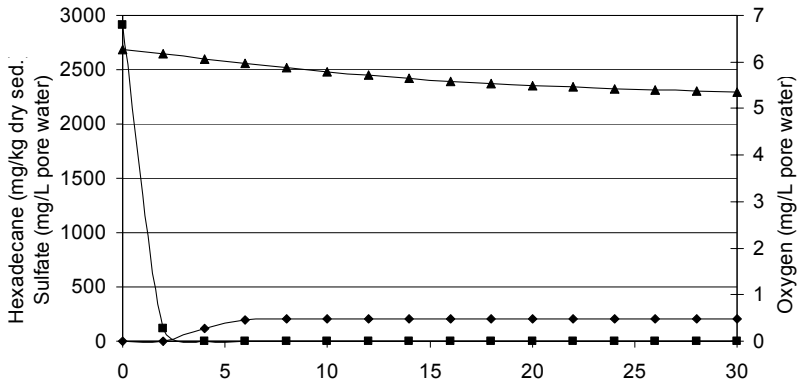
Because bioturbation is effective in the first few centimeters but decreases exponentially with depth, the effect of bioturbation is only shown in the first few centimeters in Figure 4.5. Decreasing the bioturbation coefficient from 0.033 to 0.0022 cm^2/d did not significantly change the model predictions for oxygen, sulfate or tetradecene concentration at any depth. Increasing the coefficient to 0.33 cm^2/d resulted in a prediction of higher concentrations of hexadecane in shallower sediment. Hexadecane concentration as shown in Figure 4.5 c is higher shown in Figure 4.5 a or b. The more disturbed sediment is also more homogenous throughout the depth.

4.6.2.2 Oxygen Consumption Zone

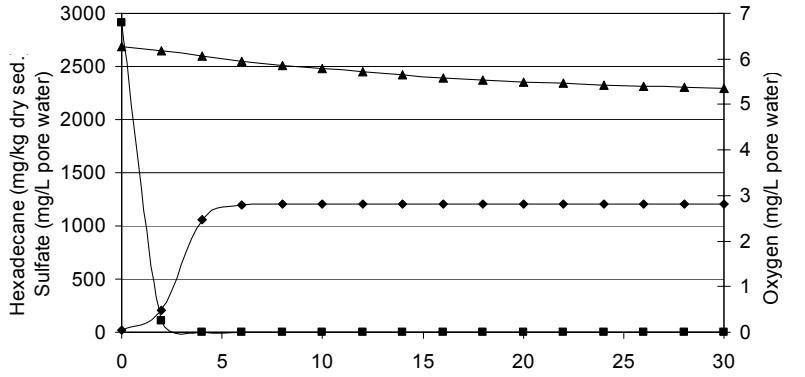
The limitation of the presence of oxygen to the first few centimeters limits the magnitude of effects that changing the parameters associated with degradation of the substrate in the oxygen zone will have on the system as a whole.

Maximum Growth rate constant (μ_1): The maximum growth rate for aerobes was decreased (Figure 4.6 a) or increased (Figure 4.6 c) by tenfold. Increasing the growth rate did not affect the consumption of hexadecane in the sediment significantly (due to oxygen limitations) but decreasing the value tenfold caused the hexadecane consumption to decrease in the first few centimeters than in the deeper sediment. This is because aerobic degradation became very slow and oxygen was inhibitory for anaerobic biodegradation of hexadecane by SRB so no degradation occurred. In Figure 4.6 a the predicted concentration of hexadecane in the aerobic zone is actually higher than predicted concentration of hexadecane in the anaerobic zone (deeper than 8 cm because the activity of the SRB was greater than the activity of the aerobes.

a) F = 500 mg hexadecane/kg dry sediment



b) F = 2000 mg hexadecane/kg dry sediment



c) F = 4000 mg hexadecane/kg dry sediment

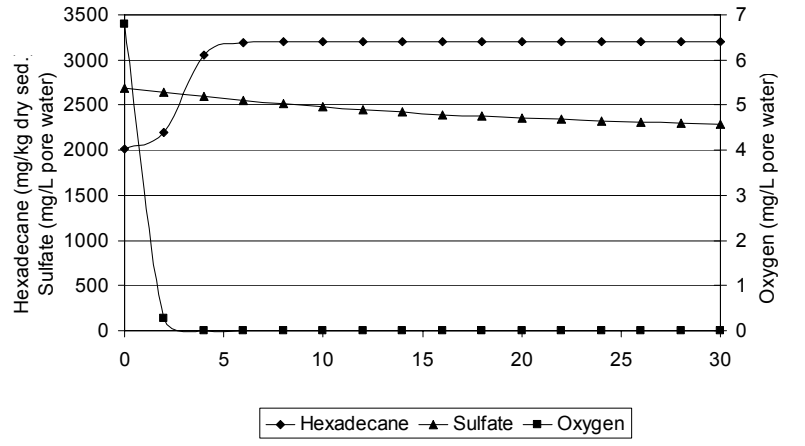
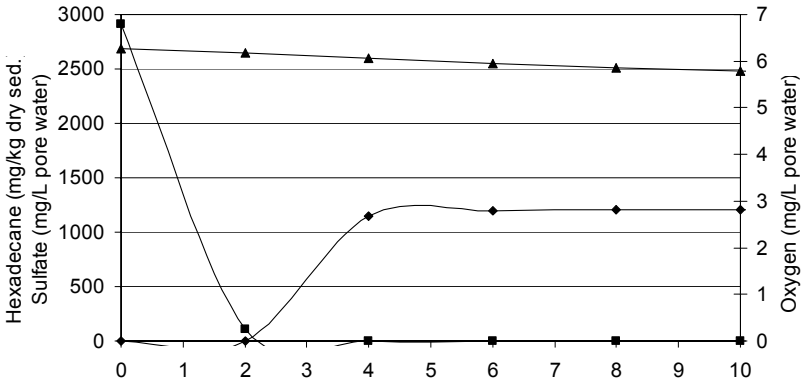
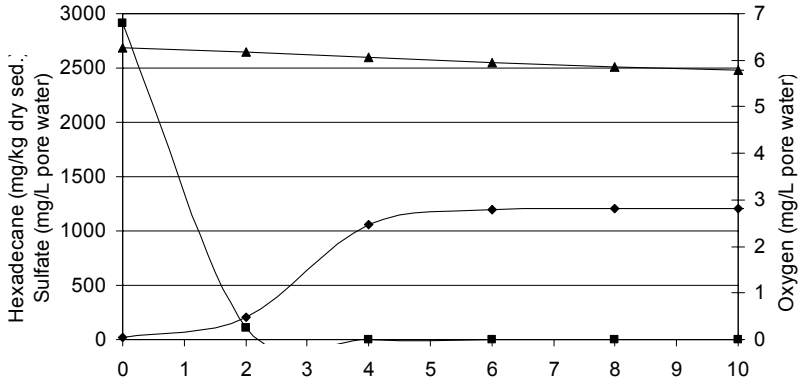


Figure 4.4. Effect of the initial hexadecane concentration on O_2, SO_4, and hexadecane concentrations in the first 30 cm after two years.

a) $D_b = 0.00022$ cm^2/ day



b) $D_b = 0.033$ cm^2/ day



c) $D_b = 0.33$ cm^2/ day

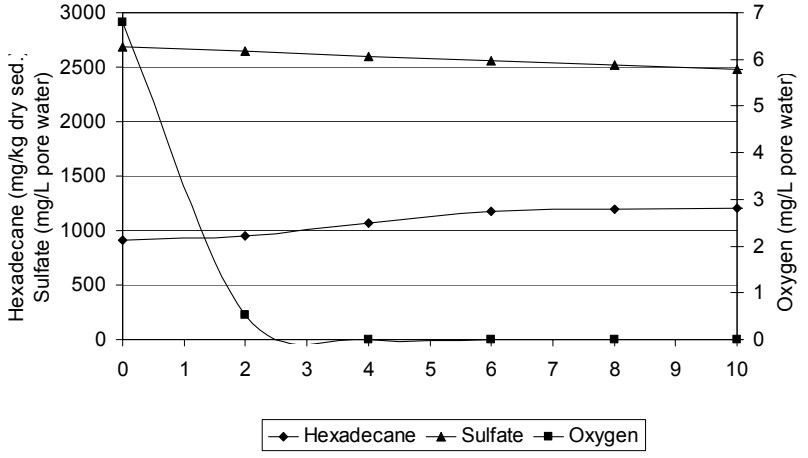
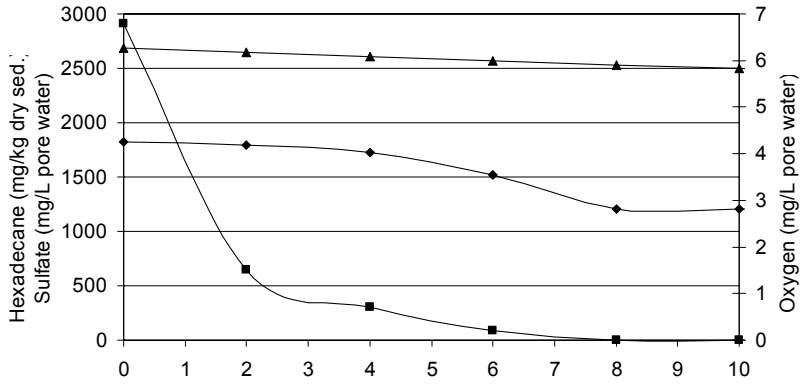
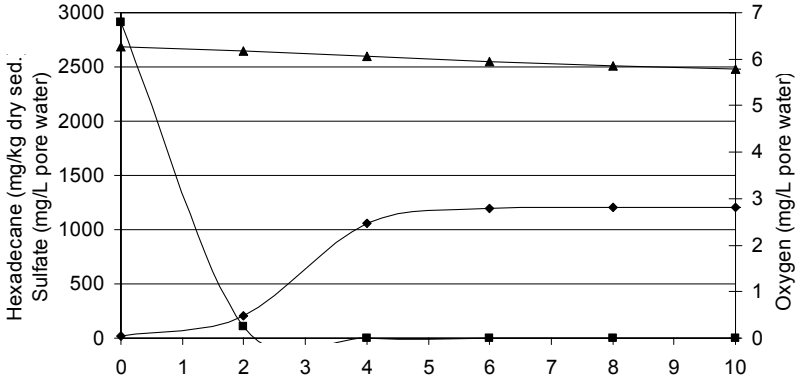


Figure 4.5. Effect of the bioturbation coefficient on O_2, SO_4, and hexadecane concentrations in the first 10 cm after two years.

a) $\mu_1 = 0.0017\ day^{-1}$



b) $\mu_1 = 0.017\ day^{-1}$



c) $\mu_1 = 0.17\ day^{-1}$

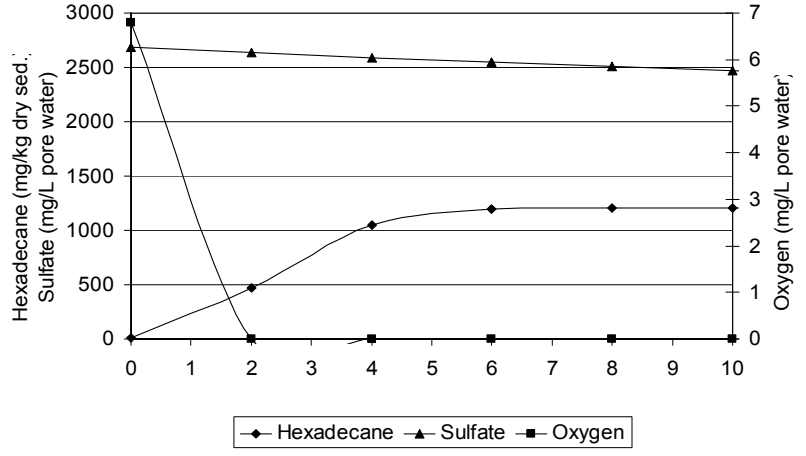


Figure 4.6. Effect of the growth rate of aerobes on O_2, SO_4, and hexadecane concentrations in the first 10 cm after two years.

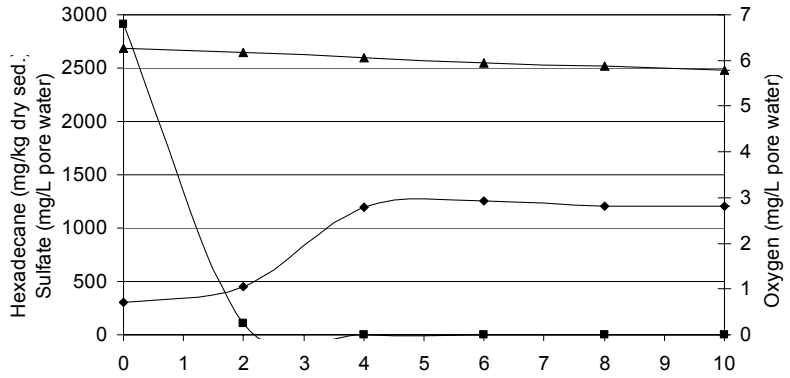
Aerobic initial cell concentration ($X_{1,0}$): When the concentration of hexadecane degrading aerobes was increased, hexadecane consumption did not change greatly (Figure 4.7 c) again due to oxygen limitations. When the initial aerobe concentration was decreased (Figure 4.7 a) the model predicted that slightly more hexadecane would be left in the sediment after 2 years of incubation. This is not a significant difference and can only be observed at the 2 cm depth.

Yield (Y_1): The calculated yield and the yield found in archives for aerobes depicted in Table 2.3 are 1.3965 and 0.3 g cells per g hexadecane consumed. In this sensitivity analysis, the calculated yield was used as a maximum yield, because this value is maximum theoretical yield. The minimum yield was chosen to be 10 fold less than the archival observed yield. When the yield was decreased (Figure 4.8 a) there was very little difference in the predicted concentration of hexadecane, sulfate or oxygen in the sediment from those observed when the archival assumption was used, again a small difference at 2 cm is visible. When the yield was increased to the theoretical value, the concentrations of O_2 and hexadecane in the first few centimeters were increased greatly (Figure 4.8 c). Increasing the yield resulted in a reduction in biodegradation activity because the population reached its maximum carrying capacity. It is unlikely that environmental organisms will ever be able to achieve theoretical yields. The major differences are observed in the first 4 cm.

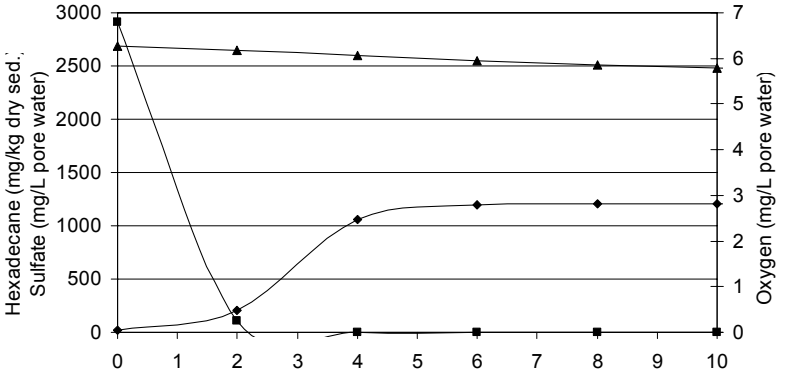
Half saturation constant for hexadecane limitation in oxygen consumption zone ($K_{S,1}$): The half saturation coefficient should be evaluated in terms of the substrate concentration. When it is low and the substrate concentration is high, it will have very little effect. As it approaches the substrate concentration (or the substrate concentration decreases), its effect becomes more important. The most observable effect here was when $K_{S,1}$ was 1190 mg/kg (Figure 4.9 c) or about ½ of the initial substrate concentration, there was still a significant amount of hexadecane remaining in the aerobic zone (first 4 cm).

Oxygen limitation coefficient ($K_{s,o}$): The oxygen limitation for degradation of hexadecane is determined by the enzymes employed in oxygenic reactions by the organisms. Typical environmental organisms will have multiple oxygen utilizing systems some with low efficiencies that are more rapid when excess oxygen is present and some with very high binding efficiencies but have slower rates of reaction. When the oxygen limitation coefficient is high, it means the enzyme has low binding capacity and the system will shut down at higher oxygen concentrations. When the limitation coefficient was increased, the model predicted the amount of hexadecane consumed would decrease as expected (Figure 4.10). It should also be noted that the decrease in aerobic activities at higher oxygen concentrations also caused and increase in the concentration of hexadecane in the SRB zone, due to the higher concentrations of oxygen in the sediment Figure 4.10 c.

a) $X_{1,0}$ = 0.2347 mg aerobes /kg dry sediment



b) $X_{1,0}$ = 2.347 mg aerobes /kg dry sediment



c) $X_{1,0}$ = 23.47 mg aerobes /kg dry sediment

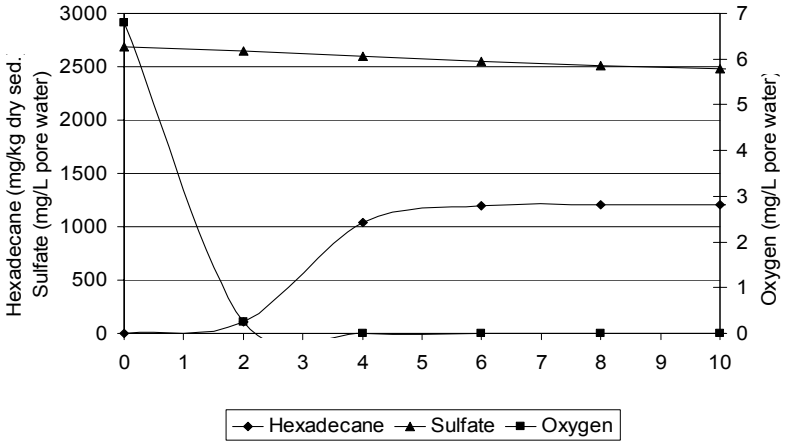
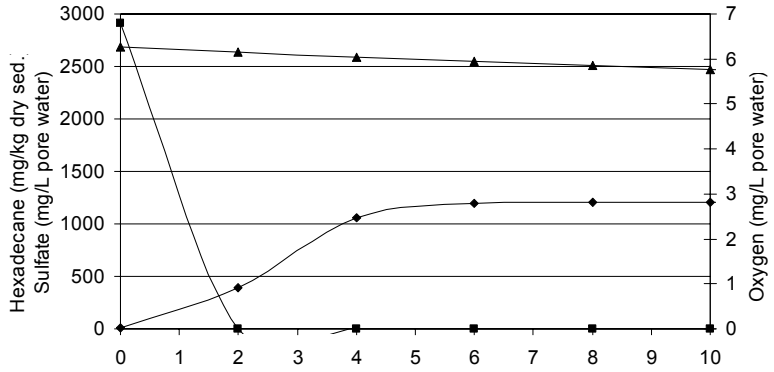
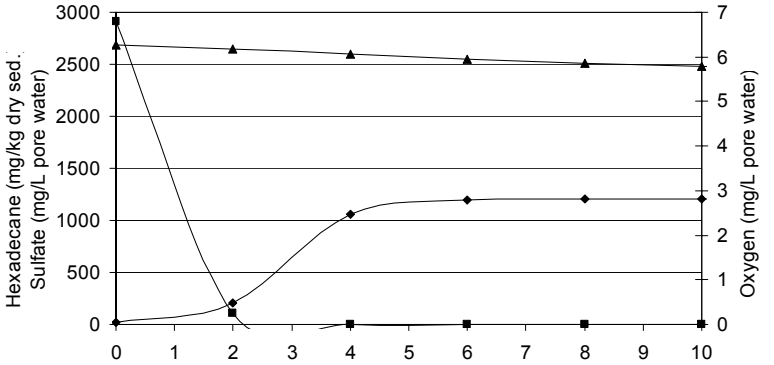


Figure 4.7. Effect of the initial concentration of aerobes on O_2, SO_4, and hexadecane concentrations in the first 10 cm after two years.

a) $Y_1 = 0.03$ g aerobes formed / g hexadecane consumed



b) $Y_1 = 0.3$ g aerobes formed / g hexadecane consumed



c) $Y_1 = 1.4$ g aerobes formed / g hexadecane consumed

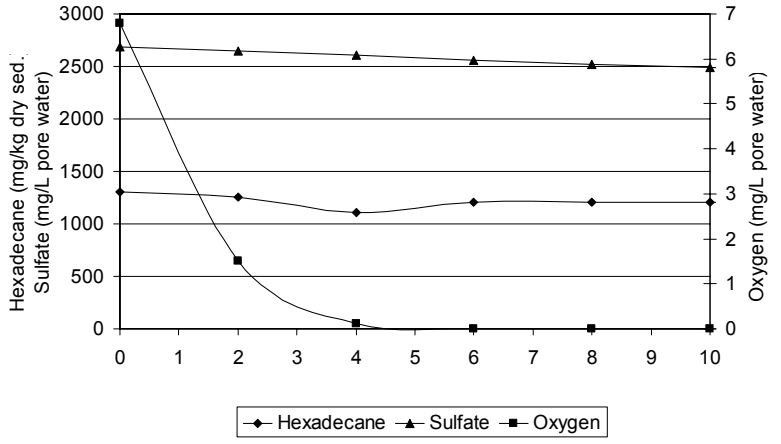
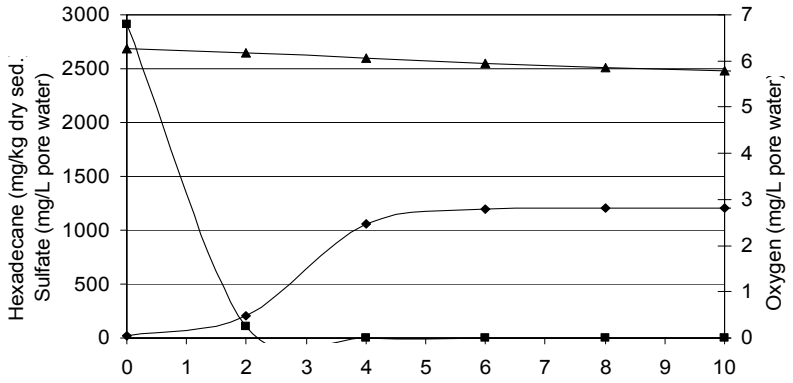
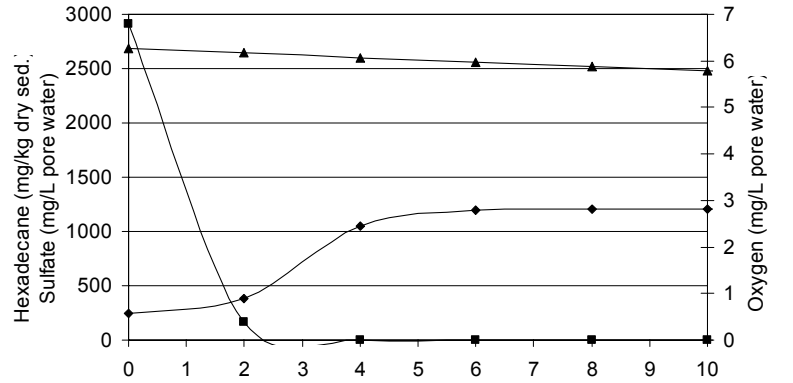


Figure 4.8. Effect of the yield value for aerobic hexadecane degradation on O_2, SO_4, and hexadecane concentrations in the first 10 cm after two years.

a) $K_{S,1}$ = 1.190 mg hexadecane / kg dry sediment



b) $K_{S,1}$ = 119.0 mg hexadecane / kg dry sediment



c) $K_{S,1}$ = 1190.0 mg hexadecane / kg dry sediment

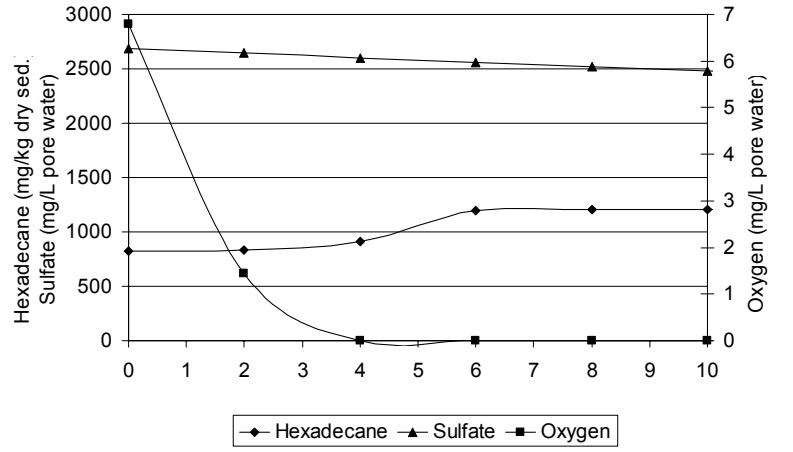
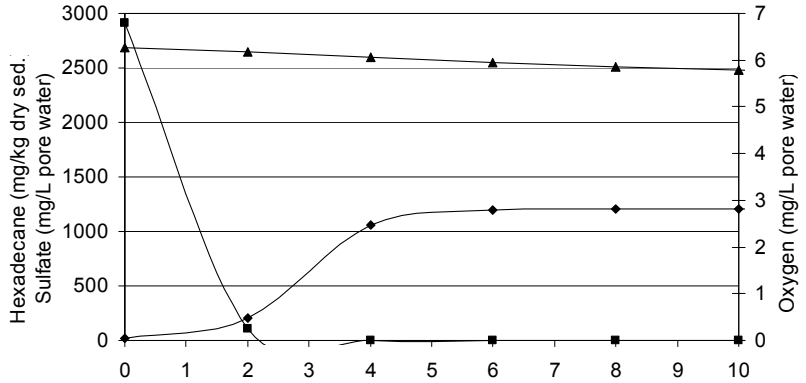
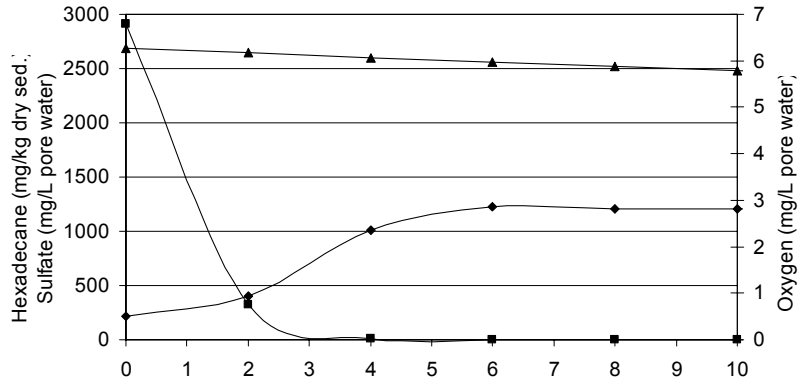


Figure 4.9. Effect of the half saturation constant for aerobes on O_2, SO_4, and hexadecane concentrations in the first 10 cm after two years.

a) $K_{S,O}$ = 0.0192 mg oxygen / L pore water



b) $K_{S,O}$ = 0.192 mg oxygen / L pore water



c) $K_{S,O}$ = 1.92 mg oxygen / L pore water

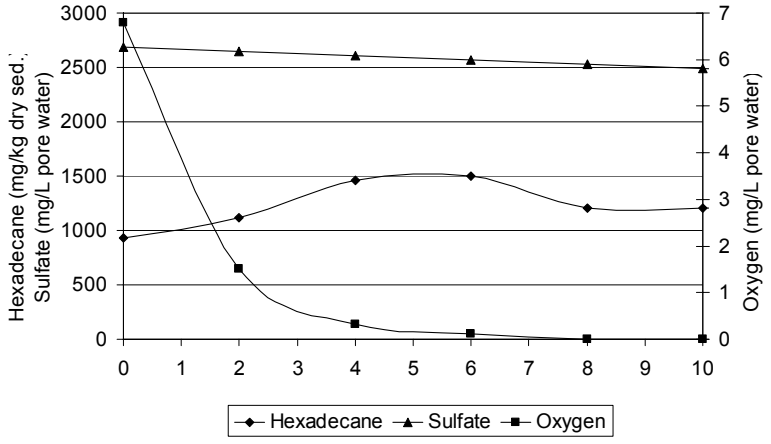


Figure 4.10. Effect of the oxygen limitation coefficient on O_2, SO_4, and hexadecane concentrations in the first 10 cm after two years.

4.6.2.3 Sulfate Consumption Zone

Since most of the hexadecane consumption is expected to occur with sulfate as the electron acceptor, the kinetic parameters for sulfate reduction should have more of an effect on the model predictions.

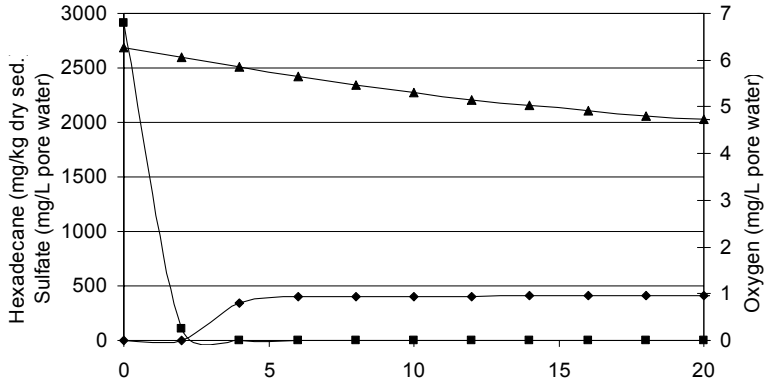
Maximum growth rate constant (μ_2): As expected the model was very sensitive to changes in the maximum growth rate constants for SRB. Even a very small increase (doubling) in μ_2 caused a large increase in hexadecane consumption (Figure 4.11 a vs b) as indicated by the decrease in hexadecane concentrations remaining after two years of incubation. Even a small decrease (halving) in μ_2 caused large decrease in hexadecane consumption (Figure 4.11 c) as indicated by the large increase in hexadecane remaining after 2 years of incubation. The changes in μ_2 also affected the sulfate concentration in the sediment (as expected). The oxygen concentrations in the sediment were not affected, also as expected.

SRB initial concentration ($X_{2,}$): When the initial SRB concentration was increased more biodegradation of hexadecane occurred as evidenced by the decreasing concentrations of hexadecane predicted by the model and shown in Figure 4.12 a through c. High initial SRB concentration would occur when sediment has been exposed to hydrocarbons or other carbon sources. High initial SRB concentration could also decrease the adaptation period for organisms to degrade hexadecane.

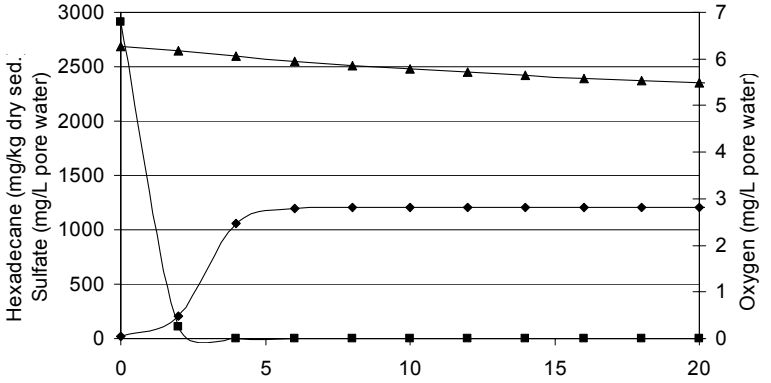
Yield (Y_2): The calculated yield and the yield found in archives for SRB were presented in Table 2.3 as 0.1446 and 0.0327 g SRB produced per g hexadecane consumed. In this sensitivity analysis the theoretical (calculated) yield is used as maximum yield and a value 10 times less than the archival yield was used as a minimum yield. In Figure 4.13 a the low yield (0.00327) of SRB caused more SO_4 to be used but was accompanied by complete removal of hexadecane in all depths within the first 2 years. This is due to two factors; 1) the increase in the rate due to an increase in yield, an intrinsic relationship in the Monod model and 2) it takes longer to reach the carrying capacity of the system. Conversely increasing the yield to the calculated theoretical yield caused less sulfate and hexadecane removal from the sediment (Figure 4.13 c).

Half saturation constant for hexadecane limitation in sulfate consumption zone ($K_{s,2}$): Because of the high hexadecane concentration in sediment, a change in half saturation constant (1000 fold up or down) had no effect on the results from the model prediction (Figure 4.14). In the sulfate reaction zone, the half saturation constant for hexadecane is the least effective parameter in the model. To have any effect the $K_{S,2}$ would have to be very near the hexadecane concentration.

a) μ_2 = 1.502E-5 day^{-1}



b) μ_2 = 7.508E-6 day^{-1}



c) μ_2 = 3.754E-6 day^{-1}

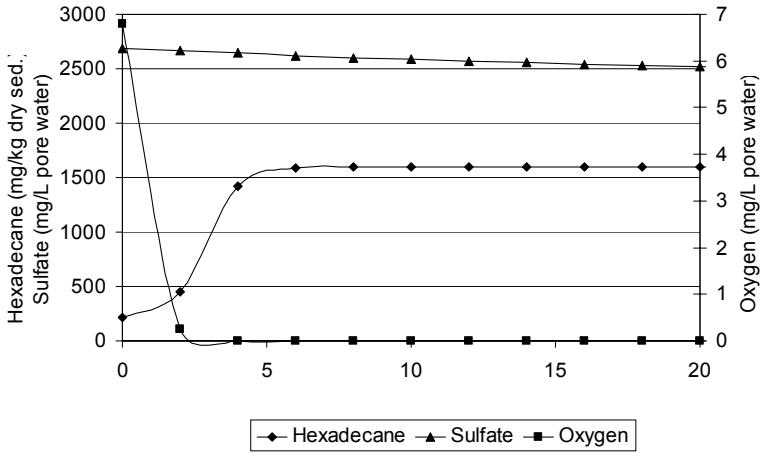
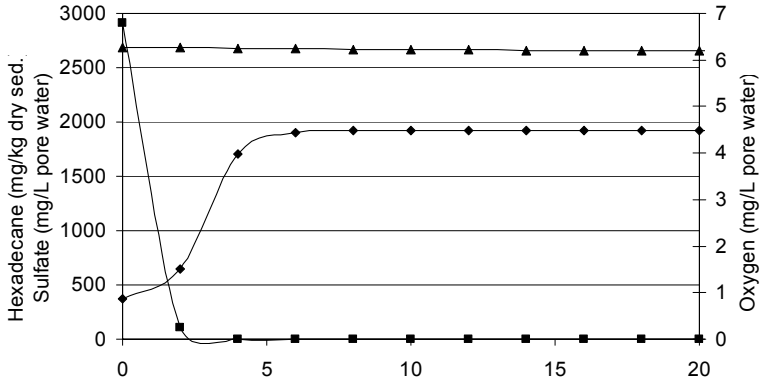
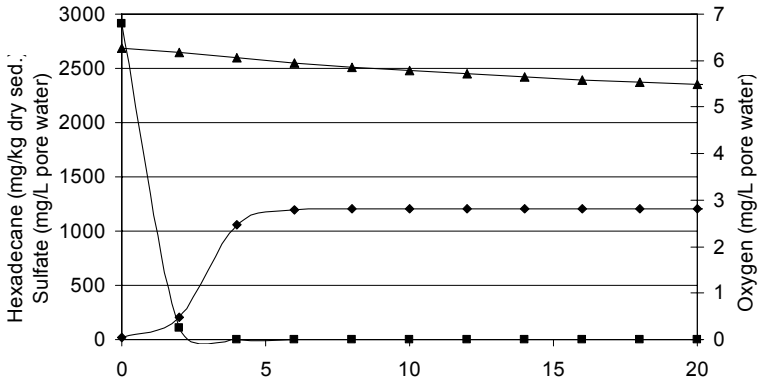


Figure 4.11. Effect of the growth rate of SRB on O_2, SO_4, and hexadecane concentrations in the first 30 cm after two years.

a) X_2 = 503.5 mg SRB / kg dry sediment



b) X_2 = 5035 mg SRB / kg dry sediment



c) X_2 = 50350 mg SRB / kg dry sediment

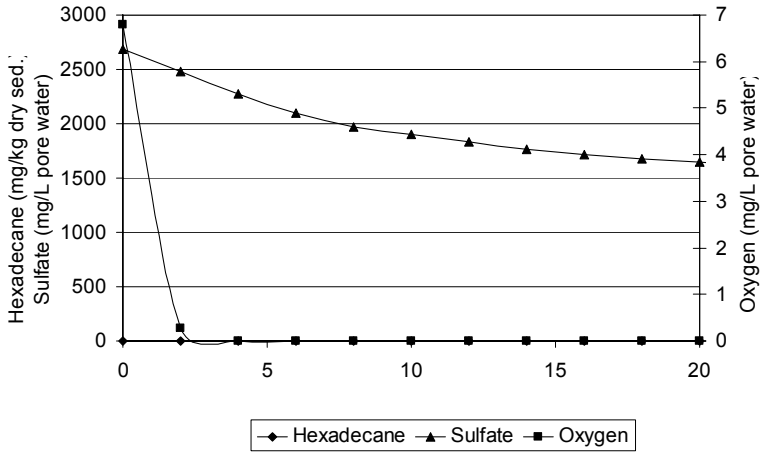
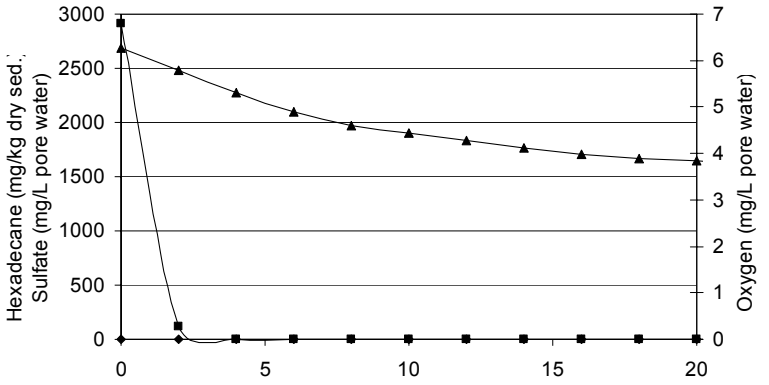
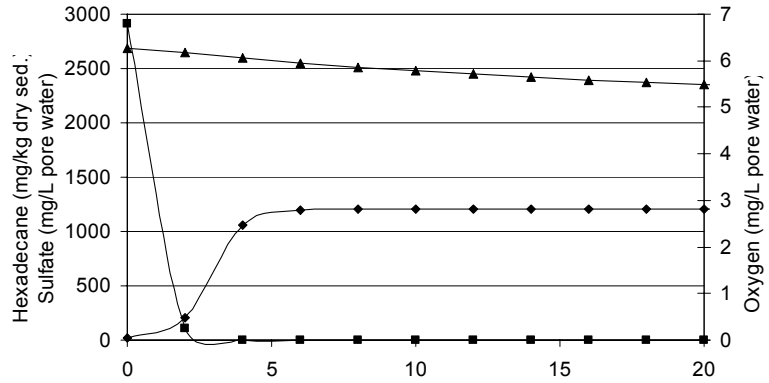


Figure 4.12. Effect of the number of SRB on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

a) Y_2 = 0.00327 g SRB formed / g hexadecane consumed



b) Y_2 = 0.0327 g SRB formed / g hexadecane consumed



c) Y_2 = 0.1446 g SRB formed / g hexadecane consumed

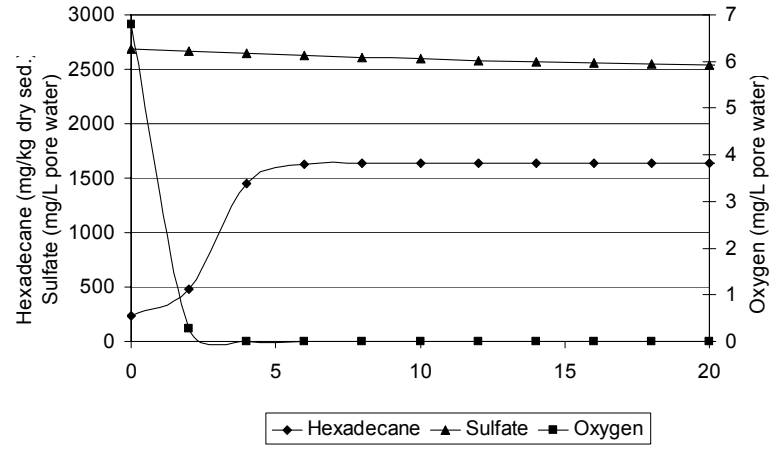
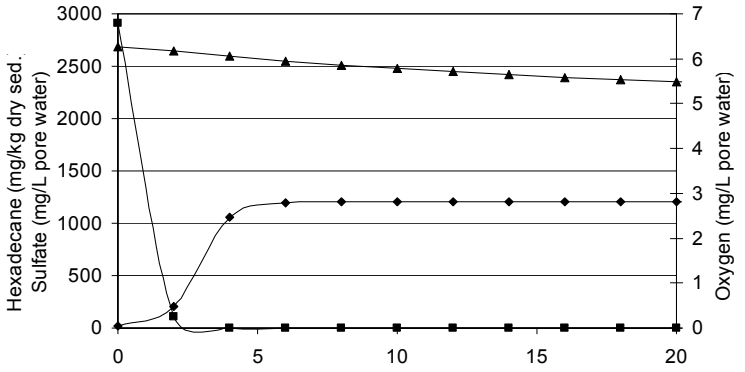
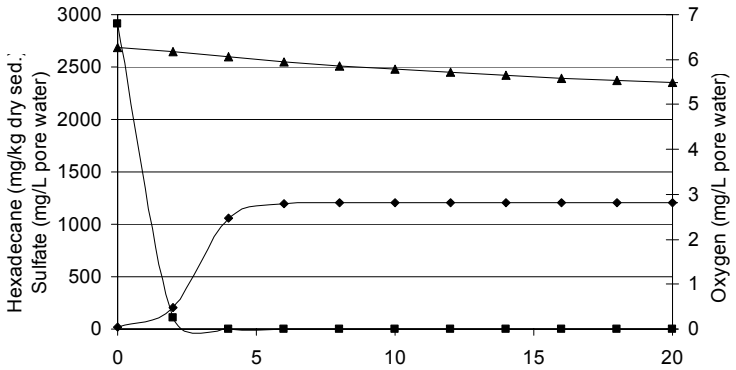


Figure 4.13. Effect of the yield of SRB on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

a) $K_{S,2} = 1.17 \times 10^{-6}$ mg hexadecane / kg dry sediment



b) $K_{S,2} = 1.17 \times 10^{-3}$ mg hexadecane / kg dry sediment



c) $K_{S,2} = 1.17$ mg hexadecane / kg dry sediment

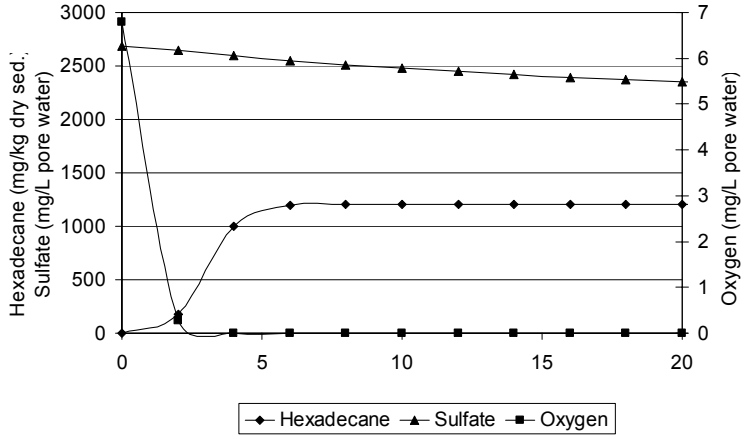


Figure 4.14. Effect of the half saturation constant for SRB on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

Half saturation constant for sulfate limitation ($K_{s,SO4}$): Because of the high sulfate concentration in sediment pore water; a change in half saturation constant (of 10 fold up or down) has little effect on the result from the model (Figure 4.15). Changing $K_{s,SO4}$ would only be effective when the concentration of sulfate was very low or the value of the constant was close to or higher than the sulfate concentration. Other than when the porosity was decreased to a very low value did the model ever predict the sulfate concentration would become limiting.

Oxygen inhibition coefficient in sulfate consumption ($K`_{O, SO4}$): Because of the low oxygen concentration in the sediment, and because oxygen can only diffuse to the first few centimeters, changing this constant had very little effect on the model predictions. Figure 4.16 demonstrates that the only visible effect of decreasing the O_2 inhibition coefficient 10X was observed as slightly higher hexadecane concentration in the first 4 cm. Increasing $K_{O,SO4}$ 10X caused a slightly lower hexadecane concentration at 4 cm.

4.6.2.4 CO_2 Consumption Zone

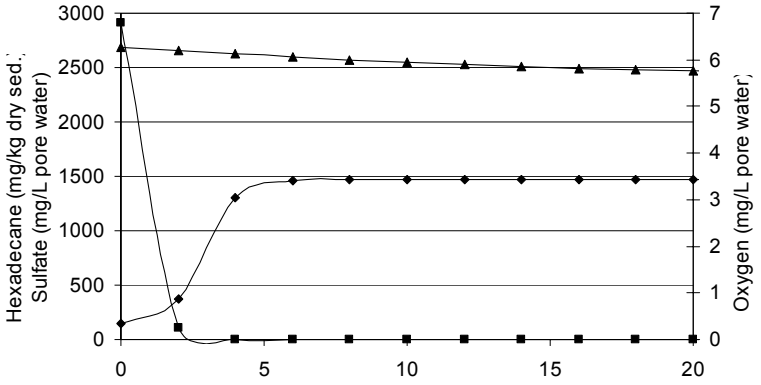
Because of high sulfate concentration and low initial methanogen concentration, methanogenesis is limited. Therefore, all parameters related to methanogens had very little effect on the model predictions. Research concerning methanogenic activities that can be found in the literature does not do more than document that this type of biodegradation may occur in deep-sea sediment. The sensitivity analysis was run for all of the methanogenic parameters, but the results will not be presented here since there were not any significant differences observed in the predictions of hexadecane, sulfate, and oxygen concentrations observed in the graphs.

4.7 CONCLUSIONS FROM INITIAL MODELING

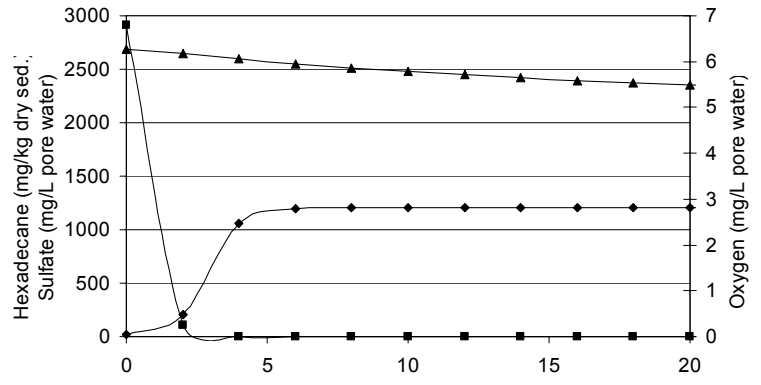
The model was developed using existing fundamental equations for modeling physical and biological rates of compound diffusion and biodegradation. The input parameters were obtained from a review of existing information from related articles and research or they were estimated based on solving for a predicted model performance. With these initial parameters, the model as developed in the spreadsheet solution performed well, in other words the predicted results compare reasonably with expectations. After all of the hexadecane was consumed in the all depths of the sediment, the model predicted oxygen and sulfate concentrations in the sediment would to slowly return to initial conditions due to the effects of diffusion and bioturbation.

The sensitivity analysis showed that the parameters the model was most sensitive to were a) porosity of the sediment, b) maximum growth rate constants for SRB and less so for aerobes, c) initial cell concentrations of SRB, and d) yields of aerobes and SRB. Sulfate was never limiting in the sediment (unless the porosity became very low) and because of the high sulfate concentration methanogens had very little impact on the biodegradation of hexadecane.

a) $K_{S,SO4}$ = 1555 mg / L pore water



b) $K_{S,SO4}$ = 156 mg / L pore water



c) $K_{S,SO4}$ = 16 mg / L pore water

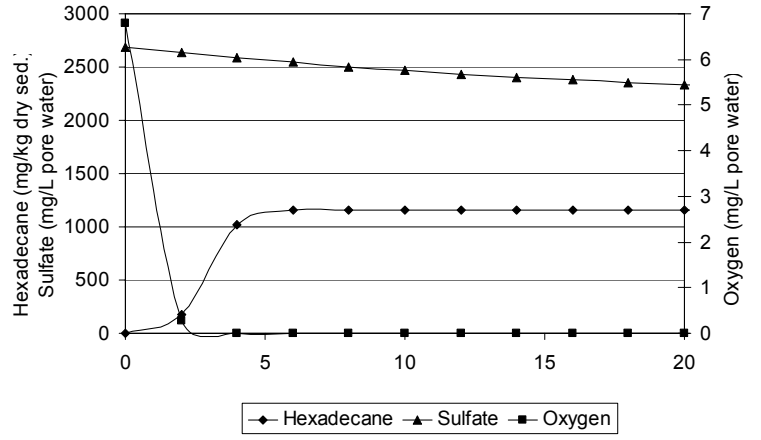
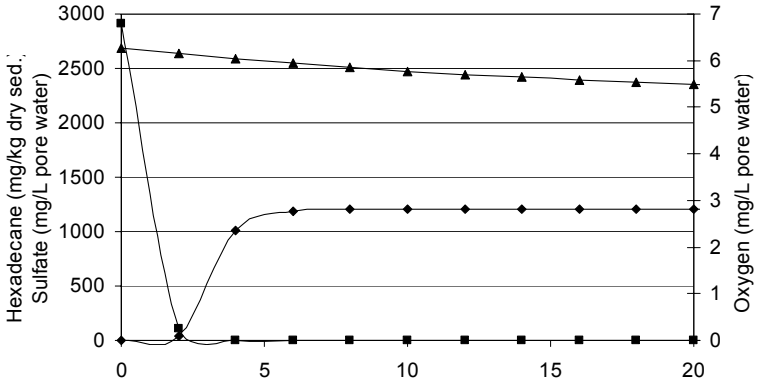
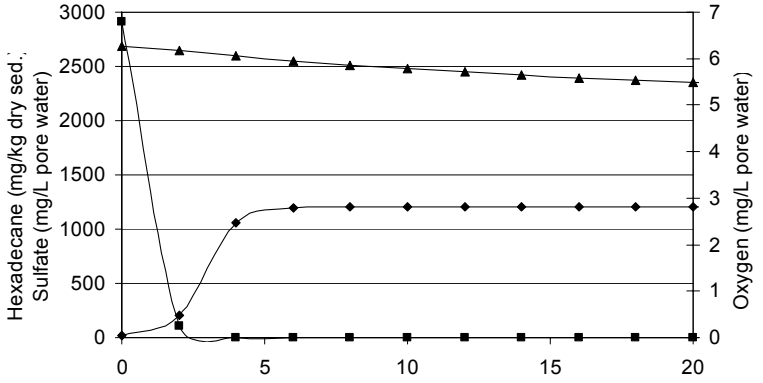


Figure 4.15. Effect of the sulfate limitation coefficient for SRB on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

a) $K`_{O,SO4}$ = 2.56 mg / L pore water



b) $K`_{O,SO4}$ = 0.256 mg / L pore water



c) $K`_{O,SO4}$ = 0.0256 mg / L pore water

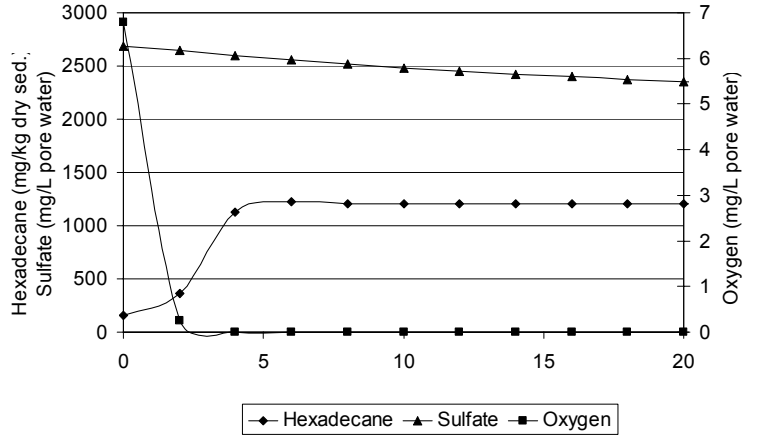
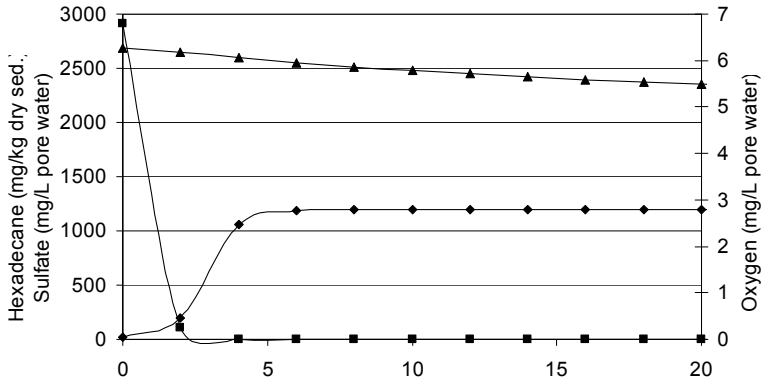
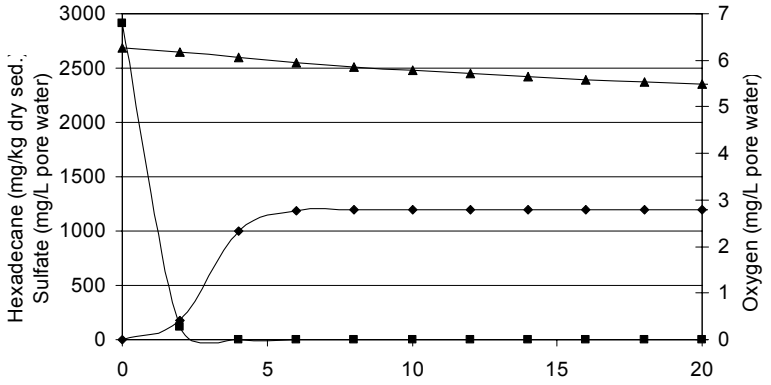


Figure 4.16. Effect of the oxygen inhibition coefficient for SRB on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

a) $K`_{O,CO2}$ = 2.56 mg / L pore water



b) $K`_{O,CO2}$ = 0.256 mg / L pore water



c) $K`_{O,CO2}$ = 0.0256 mg / L pore water

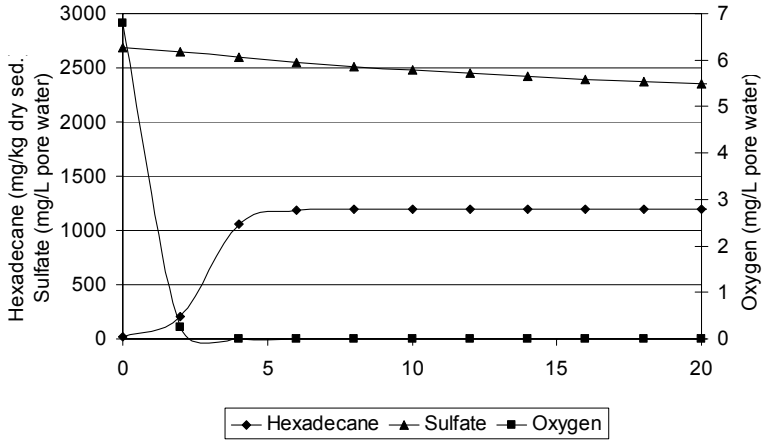
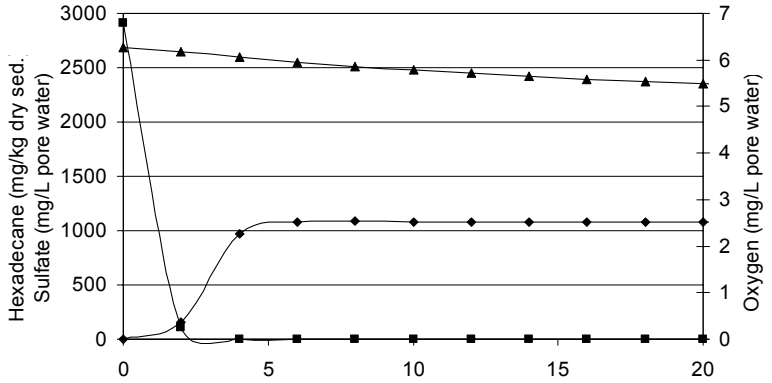
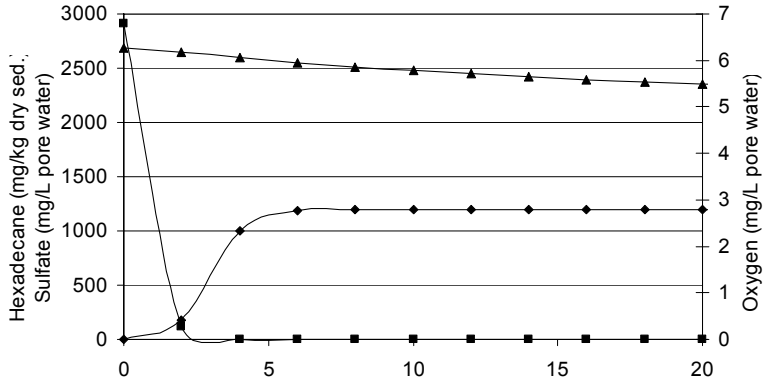


Figure 4.17. Effect of the oxygen inhibition of methanogens on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

a) $K`_{SO4,CO2}$ = 980 mg / L pore water



b) $K`_{SO4,CO2}$ = 98 mg / L pore water



c) $K`_{SO4,CO2}$ = 9.8 mg / L pore water

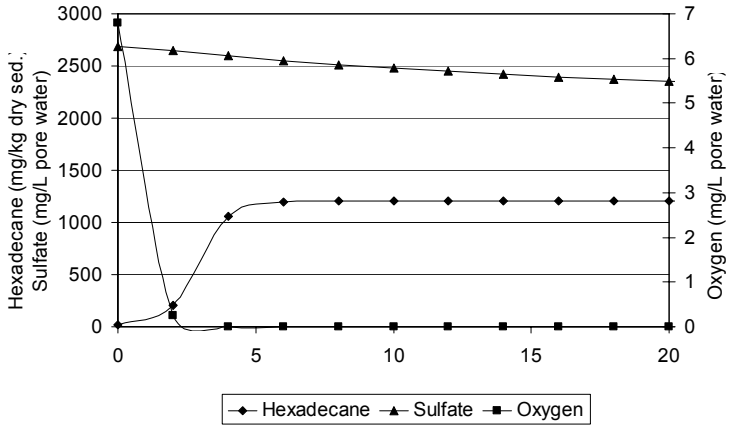


Figure 4.18. Effect of the sulfate inhibition of methanogens on O_2, SO_4, and hexadecane concentrations in the first 20 cm after two years.

CHAPTER 5 MICROECOLOGY STUDIES

5.1 METHOD DEVELOPMENT

Although the methods used in this research were not developed *de novo* for this research, a considerable effort was made to validate that the methods used were appropriate to meet the objectives of the research.

5.1.1 Separation of Cells and Sediment

The general appearance of the sediments under the microscope suggested that the sediments contained a lot of diffuse organic material that was fluorescing with the cells. Figure 5.1 presents a photomicrograph of a sediment sample magnified with the 100X objective and viewed using the DAPI filter. This is one of the better microscopic fields, and several DAPI stained cells can be seen. The cells stained with DAPI are a true blue (a few examples are highlighted with a white circle). The organic material is yellow, green, or grey and can mask the blue of the DAPI cells. This fluorescence posed problems while performing DAPI counts as well as counting through FITC and TRITC filters since there were low levels of autofluorescence in these filters as well.

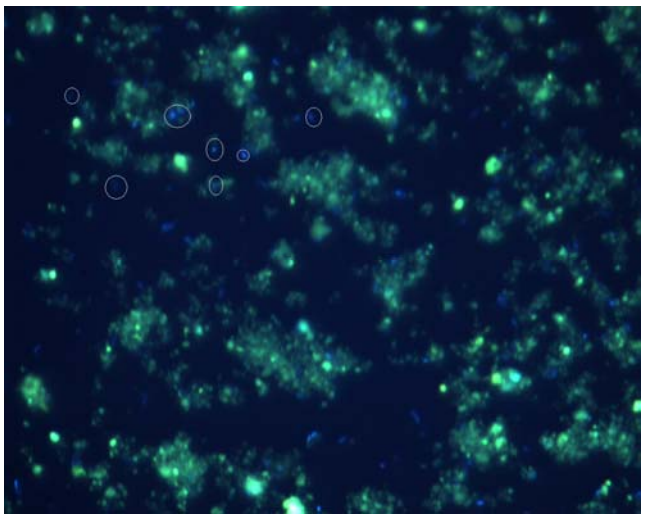


Figure 5.1. Sediment viewed using the DAPI filter (100x oil objective).

A sample of sediment was fixed and homogenized as described above (Section 3.2.3). After homogenization, the sediment slurry was disrupted by sonication three times at 2 min intervals using the sonic probe (25 kHz). The sand particles were allowed to settle out into a pellet and the supernatant was poured off carefully into two 40 ml glass centrifuge tubes. These were centrifuged slowly (300 rpm) for 2 min using a MSE GT-2 table centrifuge. This resulted in the formation of three layers. The topmost clear layer from both the tubes was separated and labeled as 1st layer. The second, cloudy, layers from both the tubes were combined in one tube. The third layer (i.e., the pellet) was added to the sand pellet.

The second layer from each tube was diluted with PBS, and the process (from slow centrifugation) was repeated twice. All the 1st (clear) layers were added together into one 1st layer and all the pellets were added into one pellet. The remaining second layer obtained after slow centrifugation and separation (3 times) was now treated as the original homogenized sediment slurry by adding some more PBS and the whole process (from sonication) was repeated. The 1st layer, 2nd layer and pellet were all separated into their respective tubes and the volumes noted. These layers were then used for DAPI staining.

Table 5.1 presents the cell counts after DAPI staining all the three layers of the sediment. Theoretically the first layer should have more cells and fewer sediment particles, so that it can be used for FISH. The other two layers were expected to be difficult to use in FISH due to the presence of interfering sediment particles. The goal was to prove that these layers could be discarded because they did not contain significant numbers of cells. The results indicate that the 2nd layer and the pellet cell counts account for a significant percentage of the total counts and that they cannot be discarded. In the case of Galveston Bay sediment the results clearly indicate that the 2nd layer has more cell numbers than the 1st layer implying that the cells cannot be separated from the sediment particles using these techniques.

Another attempt to separate the sediments and the cells was done by increasing the pH of the supernatant in an attempt to dissolve the organic material, which was thought to be humic or fulvic acids. This was accomplished by taking a sample of GC112 fixed sediment slurry and disrupting the cells by sonication three times at two-minute intervals using the sonic probe ($25H_Z$). The sand portion was then allowed to settle for less than three minutes. The supernatant was decanted into a second tube, and after removing a small sample for DAPI staining, its pH was raised to about 13 by using 2N NaOH. This basic supernatant was centrifuged for 15 minutes to remove the cells. The supernatant was then decanted from the pellet. Table 5.2 presents the results obtained after performing DAPI staining on samples of these solutions to visualize the total amount of cells present.

The results revealed that the sand pellet contained more cells than expected (25 percent of the total). This was unexpected. The amount of cells present in the first layer supernatant was 75 percent of those present in the entire solution (first layer supernatant plus the sand pellet). The cell pellet only contained 21 percent of the cells that were in the original supernatant. This is where the majority of the cells were supposed to be detected.

These results again suggest that the removal of the cells from the sediment would not be possible with these techniques. Further attempts to separate/clarify the microbial fractions of the sediment from the particles in the sediment were as follows: 1. acidification of the fixed sample before probing; 2. acidification of the sample after probing; 3. fast centrifugation to separate cells into a pellet and leave diffuse organic material in the supernatant. None of these procedures was successful and counting was the same as when just homogenization was done for 15 min with addition of 0.5 percent sodium pyrophosphate. In summary, the attempts made at cell separation were not successful. In order to count as many cells as possible, the whole sediment was used for MPN and FISH analysis. It must be kept in mind that there will be interference from natural sediment particles.

Table 5.1

Quantitative Analysis of Cell Counts in Separation Layers

Sediment	1st Layer (cells) (% of total)	2nd Layer (cells) (% of total)	Pellet (cells) (% of total)	Total Counts (cells)
Freeport				
Trial I	9.1×10^8 (65%)	2.7×10^8 (19%)	2.3×10^8 (16%)	1.4×10^9
Trial II	1.0×10^8 (18%)	3.5×10^8 (64%)	1.0×10^8 (18%)	5.5×10^8
Galveston Bay				
Trial I	1.3×10^8 (40%)	1.4×10^8 (45%)	4.9×10^7 (15%)	3.2×10^8
Trial II	7.6×10^7 (21%)	2.5×10^8 (67%)	4.5×10^7 (12%)	3.7×10^8
Trial III	7.3×10^7 (20%)	2.5×10^8 (69%)	4.1×10^7 (11%)	3.6×10^8
Sportsman Road				
Trial I	7.1×10^7 (56%)	1.9×10^7 (15%)	3.8×10^7 (29%)	1.3×10^8
Trial II	5.0×10^7 (49%)	2.6×10^7 (25%)	2.7×10^7 (26%)	1.0×10^8

Table 5.2

DAPI Counts on Separated Cells and Supernatants

Layers	DAPI Counts (cells)	Cell Recovery
Original supernatant (OS)	1.4×10^8	75% of total 1
Sand pellet	4.7×10^7	25% of total 1
Total 1	1.87×10^8	
Basic supernatant	7.3×10^7	52% of OS
Cells	3.00×10^7	21 % of OS
Total 2	1×10^8	71% of OS

5.1.2 Most Probable Number (MPN) Technique

Engineers and microbiologists have used the MPN method to enumerate microorganisms in samples since 1915 (Cochran 1950). The validation portion of this research was to ensure that the media used gave the highest number of organisms for each of the different types of nutritional groups given that the procedures have not been extensively applied to marine sediments. The majority of the media used were taken from literature sources but altered to fit the desired marine conditions and then tested to see if they were acceptable. There was no way to prove that the media was the "best" except by whether it gave higher counts than a different formulation, or whether the counts obtained could be predicted based on the expected counts from the sediment activity studies. The validation activities also allowed the selection of appropriate dilutions for future research.

Sediments collected from the shores of the Gulf of Mexico were used for the media validation studies since there were insufficient amounts of the deep Gulf sediments to use for this test. Table 5.3 presents the results obtained during the MPN validation studies. Two media formulations were examined for their ability to give the greatest number of SRB. The major differences were the use of sodium lactate or sodium acetate as carbon sources. Sodium lactate is the standard carbon source used in SRB media, but since the research goal is to study the number of organisms that could possibly be involved in hydrocarbon degradation, it was felt that acetate would be the more appropriate carbon source since this compound is a product of the β-oxidation of hydrocarbons. Lactate is an intermediate of sugar, not hydrocarbon, metabolism. The results showed that the use of acetate as an electron donor and carbon source produced higher numbers than the use of lactate did, especially in the sediment that was known to be more competent at hydrocarbon degradation (Freeport#1). The use of the lactate medium was dropped.

Table 5.3

Method Validation for MPN Media

MPN Test	Sediment	Result (MPN/g dry sediment)
SRB (acetate utilizing)	Freeport#1	1.4×10^9
	Fourchon#4	7.5×10^7
SRB (lactate utilizing)	Freeport#1	2.0×10^8
	Fourchon#4	2.6×10^7
General Anaerobes	Freeport#1	1.7×10^8
	Fourchon#4	5.3×10^7
Methanogens	Freeport#1	5.1×10^3
	Fourchon#4	3.5×10^3

The validation of the media used to enumerate other nutritional groups was primarily to show that some positive results could be produced using the specific media. Only one formulation of each medium was examined. The media for general anaerobes and methanogens both produced numbers that would be acceptable. Organisms were counted with each medium and higher numbers were found in the sediments that displayed more SBF-degrading activity (Freeport#1, see Herman and Roberts 2005). During method validation work, all the dilution sets were started at 10^{-2} and ended in 10^{-6}. The results allowed the selection of different dilution ranges for different organisms i.e., decrease dilutions (10^{-1} - 10^{-5}) for the methanogens, increase to higher dilutions (10^{-3} - 10^{-7}) for SRB and general anaerobes.

The overall numbers obtained also met theoretical expectations when compared to other nutritional groups. The SRB were higher in number than others and this is expected because of the high concentration of sulfate in seawater. General anaerobes were lower in numbers than SRB, possibly because the media has no electron acceptor and only allows fermentation. Methanogens were the lowest in numbers. They were expected to be present in low numbers because of the high concentrations of sulfate in seawater, which inhibits methanogenesis.

5.1.3 Verification of Molecular Technique

The idea that any growth medium or method, such as those described above for the MPN of different nutritional groups, would always give lower numbers than would be truly found in any environment has prompted the development of molecular techniques like FISH to allow the enumeration of organisms based on their DNA or RNA sequences. The oligonucleotide probes used in this study were purchased with fluors that produce different colored signals so that each sample could be tested with probes directed to at least two different phylogenetic groups and sample volume could be conserved. The ability to sandwich the probes (i.e. use two probes on one sample) allows more information to be obtained from each sample. The oligonucleotide probe sequences were selected to allow the enumeration of all Eubacteria (Eub 338), all Archaebacteria (Arch 915), all Clostridia (Clost I), and most SRB (SRB 385). There is no oligonucleotide probe that can be used to enumerate all SRB, but SRB 385 will bind to the majority of SRB. The Eubacterial probe was purchased with a Texas Red fluor, which fluoresces red and is observed through a filter with excitation and emission wavelengths specifically for this fluor (TRITC filter). All of the other probes were purchased conjugated to a fluorescein fluor, which is observed through a filter with excitation and emission wavelengths specifically for this fluor (FITC filter).

The main objective of the method development and validation studies was to determine a hybridization temperature (T_H) that would allow for successful hybridization of both probes and to verify that each probe was hybridizing to the correct organisms at that temperature. The objective for each probe combination was to prove that the two probes could be used simultaneously (sandwiched) at a selected T_H and maintain selectivity.

5.1.3.1 Probe Combination Eub 338 and Clost I.

The positive controls for Clost I probe were *C. alginoliticum* and *C. sordelli*, i.e. the probe should bind to these organisms, fluoresce green and be observable using the FITC filter on the microscope. The negative controls for Clost I probe were *B. subtilis, D. multivorans, D. salexigens, E. coli, P. aeruginosa,* and *S. aureus* i.e. the probe should not bind to these organisms and there should be no fluorescence observed through the FITC filter. The positive controls for Eub 338 probe were *B. subtilis, C. alginoliticum, C. sordelli, D. multivorans, D. salexigens, E. coli, P. aeruginosa,* and *S. aureus* which should be bound by the probes and thus be observable as red fluorescence through the TRITC filter. The negative controls for Eub 338 were run later (see 5.1.3.3) and gave the expected results.

For the combination of Clost I and Eub 338 probes, samples were hybridized at hybridization temperatures from 53°C to 57°C in increments of 0.5°C. The highest number of cells showing fluorescence was observed at T_H = 53.5°C. The probe combination of Clost I and Eub 338 worked as expected with all the pure cultures used. Table 5.4 presents the matrix obtained when pure cultures were probed with the two probes simultaneously at T_H = 53.5°C. All of the cultures were Eubacteria and were detected as having the Eub 338 probe hybridized to them (TRITC filter) and only the *Clostridia* were detected with the Clost I probe (FITC filter). The DAPI also stained all of the cells. The culture *C. alginoliticum* was attached to the alginate in the medium

and was therefore, clumpy. The culture *M. roseus* was also clumpy. These cultures were not used in further experiments.

Table 5.4

Probe Combination Eub 338 and Clost I Validation (T_H = 53.5°C)

Culture	DAPI	FITC	TRITC
B. subtilis	+	-	+
C. sordelli	+	+	+
C. alginoliticum	+	+	+
D. multivorans	+	-	+
D. salexigens	+	-	+
E. coli	+	-	+
M. roseus	+	-	+
P. aeruginosa	+	-	+
S. aureus	+	-	+

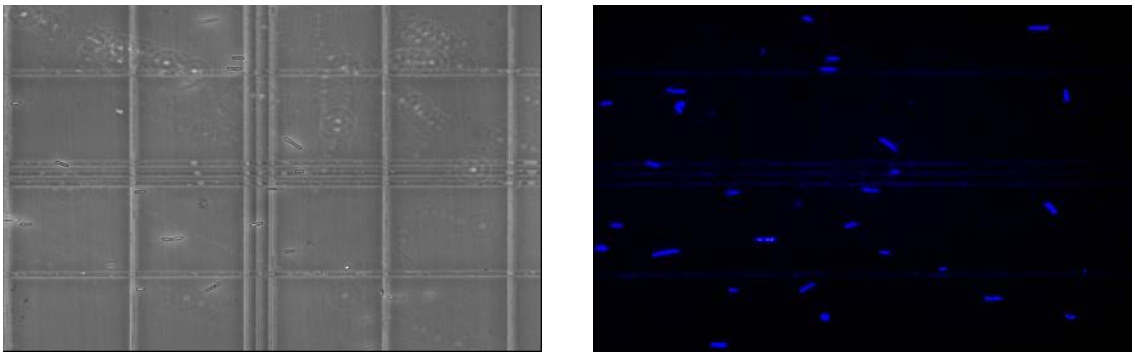
Figure 5.2 a presents photomicrographs of *C. sordelli* using phase contrast microscopy and a 40x objective after fixing and probing. Figure 5.2 b, c, and d present the same field at 40x objective through DAPI, FITC, and TRITC filters, respectively. *C. sordelli* should fluoresce through all the filters as shown in the figure. These photomicrographs were taken to represent the same observation area viewed through all the three filters consecutively.

As an example of a negative control for this probe combination Figure 5.3a-d present photomicrographs of the *S. aureus* culture after fixing and probing. *S. aureus* is a positive control for DAPI and TRITC and is a negative control for FITC. It should fluoresce blue and red through DAPI and TRITC filters, respectively but should not fluoresce at all through the FITC filter as shown.

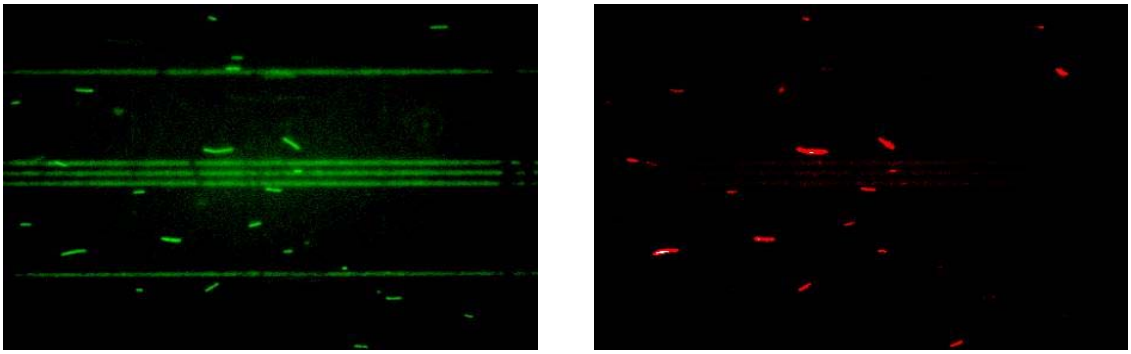
5.1.3.2 Probe Combination Eub 338 and SRB 385.

The positive controls for SRB 385 probe were *D. multivorans* and *D. salexigens* and should be observed as green fluorescence through the FITC filter. The negative controls for SRB 385 probe were *B. subtilis, C. sordelli, E. coli, P. aeruginosa,* and *S. aureus*. Hybridization was attempted at 54°C and 53.5°C. Good probing was observed at T_H = 53.5°C. This probe combination worked as expected with all the pure cultures used. Table 5.5 presents the matrix of results obtained when the two probes were used in combination at T_H = 53.5°C.

Figure 5.4a-d present photomicrographs of the *D. multivorans* culture magnified using the 100x objective with oil immersion and viewed using phase contrast as well as using the DAPI, FITC or TRITC filters. *D. multivorans* is a positive control for all the probes so it should fluoresce through all the filters as shown in the figures. These photomicrographs were taken to represent the same observation area viewed through all the three filters consecutively.

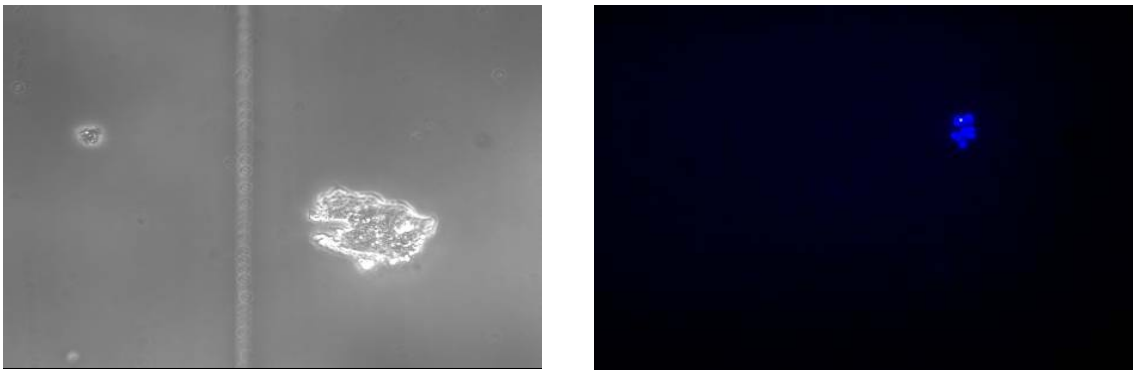


a. Phase Contrast b. DAPI filter

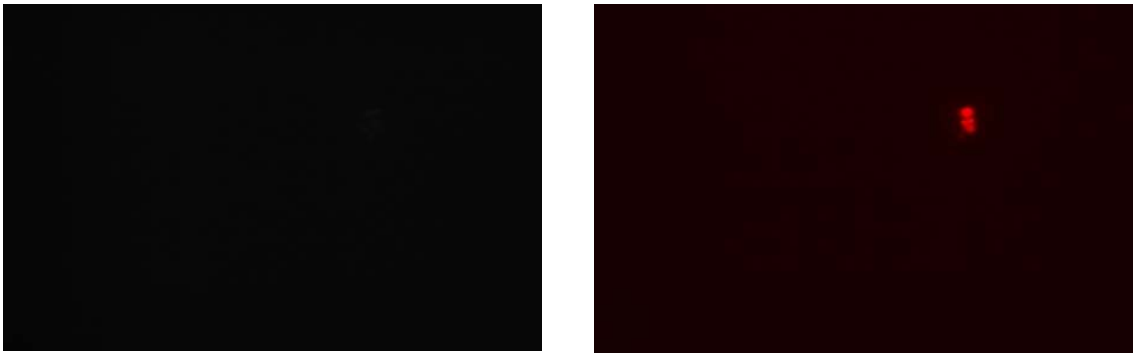


c. FITC filter d. TRITC filter

Figure 5.2. *C. sordelli* using Eub 338 and Clost I probes (40x objective).



a. Phase contrast b. DAPI filter



c. FITC filter d. TRITC filter

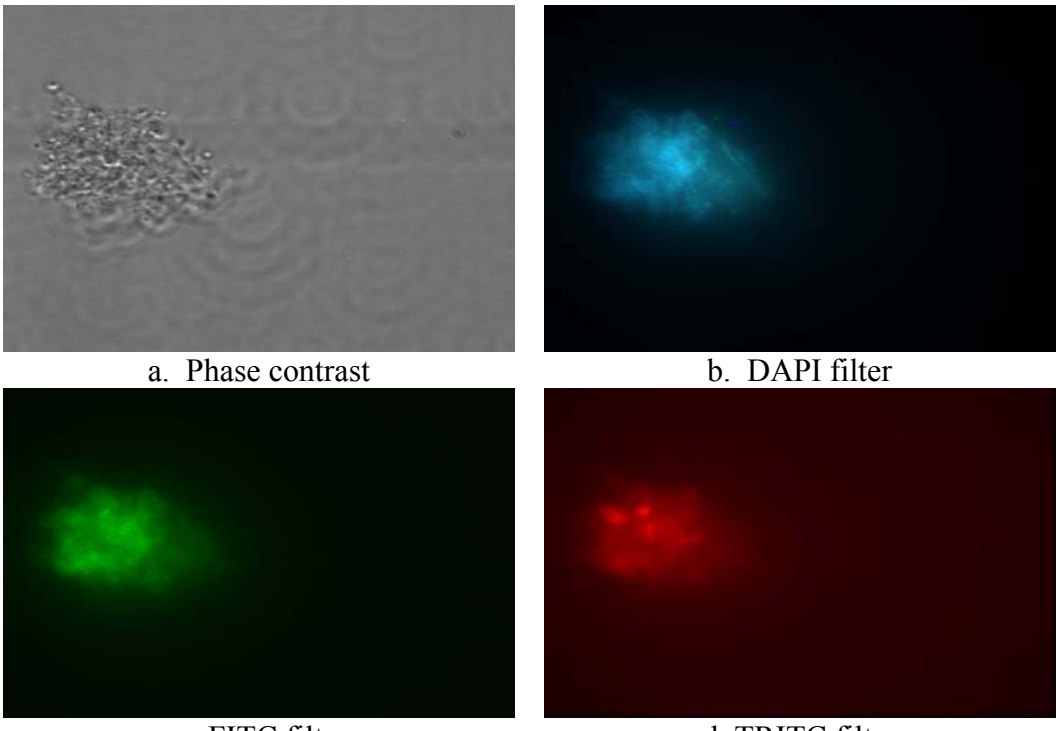
Figure 5.3. *S. aureus* using Eub 338 and Clost I probes (100x objective).

Table 5.5

Matrix Obtained for Probe Combination Eub 338 and SRB 385 (T_H = 53.5^{o}C)

Culture	DAPI	FITC	TRITC
B. subtilis	+	-	+
C. sordelli	+	-	+
D. multivorans	+	+	+
D. salexigens	+	+	+
E. coli	+	-	+
P. aeruginosa	+	-	+
S. aureus	+	-	+

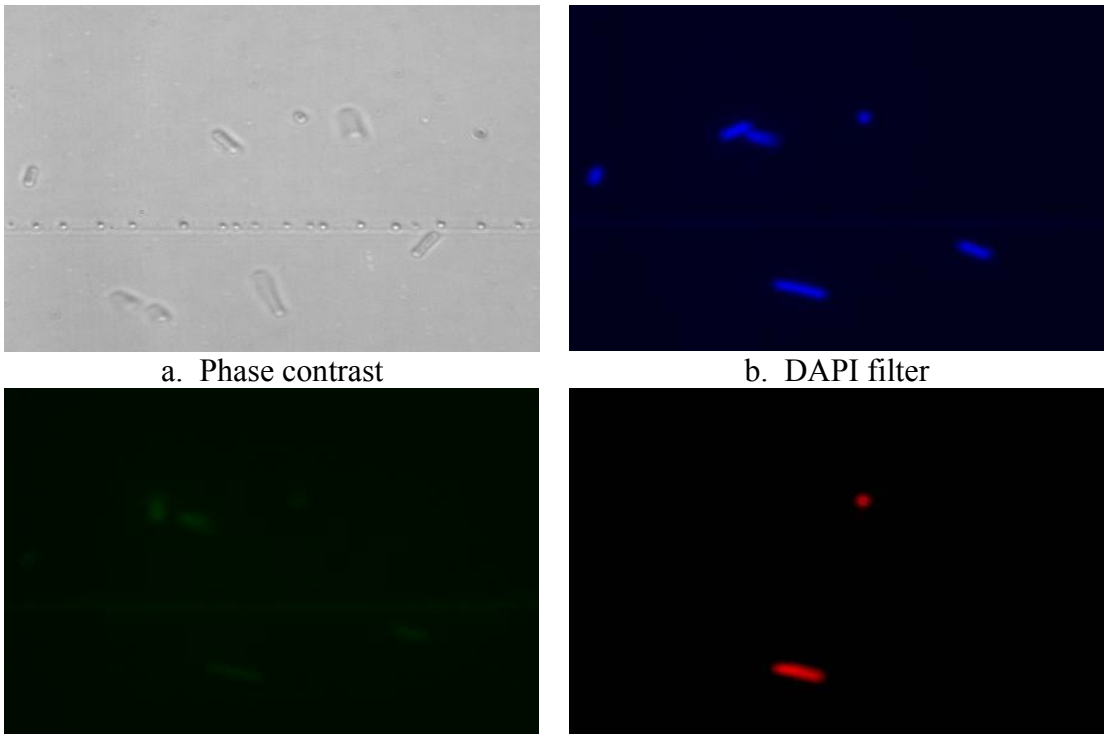
Figure 5.5 a-d presents photomicrographs of *C. sordelli* using a 100x oil objective viewed using phase contrast, as well as DAPI, FITC, and TRITC filters. *C. sordelli* is a positive control for DAPI and TRITC and is a negative control for FITC. It should fluoresce blue and red through DAPI and TRITC filters, respectively but should not fluoresce at all through the FITC filter as shown. These photomicrographs were taken to represent the same observation area viewed through all the three filters consecutively.



a. Phase contrast b. DAPI filter

c. FITC filter d. TRITC filter

Figure 5.4. *D. multivorans* using Eub 338 and SRB 385 probes (100x objective).



a. Phase contrast b. DAPI filter

c. FITC filter d. TRITC filter

Figure 5.5. *C. sordelli* using Eub 338 and SRB 385 probes (100x objective).

5.1.3.3 Probe Combination Eub 338 and Arch 915.

The positive controls for the Arch 915 probe were *H. salinarium* and *M. mazei* and so should bind the probe and be observed as green fluorescence through the FITC filter. The negative controls for Arch 915 probe were *B. subtilis, C. sordelli, D. multivorans, D. salexigens, E. coli, P. aeruginosa,* and *S. aureus* and should not bind the probe nor be observed to have green fluorescence through the FITC filter. The positive controls for Eub 338 probe were *B. subtilis, C. sordelli, D. multivorans, D. salexigens, E. coli, P. aeruginosa,* and *S. aureus*. The negative controls for Eub 338 probes were *H. salinarium* and *M. mazei*.

Initially this probe combination did not work as expected with *H. salinarium* because this culture requires a high salt concentration for cell integrity. To satisfy this, the salt concentration in the hybridization buffer was increased to 25 percent, which in turn interfered with the probing. The probe combination was then tried with *M. mazei* instead. Initially this also did not work as expected.

The next step was to try various formamide percentages to make the cells more permeable to the probe. Formamide was added to the hybridization buffer starting from 10 percent increasing in increments of 5 percent. With the addition of 30 percent formamide in the hybridization buffer, the Arch 915 probe worked as expected. However, the Eub 338 probe worked best at a maximum of 10 percent formamide at the selected T_H (53.5 °C) thus implying that the two probes cannot be sandwiched together.

The verification was then performed using the two probes separately. Table 5.6 presents the matrix of results obtained when the samples were probed with the two probes separately, Arch 915 with 30 percent formamide and Eub 338 with no formamide, at $T_H = 53.5^oC$.

Table 5.6

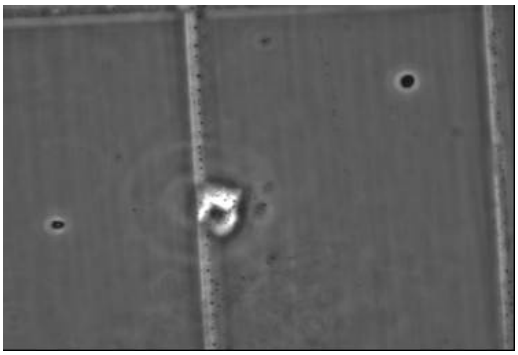
Matrix Obtained for Probe Combination Eub 338 and Arch 915 ($T_H = 53.5^oC$)

Culture	DAPI	FITC	TRITC
B. subtilis	+	-	+
C. sordelli	+	-	+
D. multivorans	+	-	+
D. salexigens	+	-	+
E. coli	+	-	+
H. salinarium	+	+	-
M. mazei	+	+	-
P. aeruginosa	+	-	+
S. aureus	+	-	+

Figure 5.6a-d present photomicrographs of the *M. mazei* culture after fixing and probing viewed using a 100x oil objective through phase contrast, DAPI, FITC, or TRITC filters. *M. mazei* is a positive control for DAPI and FITC and it fluoresced the respective colors through these filters. *M. mazei* is a negative control for Eub 338 and did not fluoresce red in TRITC at any percent of formamide.

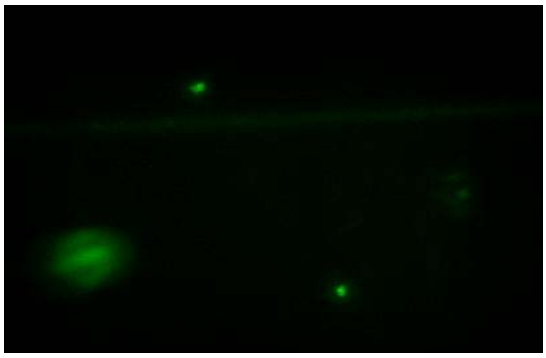
Figure 5.7a-d present photomicrographs of *C. sordelli* after fixing and probing viewed using a 100x oil objective and through phase contrast, DAPI, FITC, or TRITC filters. Since *C. sordelli* is a positive control for DAPI and TRITC and is a negative control for FITC it fluoresces blue and red through DAPI and TRITC filters respectively, but does not fluoresce through the FITC filter as shown in the figure. These photomicrographs were taken to represent the same observation area viewed through all the three filters consecutively.

Method validation experiments using pure cultures were repeated at the end of the research. In this case two probe combinations (Eub 338 and Clost I or Eub 338 and SRB 385) were verified as effective again. The results obtained for enumeration of the cells when probed with the probe combination of Eub 338 and Clost I are shown in Table 5.7. The positive control used for the Clost I probe was *C. sordelli*. The negative controls for the Clost I probe used were *D. multivorans, E. coli*, and *P. aeruginosa.* For the Eub 338 probe the positive controls that were used were *C. sordelli D. multivorans, E. coli*, and *P. aeruginosa.* The Clost I probe was working well as it did not hybridize to any of the negative controls. The results also suggest that the probes are accounting for approximately 50 percent of the total cells as detected by DAPI except in the case of *D. multivorans* in which Eub 338 only hybridized to 22 percent of the total cells. This is reasonable as the Eub 338 and Clost I probes are directed at rRNA which is only present in large amounts in active cells while DAPI will detect any cell that has DNA. The low counts for *D. multivorans* are most likely due to the clumpy nature of that organism.



a. Phase contrast

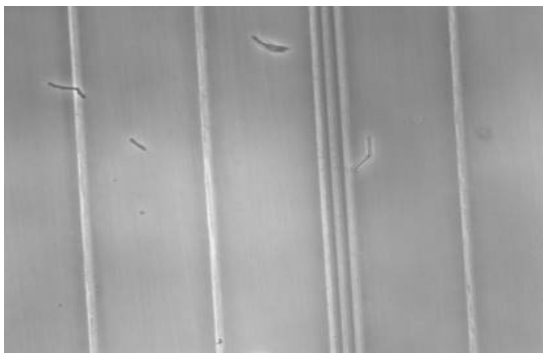
b. DAPI filter



c. FITC filter

d. TRITC filter

Figure 5.6. *M. mazei* using Eub 338 and Arch 915 probes (100x objective).



a. Phase contrast

b. DAPI filter

c. FITC filter

d. TRITC filter

Figure 5.7. *C. sordelli* using Eub 338 and Arch 915 probes (100x objective).

Table 5.7

Probe Combination of Clost I and Eub 338

Culture	DAPI (cells/ml)	FITC (cells/ml)	TRITC (cells/ml)	FITC/DAPI (%)	TRITC/DAPI (%)
C. sordelli	1.89×10^5	9×10^4	8.1×10^4	48	43
D. multivorans	3.3×10^5	-	7.2×10^4		22
E. coli	2×10^5	-	9.9×10^4		49.5
P. aeruginosa	1.8×10^5	-	9.9×10^4		55

Table 5.8 shows the results obtained for the probe combination of Eub 338 and SRB 385. The positive control used for the SRB 385 probe was *D. multivorans* so it should fluoresce green when observed through the FITC filter. The negative controls used for the SRB 385 probe were *C. sordelli, S. aureus, E. coli* and *P. aeruginosa*. The RNA probes detected about 50 percent of the organisms that DAPI detected, which is as expected even though attempts were made to fix the pure cultures in the most active state.

Table 5.8

Probe Combination of SRB 385 and Eub 338

Culture	DAPI (cells/ml)	FITC (cells/ml)	TRITC (cells/ml)	FITC/DAPI (%)	TRITC/DAPI (%)
D. multivorans	2.3×10^5	1.17×10^5	9.9×10^4	51	43
E. coli	2.2×10^5	-	9.9×10^4		45
P. aeruginosa	1.94×10^5	-	1.13×10^5		58
S. aureus	2.12×10^5	-	8.6×10^4		41
C. sordelli	2.3×10^5	-	1.1×10^5		48

5.2 RESULTS FROM SAMPLE ANALYSIS

Samples from six locations were used for the majority of the research. The sediments chosen for study were MP299 (NF and FF), GC112 (NF and FF), and VK916 (NF and FF). The sediments were chosen because they encompassed the range of depth of the samples available. FISH and MPN analysis were performed on the original sediments collected from the Gulf of Mexico (time zero samples) and on samples from the anaerobic microcosms of Gulf sediments incubated with surrogate SBF (time final samples).

5.2.1 Time Zero Samples

Both MPN and FISH analyses were performed on the original sediment samples as received (other than homogenization). These will be referred to as time zero samples to differentiate them from the samples processed after incubation in microcosms with spiked substrates or controls.

5.2.1.1 Results of FISH Analysis

Table 5.9 presents the DAPI, Archaebacterial, SRB, and Eubacterial counts obtained from the time zero enumerations of organisms in the sediments. Four of the six samples were processed in replicate to allow an estimation of the error of the procedure. The coefficient of variability for replication based on counting of three separate sub samples of the fixed sediment ranged from 14 to 87 percent. The coefficient of variability for replication based on three separate counts of the same filter of one sub sample ranged from 15 to 23 percent.

The numbers of SRB, Archaebacteria or Eubacteria detected in these samples are very high in relationship to the numbers of organisms detected with the DAPI probe. The numbers of SRB should be only a small fraction of the Eubacterial numbers, typically the percentages vary from 0.7 to 11.7 percent (Ravenschlag et al. 2000). This suggests that the DAPI probe is underestimating the numbers of organisms in the sediment, most likely due to interference from sediment detritus.

Table 5.9

Results of FISH Analysis on Time Zero Sediments (cells/g dry sediment)

Sediment	Location	DAPI	SRB	Archaebacteria	Eubacteria
MP299	NF	1.1×10^8	1.6×10^8	2×10^8	4.1×10^8
	FF[1]	$(2.9 \pm 0.4) \times 10^8$	$(1.6 \pm 0.8) \times 10^8$	$(1.2 \pm 0.2) \times 10^8$	$(3.4 \pm 1.0) \times 10^8$
GC112	NF[2]	3.5×10^8	$(3.4 \pm 0.5) \times 10^8$	2.8×10^8	$(9.8 \pm 4.2) \times 10^8$
	FF	8.8×10^7	2.7×10^8	2.1×10^8	9.1×10^8
VK916	NF[1]	$(6.7 \pm 5.8) \times 10^7$	$(1.7 \pm 0.6) \times 10^8$	$(1.6 \pm 0.5) \times 10^8$	$(4 \pm 0.7) \times 10^8$
	FF[1]	$(9.8 \pm 4.7) \times 10^7$	$(2.0 \pm 0.6) \times 10^8$	$(1.2 \pm 0.4) \times 10^8$	$(4.5 \pm 1.6) \times 10^8$

[1]Replication is based on counting of three separate sub samples of the fixed sediment.
[2]Replication is based on three separate counts of the same filter of one sub sample.

5.2.1.2 Results of MPN Analysis

MPN tubes were set up for the sediment samples at time zero containing media to support the growth of methanogens, hydrocarbon-utilizing aerobes, SRB, and general anaerobes. No methane was detected in the tubes used to enumerate methanogens even after two years of observation. The lowest dilution used for this measurement was 10^{-1}. This could be because methanogenesis is a very slow process and the organisms are only present in low numbers in the sediment. The medium contained a redox indicator and even after the long incubation periods, it indicated that the tubes were still reduced enough for methanogens to grow if they were there in

sufficient numbers. The medium controls that were spiked with near shore sediment known to contain methanogens were positive for methane production.

There was also a lack of growth of hydrocarbon-utilizing aerobes in the time zero samples. This is also unusual since one of the ingredients in the sheen screen test was tetradecene, which some microcosms proved was degraded by the sediments. An incubation period of 8-10 weeks was used for this enumeration, apparently it was too short. As is reported in Chapter 6 the lag time for tetradecene degradation was up to 22 weeks in some cases. Controls inoculated with near shore sediments did show the removal of sheen, which was the expected positive result.

The method used to grow SRB and general anaerobes did produce positive results. Table 5.10 presents the results for SRB and general anaerobes in all six sediments. The results for the time zero analysis of GC112 NF were lost. The results presented for general anaerobes in the MP299 NF and FF samples are the detection limits based on the lowest dilution (10^{-1}) and the amount of sediment that was used. No growth was observed for these two sets of tubes.

Table 5.10

Results of Time Zero MPN Enumerations

Sediment	Location*	MPN/g dry sediment	
		SRB	General Anaerobes
MP299	NF	7.2×10^6	$<7.22 \times 10^2$
	FF	9.6×10^5	$<1 \times 10^3$
GC112	NF	N/A	N/A
	FF	2.3×10^6	1.1×10^7
VK916	NF	1.7×10^7	4.8×10^8
	FF	5×10^6	2.3×10^8

5.2.1.3 Discussion of the Effect of Oil Exploration Activities on Sediment Microbial Populations

The three possible effects that could be observed from exposure to cuttings coated with SBF would be: 1) a decrease in microbial numbers and diversity due to the addition of possible toxins or sediment texture changes; 2) an increase in microbial numbers due to the input of carbon from the SBF on the cuttings; or 3) no change in microbial numbers because the numbers of organisms in the sediment are already at a maximum carrying capacity. The third assumption comes from the fact the there are physical and chemical limitations to how many organisms can be actively growing in a sediment due to the volume of pore spaces and mass transport limitations that exist. Another explanation is that the death rate and growth rate are balanced, so no net overall change in number is measured.

One of the reasons several methods of analysis were used during this study was to determine which measures or population being measured might be a discriminating factor. Two methods to measure SRB were selected because theory suggests that the SRB population would respond to the addition of carbon sources to the environment.

A comparison of the time zero results (Table 5.9, Table 5.10) for the near field samples with the far field samples should reveal if the exposure of the near field samples to the drilling cuttings coated with SBF had any effect. The numbers of organisms as determined by the MPN analysis was typically lower than the numbers of organisms as determined by the FISH analysis (Figure 5.8). It is also apparent that the numbers of organisms did not vary with water depth, no matter the enumeration method.

A statistical comparison of the data obtained for the time zero samples was performed to reveal if any significant differences in the results could be determined (Table 5.11). The MPN results for time zero are compared using the method of Cochran (1950). In this test, a Z value is computed for the data and if this is greater than 1.96, the results are significantly different.

The statistical analyses showed that for MP299 there were significantly more SRB in the NF samples than in the FF samples. The comparison could not be made for the GC112 sediment because of missing data. The lack of difference in the SRB numbers for VK916 NF and FF sediments could be due to the relatively recent exposure of this site to the SBF (Table 3.1). The cuttings were discharged during November and December 2001 and the sediment collected approximately six months later. The exposure of the organisms at this site to 2,510 bbl of SBF contaminated cuttings over such a short period may have been of such volume and so recent that the population was overwhelmed and had not had time to react to the contamination in a measurable way. The results of the anaerobic biodegradation studies (Chapter 6) also support this hypothesis.

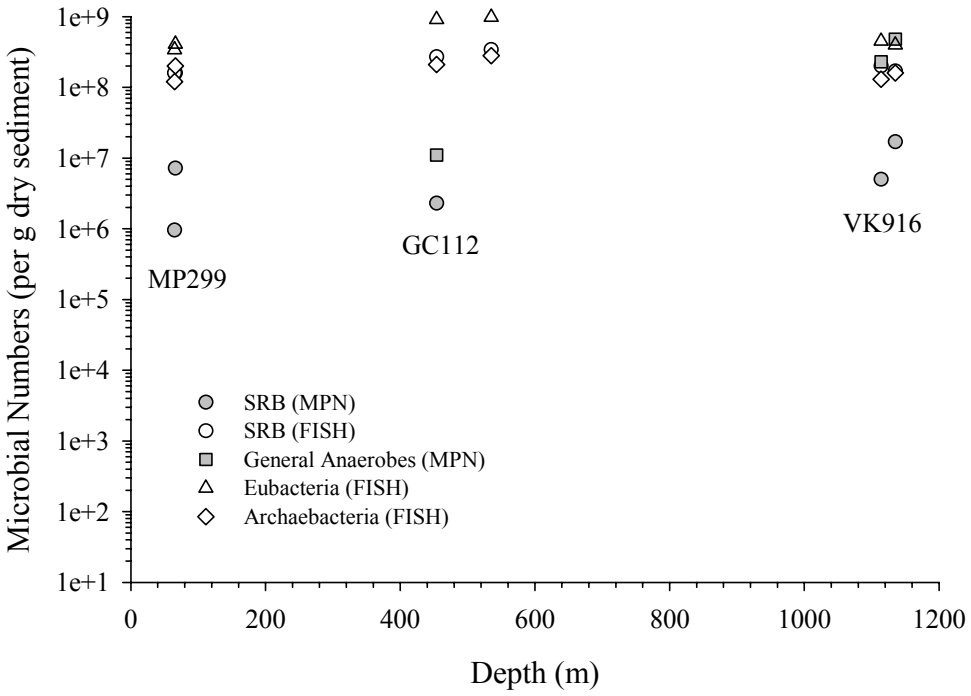


Figure 5.8. Microbial numbers in sediments at time zero.

Table 5.11

Comparison of Microbial Numbers in NF and FF Sediment (Time =0)

Sediment	SRB (MPN)	General Anaerobes (MPN)
MP299	S	nd
GC112	n/a	n/a
VK916	nd	nd

S = significantly different results were determined (NF value was significantly greater than the FF value); nd = the results were not significantly different; n/a = no comparison could be made.

5.2.2 Time Final Samples

The time final samples for the various microcosms were taken after either the tetradecene or the ethyl oleate were degraded in the microcosms as determined by GC-FID analysis. For GC112 NF the time final analyses were taken after tetradecene was removed (50 weeks). For GC112 FF the time final was also the time required for the removal of tetradecene (54 weeks). The time final for MP299 NF of 70 weeks was also the time needed to remove tetradecene. The time final for MP299 FF microcosms was the time to remove ethyl oleate (23 weeks) (and only the ethyl oleate and control microcosms were enumerated). The time to remove ethyl oleate (26 weeks) was also the factor used to determine the time final for VK916 FF sediment. Although tetradecene was removed in the last two sediments, MPN incubations were not initiated with tetradecene microcosm samples since there was not enough time for the incubations to be complete before the report was due.

5.2.2.1 FISH Analyses

The results of FISH analysis of the MP299 FF sediment sample after 23 weeks of incubation with ethyl oleate or incubation with no addition other than seawater are presented in Table 5.12. The table also presents a preliminary analysis of the numbers with the objective of determining whether they make sense within the generally accepted theory of the expected results using these techniques. It is typical to present the results from RNA probes as a percentage of the population that would be represented by the DAPI counts. It is also typical that no one population in an environmental sample would represent more than 12-20 percent of the total population unless there were extreme pressure on the organisms in the environment which would allow one population to dominate because they are the only ones that can survive. This was not expected to occur in these sediments because they are exposed to diverse carbon sources and electron acceptors. As pointed out in the discussion of Table 5.9 the FISH analysis of the original sediments showed that the numbers of SRB, Archaebacteria and Eubacteria were individually greater than the numbers of total organisms as measured by DAPI for most of the samples. Although the numbers of any one population were less than the whole population in the MP299 FF sample after incubation, the numbers of the different groups of organisms still accounted for

too high a percentage of the DAPI numbers. The lowest fraction of any population as compared to the to the DAPI counts was 56 percent for Archaebacteria in the controls. This can be accounted for by masking of the DAPI counts from sediment particles and detritus. This would provide overall lower DAPI counts and an underestimation of the total population.

It is also unusual that the SRB and Clostridia make up such a large percentage of the Eubacterial counts. These organisms are both Eubacteria but should not be such a large fraction of the total population. Adding the Clostridia and SRB counts accounts for more than 100 percent of the Eubacterial counts in both the control and ethyl oleate spiked microcosms. This again is improbable. This would suggest either the Eubacterial counts are low, the SRB or Clostridial counts are high, or a combination of both.

Table 5.12

Results of FISH Analysis of MP299 FF Time Final Sediments

Probe	Control (cells/g dry sediment x 10^8)	DAPI (%)	Eub 338 (%)	Ethyl Oleate (cells/g dry sediment x 10^8)	DAPI (%)	Eub 338 (%)
DAPI	1.6 ± 0.14	100	na	1.5 ±0.05	100	na
Eubacteria	1.3 ± 0.3	81	100	1.1 ±0.01	73	100
SRB	1.2 ± 0.3	75	92	1.3 ±0.067	87	118
Clostridium	1.01 ± 0.034	63	78	0.9 ± 0.066	61	84
Archaebacteria	0.9 ± 0.05	56	na	0.9 ± 0.035	98	na
Eubacteria+ Archaebacteria	2.2 ± 0.35	138	na	2± 0.13	133	na

na-analysis not applicable.

Table 5.13 presents the results from the FISH analysis of the GC112 FF sediment after 54 weeks of incubation in the presence of ethyl oleate or incubation with only a seawater amendment (control). The Eubacterial counts of the microcosm spiked with ethyl oleate accounted for 25 percent of the DAPI counts, which is reasonable. The Eubacteria are 50 percent of the total counts in the control microcosm, which is high. Again, the SRB and Clostridium are too large a percentage of the Eubacterial counts, they individually account for more than 100 percent of the Eubacterial counts. Since the method did not seem to be producing reliable results in the sediment samples no further FISH analyses were performed.

Table 5.13

Results of FISH Analysis of GC112 FF Time Final Sediments

Probe	Control (cells/g dry sediment x 10^8)	DAPI (%)	Eub 338 (%)	Ethyl oleate (cells/g dry sediment x 10^8)	DAPI (%)	Eub 338 (%)
DAPI	7.8 ± 0.5	100	na	13 ± 1	100	na
Eubacteria	3.9 ± 0.2	50	100	3.2 ± 0.3	25	100
SRB	4.6 ± 0.14	59	118	2.5 ± 0.2	19	78
Clostridium	4.9 ± 0.4	63	126	3.3 ± 0.2	25	103
Archaebacteria	5.4 ± 0.14	69	na	3.5 ± 0.2	27	na
Eubacteria+ Archaebacteria	9.3 ± 0.35	119	na	6.7 ± 0.52	127	na

na-analysis not applicable

5.2.2.2 MPN Analyses

As discussed above for the time zero samples, there were no positive results from the attempts to enumerate methanogens and hydrocarbon-utilizing aerobes. There was growth in the tubes used to measure general anaerobes and SRB. The results are presented in Table 5.14.

5.2.2.3 Discussion of the Effect of Anaerobic Incubations with Surrogate SBF on Microbial Populations

The determination of the response of the organisms to incubation with the surrogate SBF was accomplished by comparing the microbial number in the control microcosms with those in the microcosms spiked with ethyl oleate or tetradecene. To avoid any interference in the comparisons from artificially high numbers at time zero due to the prior field exposure of the NF sediments to SBF coated cuttings, only the analysis of samples from FF sediments was used. There was not an MPN or FISH analysis of VK916 controls because the samples were all used for GC analyses during the incubations.

The incubation of the sediment with ethyl oleate increased the number of SRB in MP299 FF sediment as determined by the MPN method (Table 5.15). Similar results were obtained for the GC112 FF sample. The incubation with ethyl oleate caused a significant increase in SRB numbers as determined by the MPN methods. The controls also showed an increase in SRB, which is possibly due to growth on natural organic material in the control sample. The SRB present in the VK916 FF sediment incubated with ethyl oleate did not increase in number.

Table 5.14

Results Time Final MPN Enumerations

Sample	SRB (MPN/g dry sediment)	General Anaerobes (MPN/g dry sediment)
MP299 NF		
Ethyl oleate microcosm	N/A	N/A
Tetradecene microcosm	3.24×10^6	5.8×10^8
Control microcosm	4.7×10^6	5.8×10^8
MP299 FF		
Ethyl oleate microcosm	1.2×10^8	1.7×10^7
Control microcosm	4.8×10^5	5.4×10^7
GC112 NF		
Ethyl oleate microcosm	2.0×10^8	1.1×10^9
Tetradecene microcosm	8.5×10^7	5.3×10^7
Control microcosm	4.6×10^7	3.3×10^9
GC112 FF		
Ethyl oleate microcosm	4.6×10^8	1.0×10^6
Control microcosm	3.7×10^8	1.9×10^5
VK916 FF		
Ethyl oleate microcosm	3.6×10^6	6.3×10^6

Table 5.15

Statistical Comparison of Time Final FF MPN Results for SRB

Sediment	T Zero vs. Control	T Zero vs. Ethyl Oleate	Control vs. Ethyl Oleate
MP299 FF	Z = 0.8 $SRB^1 = SRB^2$	Z = 3.33 $SRB^1 < SRB^3$	Z = 6 $SRB^2 < SRB^3$
VK916 FF		Z = 0.4 $SRB^1 = SRB^3$	
GC112 FF	Z=6 $SRB^1 < SRB^2$	Z=6.3 $SRB^1 < SRB^3$	Z=0.25 $SRB^2 = SRB^3$

SRB^1 = SRB at time zero, SRB^2 = SRB in controls after incubation, SRB^3 = SRB in ethyl oleate spiked microcosms after incubation. The Z-statistic is similar to the t-statistic with the population variance replacing the sample variance in the denominator of the calculation. For this data the Z at 95 percent confidence is 1.96.

All three sediments showed a difference in numbers of general anaerobes between the time zero samples and the time final samples from the microcosms spiked with ethyl oleate (Table 5.16). The general anaerobe sample at time zero for MP299 was a non-detect, thus the time final numbers (both in the range of 10^7) were significantly different. The GC112 FF samples spiked

with ethyl oleate showed an increase in general anaerobes over the time zero samples, while the controls showed a decrease in general anaerobes, which is difficult to reconcile with the increase in SRB measured in both samples. The numbers of general anaerobes also increased in the VK916 FF ethyl oleate spiked sediments over those at time zero, indicating that the general anaerobe measure may be a discriminatory measure.

Table 5.16

Statistical Comparison of Time Final FF MPN Results for General Anaerobes

Sediment	T Zero vs. Control	T Zero vs. Ethyl Oleate	Control vs. Ethyl oleate
MP299 FF	Z = 13 $GA^1 < GA^2$	Z = 12 $GA^1 < GA^3$	Z = 1.4 $GA^2 = GA^3$
VK916 FF		Z = 4.3 $GA^1 < GA^3$	
GC112 FF	Z = 5 $GA^1 > GA^2$	Z = 2.8 $GA^1 > GA^3$	Z = 2 $GA^2 < GA^3$

GA^1 = general anaerobes at time zero, GA^2 = general anaerobes in controls after incubation, GA^3 = general anaerobes in ethyl oleate spiked microcosms after incubation.

5.3 CONCLUSIONS FROM MICROECOLOGY STUDIES

Straightforward conclusions can be drawn concerning the applicability of the methods used to these types of environmental samples, but conclusions as to the meaning of the results from the samples themselves are only preliminary and should be verified by further testing and repetition. A summary of the results during method development is presented in Table 5.17.

The most obvious conclusion concerning the methods used is that the use of the FISH technique and phylogenetic probes is not recommended for these sediments. Although many others have used FISH successfully in marine sediments, these were all sandy sediments and did not present the problems associated with the sediments from the Gulf of Mexico, which had very little sand and up to 74 percent clay (Table 3.2).

No methanogens or aerobic hydrocarbon degraders were detected in this study. The presence of SRB and general anaerobes was detected. The only possible microbial response to the possible exposure of sediment to SBF and the incubation of sediment samples with surrogate SBF (ethyl oleate or tetradecene) is a possible increase in SRB (as measured by MPN) to approximately 10^8 cells/g dry sediment.

Table 5.17

Method Development Summation

Target Population	Method	Conclusion	Comment
Total Bacteria	FISH (DAPI)	Not acceptable	Sediment interference causes low numbers
SRB (acetate utilizing)	MPN	Acceptable	
SRB (lactate metabolizing)	MPN	Acceptable	Low numbers in verification test
SRB	FISH (SRB 385)	Not acceptable	Possibly high numbers
General Anaerobes	MPN	Acceptable	
Methanogens	MPN	Acceptable	
Eubacteria	FISH (EUB 338)	Not acceptable	Possibly high numbers
Clostridia	FISH (Clost I)	Not acceptable	Possibly high numbers
Archaebacteria	FISH (Arch 915)	Not acceptable	Possibly high numbers

CHAPTER 6 RESULTS OF BIODEGRADATION STUDIES

6.1 METHOD DEVELOPMENT

6.1.1 Pressure Chamber Development

The need to incubate the sediments spiked with surrogate SBF at the higher than atmospheric pressures the microorganisms would be exposed to in their natural environment required that the traditional incubation vessels for anaerobic testing (serum bottles) be changed to flexible walled vessels that would allow the transfer of pressure from the incubation vessels into the cultures. Plastic bags were considered as the new incubation vessels. To prove that the spiked compounds would not leak out of the bags, sediments from Galveston Bay that had been sterilized by autoclaving were spiked with ethyl oleate or tetradecene and placed in serum bottles or the heat seal bags and incubated under atmospheric (bottles) or pressurized to 790 psi. The concentrations of the compounds were monitored over seven months. The results indicate that there was very little is any leakage of ethyl oleate or tetradecene from the bags (Figure 6.1).

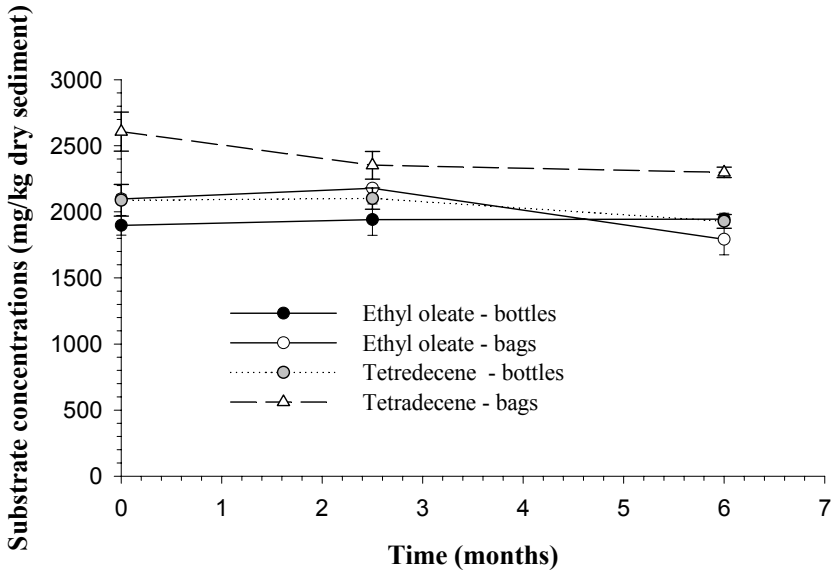


Figure 6.1. Effect of incubation vessel on concentration of surrogate SBF in sterile sediments.

To prove that a change in the incubation vessel would not affect the organisms in the sediments, inoculum from Galveston Bay shoreline sediment was set up in both bags and serum bottles. The bags were incubated in the pressure vessel (filled with water but not pressurized since these were near shore sediments). The serum bottles were incubated alongside the pressure vessel. Figure 6.2 presents the concentrations of sulfate in control and tetradecene-spiked sediments incubated in serum bottles or bags. The data points represent the average sulfate concentration of three replicate cultures. The error bars represent one standard deviation. The removal of sulfate in microcosms spiked with tetradecene and not in the controls indicates anaerobic microbial activity linked to tetradecene removal. The fact that the sulfate in the serum bottle

microcosms spiked with tetradecene was removed at the same rate as in the bag suggests that both incubation methods provide equivalent environments for the activity of marine tetradecene oxidizing, SRB.

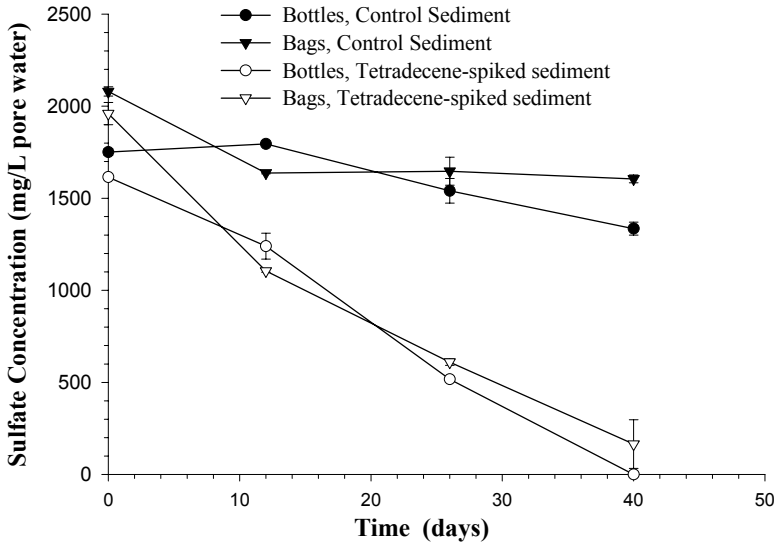


Figure 6.2. Sulfate removal in incubation vessel tests.

These results demonstrate that the closed bottle test as developed for the API and EPA can be converted for use in incubating sediments in plastic heat seal bags in a pressure chamber. The depletion of sulfate and surrogate SBF (ethyl oleate or tetradecene) are monitored to indicate culture activity.

6.2 ANAEROBIC INCUBATIONS

For each sediment, the first experiment was designed to determine whether the organisms present in the sediment required high pressure for SBF degrading activity. Samples of near field sediment from each location were spiked with 2,000 mg ethyl oleate/g dry sediment or 2,000 mg tetradecene/g dry sediment and mixed with additional synthetic seawater to replenish the sulfate concentration, or just mixed with seawater (control). Samples of these were then incubated in serum bottles at room temperature or in bags pressurized to the pressure at the depth the sediment was exposed to when it was collected.

In all of the figures presented below the symbols represent the average of the analysis of three samples; the error bars represent one standard deviation. The curves overlain on the data were generated using the first order with time delay equation that is presented later (Section 6.3).

6.2.1 MP299 Site

The MP299 samples represent the shallowest depth sampled (65 m). Sediment from this site (NF and FF) did not contain any detectable peaks in the GC-FID analysis of extracts (Table 3.1). The

NF sediment was exposed to drilling fluids from 1963-2000. The 966 bbl of either IO or LAO cuttings would have been discharged from the mid-1990's to 2000. This would constitute chronic exposure to low levels of SBF. It is entirely possible that the site has recovered from exposure. The NF site still had elevated levels of SRB over those in the FF site. The NF sample had the highest clay content (74 percent) of all of the sediments tested and the highest phosphorous concentrations (Table 3.2). NF sediment samples were incubated both at 97 psi hydraulic pressure (in bags) and at atmospheric pressure (in bottles) in a cold room at 4°C. Replicate samples were sacrificed periodically and analyzed to determine the sulfate concentration in the pore water and the concentration of surrogate SBF in the sediment. Figure 6.3 presents the results of the degradation of tetradecene and ethyl oleate at atmospheric pressure and 97 psi. As expected ethyl oleate was degraded more rapidly and with less of a lag than tetradecene. This is typical and has been observed in all of the studies using shoreline sediments. The figure also demonstrates that the organisms in the MP299 sediment did not perform any differently when they were incubated under pressure than they did at atmospheric pressure whether degrading ethyl oleate or tetradecene.

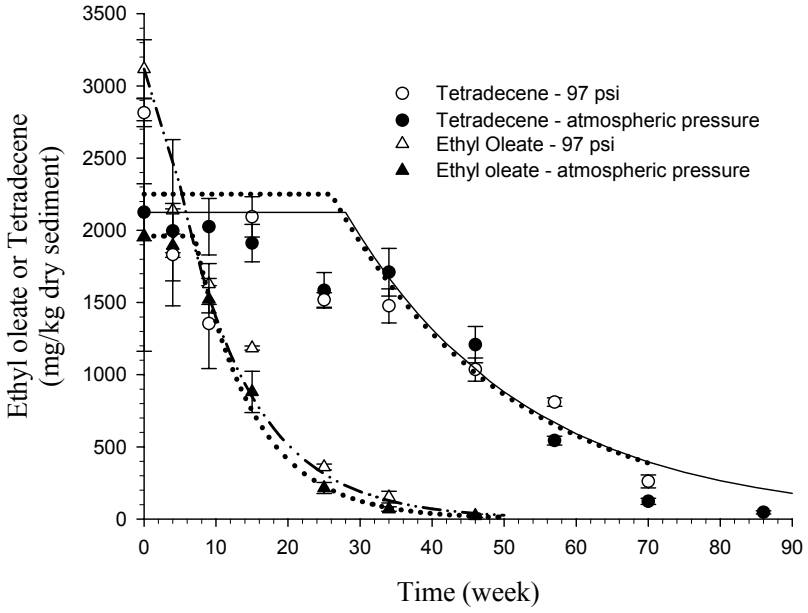


Figure 6.3. Ethyl oleate and tetradecene concentrations in MP299 NF sediment incubated at 97 psi or atmospheric pressure.

The concentrations of tetradecene and sulfate in tetradecene spiked microcosms are presented in Figure 6.4. Tetradecene degradation and sulfate removal began at very close to the same time and the two compounds were removed at very similar rates; strongly supporting the conclusion that sulfate was the terminal electron acceptor during tetradecene degradation. The lines overlaying the data were generated using first order kinetics delayed by a lag time. The determination of the lag time and the kinetic coefficients used for each dataset are explained below (Section 6.3). The link to sulfate depletion is even more apparent for ethyl oleate degradation (Figure 6.5). The only difference in the curves for sulfate and ethyl oleate

degradation is that the initial concentration of sulfate in the microcosms was greater than that of ethyl oleate. The control samples that were not spiked with substrate did not remove significant amounts of sulfate, again supporting the hypothesis that the degradation of the substrate was linked to sulfate reduction.

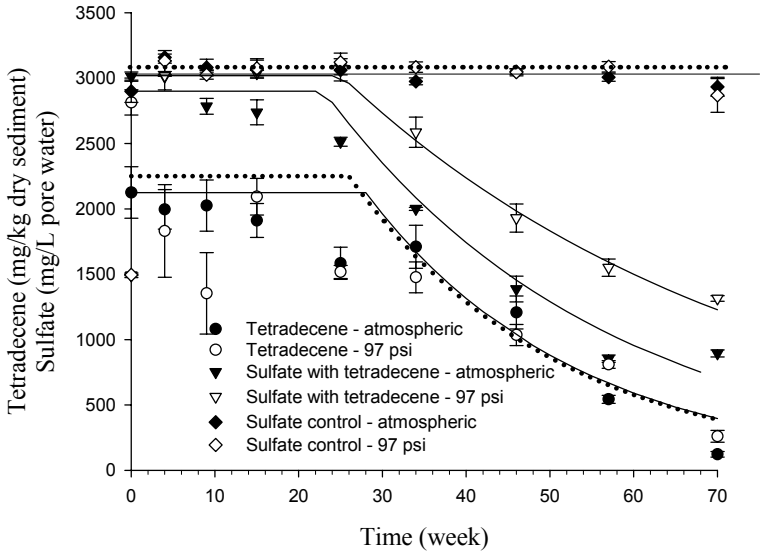


Figure 6.4. Tetradecene and sulfate concentrations in MP299 NF sediment incubated at 97 psi or atmospheric pressure.

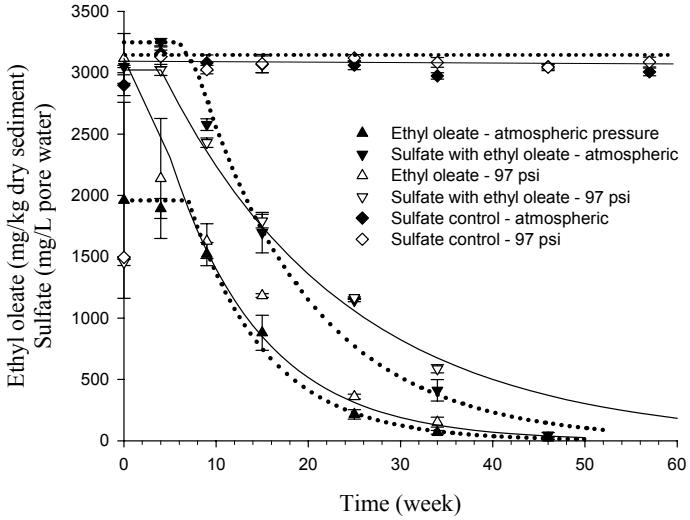


Figure 6.5. Concentrations of sulfate and ethyl oleate in MP299 NF sediment incubated at 97 psi or atmospheric pressure.

Incubations using the far field sediment were initiated as soon as the data suggested that pressure had no effect on substrate degradation. Samples were only incubated at atmospheric pressure. The original hypothesis was that since the far field samples were not previously exposed to SBF

they would degrade the substrates more slowly so sampling events were scheduled less often. Unexpectedly, this was not the case. New microcosms were set up and sampled more frequently. The data for the removal of ethyl oleate and tetradecene as well as the sulfate in these microcosms is presented in Figure 6.6. Lag times were observed before both ethyl oleate and tetradecene were degraded. Ethyl oleate was again removed sooner than the tetradecene, mostly due to a much shorter lag time (6 weeks). The link between ethyl oleate degradation and sulfate removal is again apparent. Tetradecene degradation began after a long lag period but then proceeded at a rapid rate. Sulfate removal lagged behind tetradecene degradation. Sulfate was not removed in controls that were not spiked with surrogate SBF.

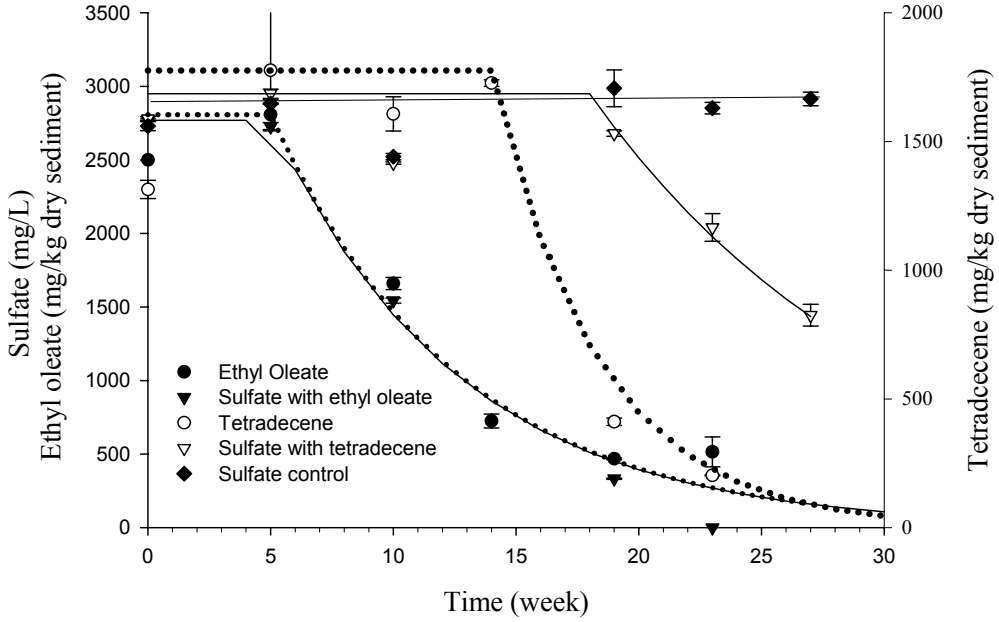


Figure 6.6. Concentrations of sulfate, ethyl oleate, and tetradecene in MP299 FF sediment incubated at atmospheric pressure.

6.2.2 GC112 Site

The GC112 sediment was obtained from a depth of 535 m. GC-FID analysis of sediment extracts showed peaks with retention times similar to commercially available SBF and thus this sediment was still impacted from drilling operations. Although the last discharge of cuttings was in 1997, over 5,000 bbl of cuttings were discharged and the site has not recovered yet.

Incubations of the near field sediment at atmospheric pressure and at 790 psi were conducted. The concentrations of tetradecene and ethyl oleate in the extracts of samples sacrificed during the incubations are presented in Figure 6.7. There was no discernable lag time before ethyl oleate degradation began and only a very short lag time before tetradecene degradation began. Ethyl oleate was degraded at a faster rate than tetradecene was. There was no significant difference in the results from incubations at atmospheric pressure to those at 790 psi. Organisms in these sediments did not require pressure to metabolize the substrates.

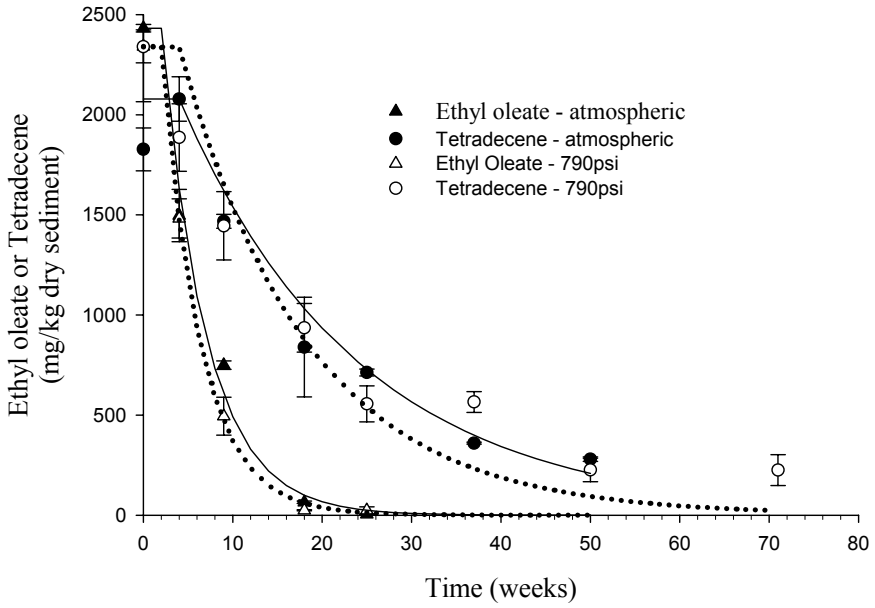


Figure 6.7. Ethyl Oleate and tetradecene concentrations in GC112 NF sediment incubated at 790 psi or atmospheric pressure.

The dependence of substrate degradation on sulfate is presented in Figure 6.8 and Figure 6.9. As the sulfate is used up (around 37 weeks) the tetradecene degradation slows down, possibly stopping altogether (Figure 6.8). The control samples that were not spiked with surrogate SBF removed some sulfate, probably due to the metabolism of the SBF present in the sediment. The ethyl oleate and sulfate were exhausted at the same time (Figure 6.9).

As soon as it was determined that samples could be incubated at atmospheric pressure, the far field incubations were started using only atmospheric incubations. As shown in Figure 6.10 the ethyl oleate was degraded without any observable lag and this was accompanied by a depletion of sulfate from the microcosms. The degradation of tetradecene occurred after a short lag, and proceeded at a similar rate to that observed for ethyl oleate removal. The sulfate was only slowly removed in the microcosms spiked with tetradecene, and sulfate removal did not continue after the tetradecene was removed, and since the sulfate in the tetradecene spiked microcosms is very similar in concentration to that in the control microcosms it is possible that sulfate was not used as an electron acceptor for tetradecene in this sediment. The sediment was uncontaminated and aerobic in nature. The sediment appeared a creamy brown, rather than the grey black of the NF sediments. It is possible that the electron acceptor used in these microcosms was oxygen.

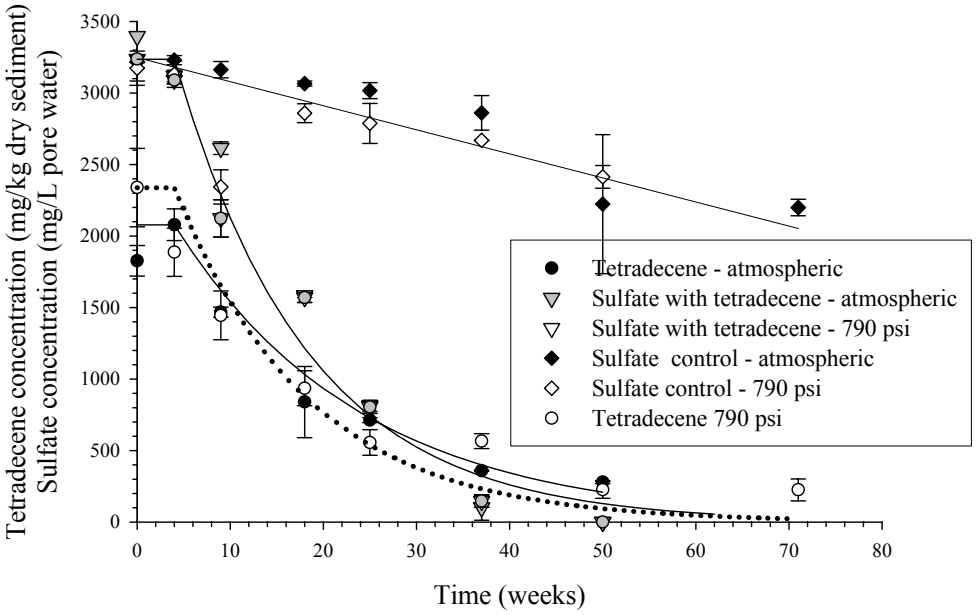


Figure 6.8. Tetradecene and sulfate concentrations in GC112 NF sediment incubated at 790 psi or atmospheric pressure.

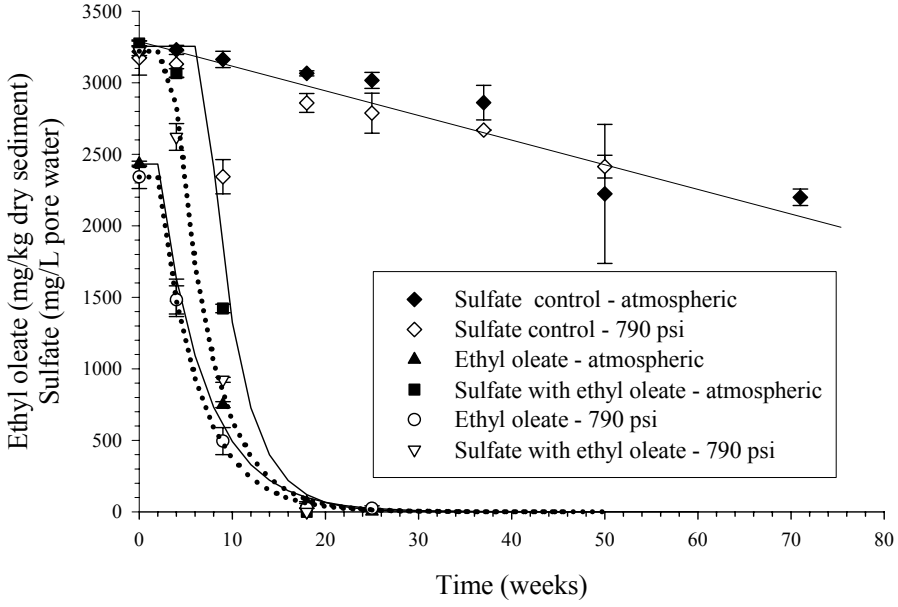


Figure 6.9. Ethyl Oleate and sulfate concentrations in GC112 NF sediment incubated at 790 psi or atmospheric pressure.

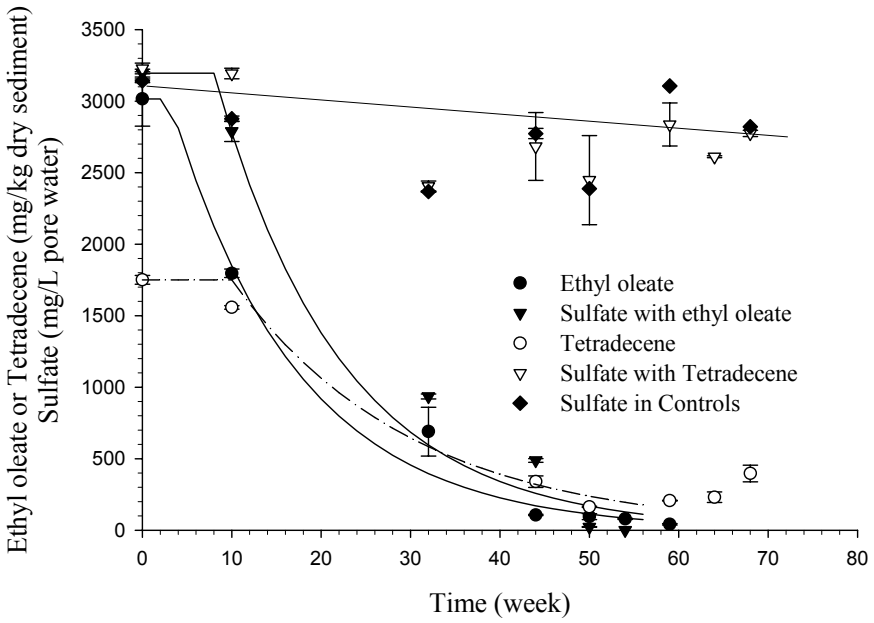


Figure 6.10. Concentrations of sulfate, ethyl oleate, and tetradecene in GC112 FF sediment incubated at atmospheric pressure.

6.2.3 VK916 Site

The sediment from VK916 represents the deepest site (1135 m) that was sampled for this study. A GC-FID analysis of extracts of the near field samples revealed peaks at retention times similar to commercial SBF, indicating the sediment was impacted by drilling operations (Table 3.1). Sediment samples spiked with tetradecene, ethyl oleate, or unspiked controls were incubated at 1700 psi. The initial sulfate concentration in the microcosms set up with near field sediment was much lower than expected (Figure 6.11), even though excess sulfate was added into the sediment with seawater. This was the second incubation of this sediment to show this. In the initial incubation that was set up with this sediment, the sulfate was depleted so quickly it was gone by the first sampling. In the second incubation, sulfate was also removed very quickly and within 12 weeks it was depleted in the ethyl oleate spiked samples. The sulfate controls also showed low initial concentrations of sulfate and evidence of sulfate depletion. This is not surprising because of the high concentrations of SBF in this sediment (Table 3.1). Fewer controls were set up for this incubation since it was a second incubation and there was not sufficient sediment, so there were no control samples after 12 weeks of incubation.

The ethyl oleate spike in this sample was measured to be almost three times the intended concentration of 2000 mg/Kg dry sediment. This could be due to analytical interference from SBF in the sediment. Ethyl oleate was initially degraded rapidly in this sediment, but after 12 weeks ethyl oleate degradation slowed. This occurred at the same time that the sulfate was depleted from the sediment, indicating that the rapid ethyl oleate depletion rate was coupled to sulfate metabolism.

Tetradecene was not removed in this incubation of VK916 NF sediment even though tetradecene depletion was apparent during the first few sampling periods of the initial incubation. The sulfate was also not removed until after 20 weeks in the microcosms spiked with tetradecene but then at a very rapid rate (Figure 6.11). This suggests that the addition of tetradecene inhibited the sulfate reducers in some way, and that when they recovered there was a lot of easily degraded material for them to use, thus the rapid degradation. This sediment showed the highest contamination of SBF and undoubtedly, this had an affect on how the sediment behaved in the test.

The VK916 FF sediment was also used to inoculate microcosms spiked with ethyl oleate, tetradecene, or controls and incubated at 1700 psi. This sediment contained the usual concentration of sulfate initially. The ethyl oleate and sulfate were removed simultaneously; again, sulfate appears to be the electron acceptor for the degradation of ethyl oleate.

The results for tetradecene analysis in this sediment are the least reproducible of any results in this study. The dotted line is a best approximation of the possible curve for tetradecene degradation extrapolated from these data. There are two data points that are not well represented by the line. It is also interesting that sulfate was not degraded in the microcosms that were spiked with tetradecene even though tetradecene was removed in these microcosms.

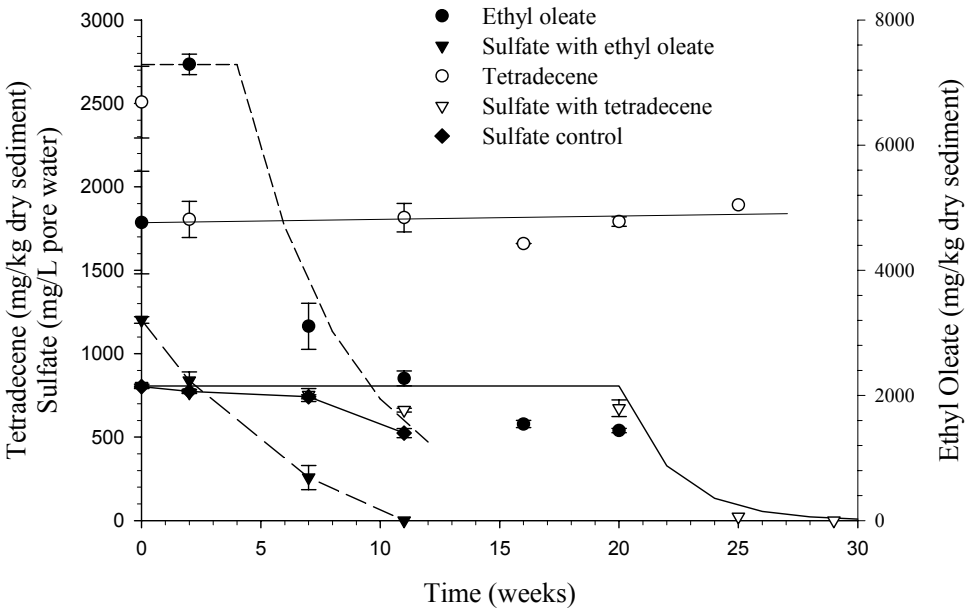


Figure 6.11. Concentration of sulfate, ethyl oleate, and tetradecene in VK916 NF sediment microcosms incubated at 1700 psi.

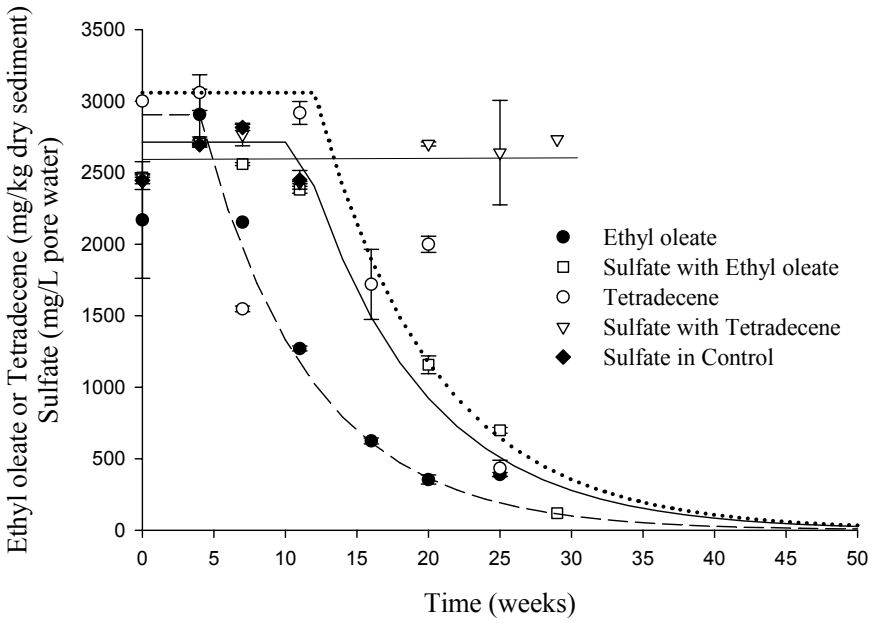


Figure 6.12. Concentrations of sulfate, ethyl oleate, and tetradecene in VK916 FF sediment microcosms incubated at 1700 psi.

6.3 INTERPRETATION OF ANAEROBIC INCUBATION DATA

6.3.1 Determination of the Decay Coefficient from Anaerobic Degradation

The major objectives of performing the anaerobic incubations were to provide direct evidence that organisms present in Gulf of Mexico sediments were capable of metabolizing SBF and to determine the kinetic coefficients that describe this degradation mathematically. The first objective was accomplished and as described above each of the sediments contained organisms that were capable of degrading ethyl oleate and tetradecene. The second objective is achieved by a more in depth analysis of the data.

The rate of change in substrate concentration in the microcosms spiked with ethyl oleate or tetradecene shown in Figures 6.3 to 6.12 was observed to follow first order kinetics after a period of initial adaptation (lag phase). First order kinetics suggest that the rate of change in substrate concentration was dependant on the substrate concentration itself. The equation describing first order kinetics describes the rate of change in terms of the concentration and a kinetic coefficient or k as shown in Equation 6.1. There is no currently accepted method of predicting the lag time before microbial degradation begins.

$$\frac{dC}{dt} = kC \qquad \textbf{Equation 6.1}$$

Where C is the concentration of the substrate, k is the kinetic coefficient and t is time.

A rigorous approach was taken to determine the value of the coefficient k from the anaerobic incubations. The determination of k depends quite heavily on the determination of the end of the lag time before substrate degradation begins, and on the determination of a stopping point of degradation. Initially the data points in the first-order decay region of the curve (i.e. after any lag time was complete) were linearized to generate a system of linear equations. The decay coefficients were then estimated by finding the least squares solutions to this system of linear equations. The estimated decay coefficients were then used as parameters in a nonlinear system (which included both lag and decay phases) to determine the exact coefficient value in an iterative manner. The determination of the lag time was initially performed by insinuating the decay curve over the data and determining the time point at which the curve intersected the initial concentration. Later a mathematical expression was developed from the theory of microbial growth and substrate degradation.

A summary of the degradation kinetics as determined from the experimental data for ethyl oleate degradation is presented in Table 6.1. Statistical analyses were performed to determine if the predicted exposure of the samples (i.e. near field vs. far field) had an effect, and the results were not different at the 95 percent confidence limit. When the data were organized based on whether contamination was detected in the sediment by extraction and GC analysis (i.e. MP299 NF was moved to the uncontaminated category) there was a significant difference in the k values. This supports the theory that pre exposure to SBF (even though the exposure was to IO's not to esters) caused an increase in the microbial population and thus an increased degradation rate. Averaging the two sets of data reveal that the removal of ethyl oleate from a pre-exposed site could be described using a k value of -0.22 ± 0.02 week^{-1}. For a model that assumes no pre-exposure, the more important data would be that the removal of the substrate from an uncontaminated site would occur with a k value of -0.11 ± 0.02 week^{-1}. The lag time is another important factor in the fate model. Ethyl oleate degradation at most sites occurred with at most 11 weeks lag. The detection of SBF in the sediment sample also had an effect on the lag time. The lag times for ethyl oleate degradation in sediments that contained SBF (GC112 and VK916 NF) were significantly ($p=0.05$) shorter (average of 3 weeks) than those that did not contain detectable amounts of SBF (average of 4 weeks).

The degradation of tetradecene by organisms in the sediment samples obtained was much more complicated to analyze. The degradation appeared to be different in the true far field samples from the near field samples (Table 6.2). The degradation of tetradecene did not appear to be linked to sulfate reduction in the far field samples, presumably due to the aerobic nature of the sediments. The FF sediments were all a cream to brown color, not the grey black that would be associated with anaerobic sediments. A t-test comparing tetradecene degradation results in the NF sediments revealed that there was no significant difference in the k values determined from the substrate decay curves. The average k value for the removal of tetradecene linked to sulfate reduction was -0.05 ± 0.01 week^{-1}.

Table 6.1

Kinetic Data for Ethyl Oleate Degradation

Microcosm	Substrate	C_o (mg/kg dry sediment)	k (week^{-1})	Correlation	T_{lag} (week)
GC 112 NF 790 psi	ethyl oleate	2341	-0.23	1	2
GC 112 NF atm.	"	2432	-0.2	.995	2
VK 916 NF	"	7289	-0.22	.929	4
Average			-0.22 ± 0.02		2.7 ± 1
MP299 NF 97 psi	"	3117	-0.1	.982	2
MP299 NF atm.	"	1960	-0.12	.998	7
MP299 FF	"	2807	-0.13	.986	5
GC 112 FF	"	3015	-0.07	.991	3
VK 916 FF	"	2905	-0.13	.965	4
Average			-0.11 ±0.02		4.2 ± 2
GC 112 NF 790 psi	SO_4^{-2} with	3220	-0.25	.997	3.5
GC 112 NF atm.	ethyl oleate	3255	-0.3	.997	7
VK 916 NF	"	1202	-0.22	.996	0
Average			-0.26 ± 0.04		3.5 ± 3.5
MP299 NF 97 psi	"	3023	-0.05	.751	4
MP299 NF atm.	"	3249	-0.08	.989	7
MP299 FF	"	2769	-0.13	.997	5
GC 112 FF	"	3195	-0.07	.994	8
VK 916 FF	"	2713	-0.12	.984	11
Average			-0.09 ± 0.03		7 ± 2.7

The lag times for tetradecene degradation were in the ranges of 4 to >30 weeks. The lag time for sulfate reduction was often longer than for tetradecene removal and sulfate reduction was slower than tetradecene removal. As mentioned earlier, it is also possible that tetradecene was inhibiting the SRB (and thus sulfate reduction) because in VK916 NF and VK916 FF sulfate removal either did not occur or occurred after a very long lag. In GC112 FF sulfate was never removed, even though tetradecene was.

Table 6.2

Kinetic Data for Tetradecene Degradation

Microcosm	Substrate	C_o (mg/kg dry sediment)	k (week^{-1})	Correlation (r^2)	T_{lag} (week)
GC 112 NF 790 psi	tetradecene	2338	-0.07	.983	4
GC 112 NF atm.	"	2078	-0.05	.991	4
VK 916 NF	"	2507	-	-	>30
MP299 NF 97 psi	"	2250	-0.04	.829	26
MP299 NF atm.	"	2124	-0.04	.958	28
Average			-0.05 ± 0.01		
MP299 FF	"	1776	-0.23*	.971	14
GC 112 FF	"	1750	-0.05*	.984	10
VK 916 FF	"	3059	-0.12*	.768	12
GC 112 NF 790 psi	SO_4^{-2} with	3236	-0.07	.990	4
GC 112 NF atm.	tetradecene	3395	-0.07	.986	4
VK 916 NF	"	-	-	-	-
MP299 NF 97 psi	"	3019	-0.02	.754	25
MP299 NF atm.	"	2900	-0.03	.986	23
Average			-0.05 ± 0.03		
MP299 FF	"	2950	-0.08	.799	18
GC 112 FF	"	3227	-	-	10
VK 916 FF	"	3000	-	-	>30

* These k values are most likely due to aerobic degradation and were not used to determine the k for sulfate reduction.

6.4 AEROBIC BIODEGRADATION RESULTS

Although an aerobic biodegradation study was not part of the original objectives for this research, a limited study was performed to provide at least one data point for the aerobic terms in the fate model. The study was conducted using sediment from the MP299 far field site. The first order decay coefficient for the natural aerobic biodegradation of NOM was determined to be approximately k_{NOUR} = -0.0018 h^{-1}, or equivalently k_{NOUR} = -0.3 week^{-1}. The first order decay coefficient for the aerobic biodegradation of tetradecene was approximately $k_{F, O2}$ = -0.0009 h^{-1}, or equivalently $k_{F, O2}$ = -0.15 week^{-1}.

CHAPTER 7 FINAL MODEL DEVELOPMENT

7.1 OVERVIEW OF THE FATE MODEL

As discussed in Chapter 4 the model was developed to predict the fate of SBF, O_2, and SO_4^{-2} after discharge of cuttings to the sediment is completed. The main mechanism for removal of SBF from the sediment is through microbial degradation. In addition to biodegradation, the concentrations of the SBF, O_2, and SO_4^{-2} are also affected by resuspension, bioturbation, sedimentation, compaction, and diffusion. Sediment mixing by organisms (bioturbation) and resuspension by bottom currents are two processes which dilute and disperse contaminants that initially settle on the seafloor. In the case of sediments affected by cuttings deposition, the results from the study by Continental Shelf Associates (2004) suggest that the redox potential discontinuity (RPD) was observed at less than 1 cm in most of the near-field sediments. This indicates that bioturbation does not play a significant role in impacted sediments since bioturbation only plays a role in aerobic sediments. In order to make the model conservative, the impact of bioturbation and resuspension in the sediments will be neglected. Sediment compaction is assumed to occur as the cuttings are deposited. Further compaction of the sediment after cuttings discharge is neglected in this model. The model as developed resolves only vertical gradients.

The equations as presented in Chapter 4 reflected theoretically predicted Monod kinetics, which the data obtained and presented in Chapter 5 and Chapter 6 do not support. Attempts to fit the data to Monod kinetics using the data from Chapters 5 and 6 were unsuccessful. The expected changes in numbers of microorganisms that would occur if true growth were occurring were not measured. This could be because the techniques used to measure the microbial numbers were inadequate, or there was not a large amount of growth of organisms.

The ability to fit the data for substrate degradation to a first order decay model indicates that there was not much growth after observable substrate degradation began. In a growing system, the rate of degradation would become faster and faster as the number of organisms increased. In this case the rate decreased with predictable first order kinetics, suggesting a population that is staying constant (at least once degradation starts) and is operating under diffusion limitations, thus first order kinetic are measured.

The equations presented in Chapter 4 were rewritten as Equations 7.1 -7.3 to reflect first order kinetics as determined in Chapter 6. The term SC has been added as an input parameter to represent the stoichiometric coefficient that can be used to represent the stoichiometry of sulfate or oxygen usage due to substrate removal. In Chapter 4 this was presented as 2.6 g sulfate for each g of hexadecene, or 1.7 g O_2 for each g of hexadecene consumed. The stoichiometry of sulfate usage for each substrate tested in this research (tetradecene or ethyl oleate) was determined by determining the stoichiometric coefficient needed to predict the sulfate concentrations in the samples from the ethyl oleate or tetradecene data. A summary of these coefficients is presented in Table 7.1.

Table 7.1

Stoichiometric Coefficients for Sulfate Usage (g SO_4/g substrate)

Sediment Test	Ethyl Oleate	Correlation (r^2)	Tetradecene	Correlation (r^2)
Predicted	4.4		4.9	
MP299 NF Atm.	1.38[1]	0.994	1.33 (LA)[3]	0.991
MP299 NF 97 psi	0.72[1] (LA)[3]	0.976	0.91 (LA)[3]	0.997
MP299 FF	0.99	1.000	NL[2]	0.979
GC112 NF Atm.	1.37 (LA)[3]	0.996	1.75	0.995
GC112 NF 790 psi	1.38 (LA)[3]	0.999	1.38	1.000
GC112 FF	1.06 (LA)[3]	1.000	NL[2]	0.976
VK916 NF1700 psi	0.165[5] (LA)[3]	1.000	NA[4]	NA[4]
VK916 FF 1700 psi	0.923 (LA)[3]	0.999	NL[2]	NA[4]
Mean (SD)	1.12 ± 0.26		1.34 ± 0.34	
Pooled Mean	1.2 ± 0.3			

[1] These coefficients gave good matches to initial curves but not the tails of the curves.
[2] NL – it was determined that for these sediments sulfate reduction was not linked to tetradecene removal.
[3] LA – the lag times for substrate and sulfate degradation were adjusted to be equal for this analyses.
[4] NA – Not Available
[5] not used in average

The predicted stoichiometric coefficients were calculated assuming that all of the reactions are directed towards the electron acceptor and none towards growth. This is an overestimation of the actual requirement for electron acceptor. It was also apparent that the degradation of the electron acceptor sulfate could not be tied directly to substrate removal in every case. This suggests that the substrate, ethyl oleate or tetradecene, could be degraded to an intermediate, such as acetate, by a fermentative organism and that intermediate would then degraded by the SRB. Since the removal of sulfate could be modeled quite well using first order kinetics with regards to its own concentration (Table 6.1), it is likely that the sulfate was the limiting nutrient for sulfate reduction, and not the SBF, spiked substrate or intermediate present in these experiments.

Equation 7.1, Equation 7.2, and Equation 7.3 are the stoichiometric equivalent forms of the three governing equations of the fate model. The advantage of using these equivalent forms is that the degradation of the electron acceptors can be controlled directly by the availability of SBF. As the concentration of SBF in the system is depleted, the reactive terms will also vanish, thus allowing the model to approximate the time taken for sulfate and oxygen replenishment by

diffusion. An additional oxygen utilization term was added to take into account the natural oxygen uptake rate in the sediments (NOUR).

Equation 7.1

$$\frac{\partial S}{\partial t} = \frac{D_{SO4}^{T}}{\theta^2}\left(\frac{\partial^2 S}{\partial z^2}\right) - SC \cdot \left((k_{F,SO4} \cdot F)\left(\frac{S}{K_{s,so4} + S}\right)\left(\frac{K'_{O,SO4}}{K'_{O,SO4} + O}\right)\right)$$

Equation 7.2

$$\frac{\partial \mathrm{O}}{\partial t} = \frac{\mathrm{D}_{\mathrm{O2}}^{\mathrm{T}}}{\theta^2}\left(\frac{\partial^2 \mathrm{O}}{\partial z^2}\right) - SC \cdot \left(k_{F,O2} \cdot F\left(\frac{\mathrm{O}}{\mathrm{K_{s,O}} + \mathrm{O}}\right)\right) - NOUR$$

Equation 7.3

$$\frac{\partial F}{\partial t} = \frac{\mathrm{D}_{\mathrm{F}}^{\mathrm{T}}}{\theta^2}\left[\frac{\partial^2 F}{\partial x^2}\right] - k_{F,O2}\, F\left(\frac{O}{K_{s,O} + O}\right) - k_{F,SO4}\, F\left(\frac{S}{K_{s,SO_4} + S}\right)\left(\frac{K'_{O,SO_4}}{K'_{O,SO_4} + O}\right)$$

Where SC = the stoichiometric coefficient (g electron acceptor/g electron donor), $k_{F,SO4}$ = the first order decay coefficient for fluid in the SRB zone (wk^{-1}), $k_{F,O2}$ is the first order decay coefficient in the oxygen zone (wk^{-1}), NOUR = the natural oxygen uptake reactive term in the sediment (due to other organic compounds present in the sediment) and all other symbols have been defined previously.

7.2 NUMERICAL SOLUTION FOR THE FATE MODEL

Due to the mathematical complexities involved in solving Equation 7.1, Equation 7.2, and Equation 7.3 simultaneously, a closed-form analytical solution was beyond the scope of this research. The solution method used was an explicit, forward-in-time, finite-difference approximation to the system of differential equations (Lapidus & Pinder, 1982). This is relatively straightforward and can be done using spreadsheet applications. The discretized forms of the partial equations of the model at time interval n and n + 1 are presented in Equation 7.4, Equation 7.5, and Equation 7.6.

Equation 7.4

$$\frac{S_i^{n+1} - S_i^n}{\Delta t} = D_S \cdot \frac{S_{i+1}^n - 2\,S_i^n + S_{i-1}^n}{(\Delta z)^2} - SC \cdot k_F\, F_i^n \left(\frac{S_i^n}{K_{S,SO4} + S_i^n}\right)\left(\frac{K_{O,SO4}}{K_{O,SO4} + O_i^n}\right)$$

Equation 7.5

$$\frac{O_i^{n+1} - O_i^n}{\Delta t} = D_O \cdot \frac{O_{i+1}^n - 2\,O_i^n + O_{i-1}^n}{(\Delta z)^2} - SC\, k_F\, F_i^n \left(\frac{K_{s,O}}{K_{s,O} + O_i^n}\right) - k_{NOUR}\, O_i^n$$

Equation 7.6

$$\frac{F_i^{n+1} - F_i^n}{\Delta t} = D_F \cdot \frac{F_{i+1}^n - 2F_i^n + F_{i-1}^n}{(\Delta z)^2} - k_{F,O2}\, F_i^n \left(\frac{O_i^n}{K_{S,O} + O_i^n}\right) - k_{F,SO4}\, F_i^n \left(\frac{S_i^n}{K_{S,SO4} + S_i^n}\right)\left(\frac{K'_{O,SO_4}}{K'_{O,SO_4} + O_i^n}\right)$$

The solutions to equations 7.4-7.6 were developed as Equation 7.7, Equation 7.8, and Equation 7.9 were written into a VBA module within an Excel Workbook.

$$S_i^{n+1} = \lambda \cdot S_{i+1}^n + (1-2\lambda)\cdot S_i^n + \lambda \cdot S_{i-1}^n - \Delta t \cdot SC \cdot k_F \cdot F_i^n \cdot \left(\frac{S_i^n}{K_{S,SO4} + S_i^n}\right)\left(\frac{K_{O,SO4}}{K_{O,SO4} + O_i^n}\right)$$ **Equation 7.7**

$$O_i^{n+1} = \lambda \cdot O_{i+1}^n + (1-2.\lambda - \Delta t \cdot k_{NOUR})\cdot O_i^n + \lambda \cdot O_{i-1}^n - \Delta t \cdot SC \cdot k_F \cdot F_i^n \cdot \left(\frac{K_{s,O}}{K_{s,O} + O_i^n}\right)$$ **Equation 7.8**

$$F_i^{n+1} = \lambda \cdot F_{i+1}^n + (1-2.\lambda - \Delta t \cdot \left[k_{F,O2}\left(\frac{O_i^n}{K_{S,O} + O_i^n}\right) - k_{F,SO4}\left(\frac{S_i^n}{K_{S,SO4} + S_i^n}\right)\left(\frac{K'_{O,SO_4}}{K'_{O,SO_4} + O_i^n}\right)\right]) \cdot F_i^n + \lambda \cdot F_{i-1}^n$$ **Equation 7.9**

Where: $\lambda = D\ \frac{\Delta t}{(\Delta z)^2}$

The VBA model input section is presented in Figure 7.1. The initial and boundary conditions were simplified from those used in Chapter 4. When the program is executed the concentrations of oxygen, sulfate, and SBF are calculated for each time and each depth and these are used to populate three output result matrixes, (SBF, sulfate and oxygen concentrations) which are used to create graphic output.

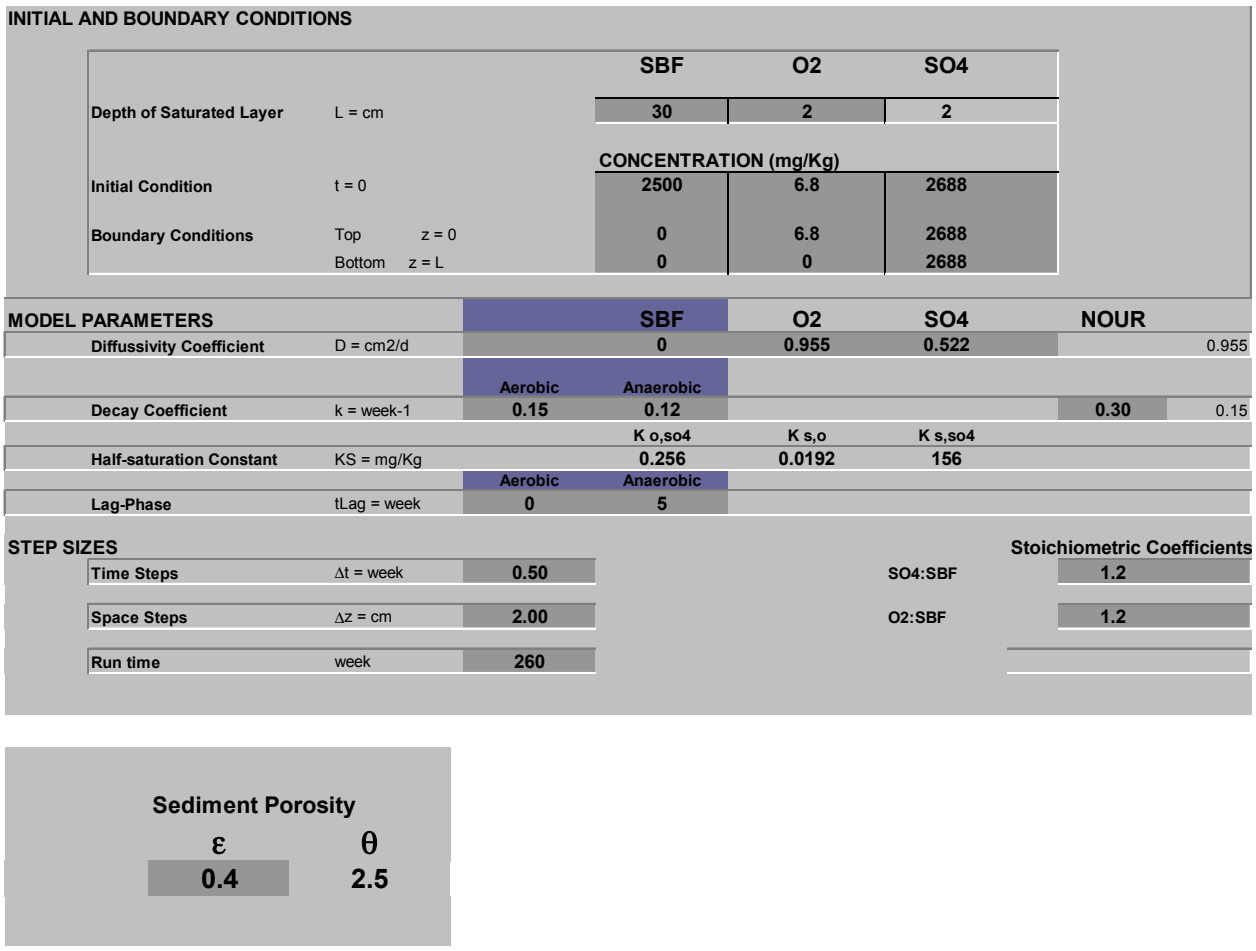
7.3 Explanation of Model Input

All of the model input parameters are contained within the input table (Figure 7.1) and can be changed depending on the scenario modeled. The summary below explains the parameters and the effects of changing those parameters in a sensitivity analysis. The results of the sensitivity analysis are not presented, but section 7.4 provides example model output designed to show the effectiveness of the specific parts of the model.

7.3.1 Initial and Boundary Conditions

7.3.1.1 Depth of the Saturated Layer

The depth of the saturated layer parameter establishes the extent of contamination of sediment with SBF, oxygen or sulfate. The SBF saturation was initially set to 30 cm to allow visualization of a large layer of sulfate degradation results. If the depth of the contaminated layer is set to a lesser value (i.e. 10 cm) then the SBF is removed from all layers of the sediment more rapidly. The input for the depth of saturated layer of sulfate and oxygen are dependant on the space step chosen. The two values must be equal.



INITIAL AND BOUNDARY CONDITIONS

			SBF	O2	SO4
Depth of Saturated Layer	L = cm		30	2	2
			CONCENTRATION (mg/Kg)		
Initial Condition	t = 0		2500	6.8	2688
Boundary Conditions	Top	z = 0	0	6.8	2688
	Bottom	z = L	0	0	2688

MODEL PARAMETERS			SBF	O2	SO4	NOUR	
Diffussivity Coefficient	D = cm2/d		0	0.955	0.522		0.955
		Aerobic	Anaerobic				
Decay Coefficient	k = week-1	0.15	0.12			0.30	0.15
			K o,so4	K s,o	K s,so4		
Half-saturation Constant	KS = mg/Kg		0.256	0.0192	156		
		Aerobic	Anaerobic				
Lag-Phase	tLag = week	0	5				

STEP SIZES				Stoichiometric Coefficients	
Time Steps	Δt = week	0.50	SO4:SBF	1.2	
Space Steps	Δz = cm	2.00	O2:SBF	1.2	
Run time	week	260			

Sediment Porosity	
ε	θ
0.4	2.5

Figure 7.1. Model input table.

7.3.1.2 Initial Concentration

The VBA model has the ability to set upper and lower boundary conditions separate from the contaminated zone. This gives the ability to model diffusion of oxygen SBF and sulfate into the contaminated zone from both above and below.

The boundary conditions set the initial SBF amount in the entire contaminated sediment zone to 2500 mg /Kg dry sediment. Because the quality of the cuttings cleaning processes and the amount of cuttings discharged is variable from drill site to drill site, the initial SBF concentration can be very low or very high. A sensitivity run of the model indicated that there was enough sulfate in the sediment to support the anaerobic degradation of SBF to completion in 2 years when the initial concentration was 500 and 2000 mg/Kg. However, anaerobic degradation of 4000 mg/Kg SBF slowed after the first year due to depletion of sulfate. The anaerobic degradation of SBF resumed when sufficient sulfate had diffused back into the sediment. The model predicted that the complete removal of 4000 mg/Kg of SBF in sediment would take approximately 5 years. The concentration of SBF in the boundaries to the contaminated zone was set to zero to reflect that the sediment above and below the cuttings zone is not contaminated with cuttings.

The initial concentration of oxygen was set to 6.8 mg/L pore water and it was present in only the upper boundary of the contaminated zone. Most analyses of both contaminated and uncontaminated sediments (e.g. Continental Shelf Associates 2004) showed that oxygen was only present in the first few centimeters of sediment, thus it is not expected to diffuse into the cuttings zone from below.

The initial concentration of sulfate was set to 2688 mg/L pore water. Sulfate was set to be present in both the upper and lower boundaries. Sulfate is rarely limiting in natural sea water and should be able to diffuse into the cuttings layer from both the top and bottom boundaries.

7.3.2 Diffusion and Decay Parameters

7.3.2.1 SBF Diffusion Coefficient

The SBF are typically assumed to have such low solubilities that the diffusion coefficient is effectively zero. The model assumes that the SBF are degraded in the bulk phase and do not have to diffuse into the aqueous phase before they can be degraded. The only results of diffusion would be to diffuse from the center of the contaminated zone out to the edges as the contaminant is removed from the edges. The ability to input an SBF diffusion parameter is retained in the model to allow versatility in the case where there is a need to model the fate of a diffusible SBF.

7.3.2.2 Oxygen Diffusion Coefficient

As discussed in Chapter 4, the diffusion coefficient was calculated to reflect the temperature and dynamic viscosity on the sea floor. As was reported in Chapter 4 changes in the O_2 diffusion coefficient did not significantly change the overall prediction of SBF degradation. The VBA model predicted an improvement in the rate of oxygen diffusion back into the sediment after aerobic degradation was completed. This shows that the VBA model was somewhat sensitive to the oxygen diffusion coefficient but in the possible temperature ranges, there was no significant effect of oxygen diffusion coefficient on the overall biodegradation process.

7.3.2.3 Sulfate Diffusion Coefficient

The sulfate diffusion coefficient was also calculated according to changes in temperature. With an initial SBF concentration of 2500 mg/kg changes in the diffusion coefficient did not change the fate of SBF in the sediment significantly. Increasing the diffusion coefficient from 0.422, 0.522 and 0.823 cm^2/day accelerated the rate of sulfate diffusion back into the sediment after the anaerobic degradation was completed.

7.3.2.4 Aerobic Decay Coefficient

The aerobic decay coefficient was determined by incubating one set of triplicate samples of MP299 FF sediment that had been spiked with tetradecene in a respirometer. The result was $k_{F,O2} = 0.15$. As discussed in Chapter 4 the model was not sensitive to this parameter because oxygen was limited to the first few centimeters of sediment. The sensitivity analysis in Chapter

4 was repeated and the results showed the oxygen profiles in the sediment after 2 years were similar for all cases, $k_{F,O2} = 0.02$, $k_{F,O2} = 0.15$ and $k_{F,O2} = 0.80$.

The $K_{S,O}$ allows the ability to turn off the oxygen linked SBF degradation parameter when oxygen becomes limiting in the sediment pore water. This parameter was set to 0.0192 which was taken from the literature (Chakravarty 1975).

7.3.2.5 Anaerobic Decay Coefficient

The anaerobic decay coefficients were determined experimentally and reported in Chapter 6. They ranged from $k_{F,SO4} = 0.04$ to $k_{F,SO4} = 0.22$. For an arbitrary initial concentration of SBF, C_0 = 2500 mg/Kg, the consumption of sulfate due to SBF degradation never lead to sulfate depletion. The faster the anaerobic degradation was completed, the faster the sulfate was replenished through diffusion. As a result, the sulfate profile in the sediment showed higher sulfate concentrations after 2 years for the higher anaerobic decay coefficients.

The $K_{o,SO4}$ allows the ability to turn off the sulfate degradation parameter when oxygen is present in the sediment pore water. This parameter was set to 0.256 which was taken from the literature (Van Capellan and Wang 1995).

The $K_{S,SO4}$ allows the ability to turn off the sulfate linked SBF degradation parameter when sulfate becomes limiting in the sediment pore water. This parameter was set to 156 which was taken from the literature (Boudreau and Westrich 1984).

7.3.2.6 NOUR Decay Coefficient

The natural oxygen uptake rate (NOUR) was measured by incubating one set of triplicate samples of MP299 FF sediment in a respirometer and measuring the oxygen uptake rate. The result was a $k_{NOUR} = 0.3$. Appling this value of the NOUR parameter in the model yielded similar oxygen profile to that measured in MP299 FF sediments during Sampling Cruise 1 (Continental Shelf Associates 2004).

7.3.2.7 Stoichiometric Coefficient and Lag Time

The stoichiometric coefficient was explained in section 7.1. The value was set to 1.2 for both oxygen and sulfate. The experimentally determined lag time for anaerobic degradation varied widely. When the model was run using a lag time of 0, 10 and 30 weeks, the SBF removal profile was delayed but the curve shape once degradation started was not different. A lag time of 5 weeks was used for most model runs.

7.3.2.8 Step Sizes.

The model solution is very sensitive to the step sizes. As mentioned above the space step must be equal to the depth of saturated layer for oxygen and sulfate. The model predictions become unstable if the time step $\leq$ 1/the highest decay rate used and $\leq$ 0.5*the $(\text{space step})^2$/maximum

diffusion coefficient used. The run time selected relates to the time and space steps and the time available for computations.

7.3.2.9 Porosity and Tortuosity of the Sediment

The porosity term did not have as much effect on the model predictions in the VBA model. There was enough sulfate in the sediment to support the anaerobic degradation of 2500 mg/Kg SBF during the course of two years. The rate of sulfate replenishment in the sediment after SBF degradation was complete was faster as the porosity of the sediment increased (lower turtuosity). The rate of SBF degradation was similar in all cases since sulfate was not limited.

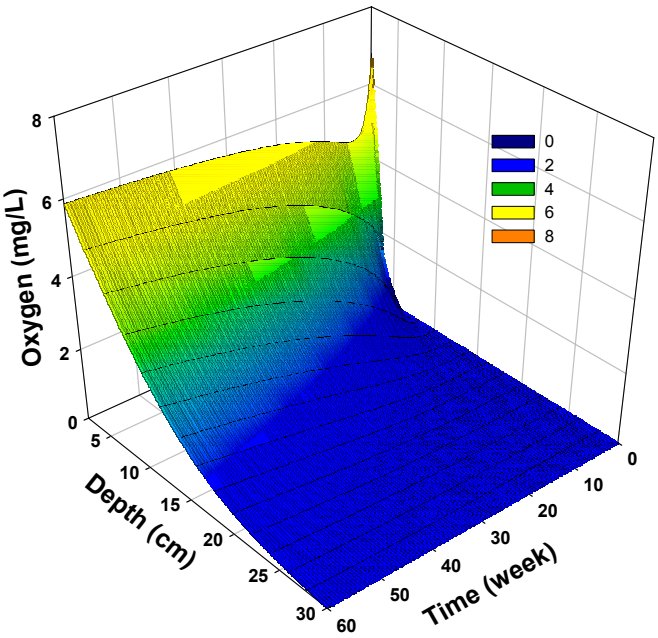
7.4 VERIFICATION OF MODEL PERFORMANCE

Several scenarios were applied to the model to verify performance. Certain parameters were turned off to observe their effects on the model predictions. These include removing all degradation terms so the diffusion terms and natural oxygen uptake rate terms could be evaluated since these were new to the model. When each term was adjusted the model responded predictably, suggesting that the code is working as predicted. The following sections summarize the more important model responses.

7.4.1 Simulation of the Natural Oxygen Profile

The ability of the model to predict the natural oxygen profile in the sediment was evaluated by turning off all of the degradation kinetics as well as the sulfate diffusion terms. Thus the only modules of the model that were running were oxygen diffusion and natural oxygen uptake rate (NOUR). When the oxygen diffusion coefficient was set to 0.955 and the NOUR was turned off, the model predicted that oxygen would diffuse in to the sediment with time Figure 7.2a. This is what would be expected when no reactive terms are incorporated into the model. When the NOUR of 0.3 wk^{-1} obtained from a preliminary aerobic incubation of MP299 FF sediment were input to the model it was able to predict a profile very similar to that found by Continental Shelf Associates (2004) (Figure 7.2b). The model is able to predict a believable diffusion of oxygen and use of this with a natural oxygen uptake rate.

a



b

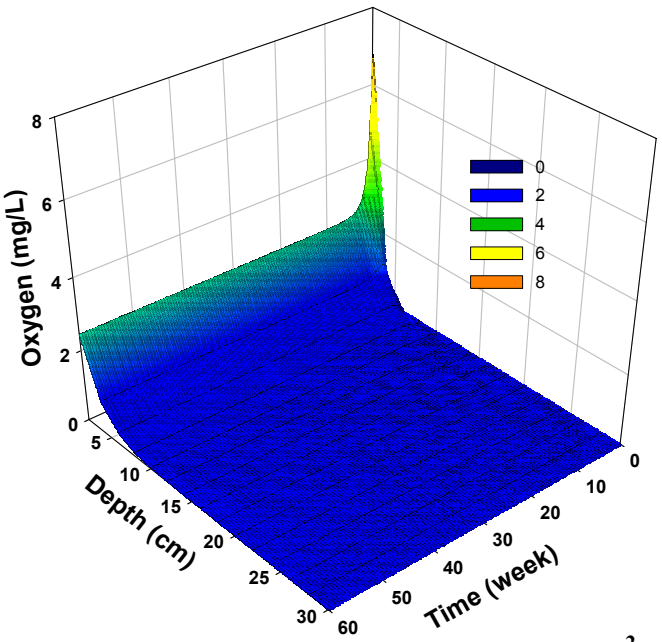


Figure 7.2. Oxygen profile with oxygen diffusion (D=0.955 cm^2/sec) with a) no reactive terms and b) with NOUR (0.3 wk^{-1}).

7.4.2 Simulation of Aerobic SBF Degradation

To verify the aerobic degradation portions of the equations were functioning as expected the model was run with all parameters off except the aerobic degradation decay coefficient (0.15 wk^{-1}) and with the aerobic parameters on including oxygen diffusion. The initial and upper boundary oxygen concentrations were set to 6.8 mg/L, the NOUR was set to 0.3 and the initial SBF concentration was 2500 mg/kg. When the diffusion term was left at zero (Figure 7.3a) the SBF profile shows that very little SBF is used when oxygen cannot diffuse into the sediment as expected. With the full aerobic degradation parameters (initial and upper boundary O_2=6.8, O_2 diffusion coefficient 0.955, an aerobic decay coefficient of 0.15, and an NOUR of 0.3) input into the model the SBF profile changes only slightly from when oxygen diffusion is not operative (Figure 7.3b).

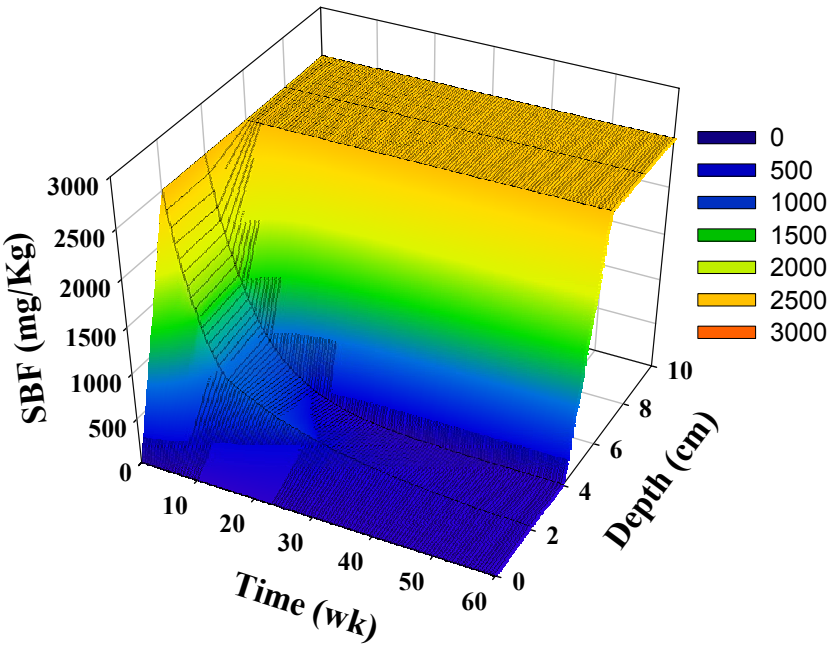
The predicted oxygen profile shows that the oxygen is completely removed from all depths of the sediment rapidly in the absence of diffusion Figure 7.4a. The typical diffusion is too slow to have a visible effect on SBF consumption. The oxygen profile is somewhat different when diffusion and degradation are both turned on. Slightly higher levels of oxygen are observed deeper into the sediment in Figure 7.4a than in Figure 7.4b.

7.4.3 Simulation of Anaerobic Degradation

To test the model's ability to predict the anaerobic degradation of SBF all of the aerobic parameters were turned off, the sulfate was set to 2688 mg/L initially and at the top and bottom boundary layers. The anaerobic decay coefficient was set to 0.12 and the lag time was set to 5 weeks. The anaerobic degradation of SBF removes the majority of the SBF present in the sediment at most depths because the amount of sulfate originally in the sediment supports the degradation of most of the SBF present (Figure 7.5a). When the diffusion of sulfate into the sediment from the top and bottom boundary layer is allowed, then even more SBF is removed, most noticeably the SBF in later times and the middle depths are different between Figure 7.5 a and b.

The sulfate profile shows more marked differences when diffusion of sulfate is on or off (Figure 7.6a and b). The sulfate concentrations are increasing with time and from the boundaries in, indicating the model is predicting that after the SBF is removed, the sulfate will diffuse back into the sediment as it is expected to.

a



b

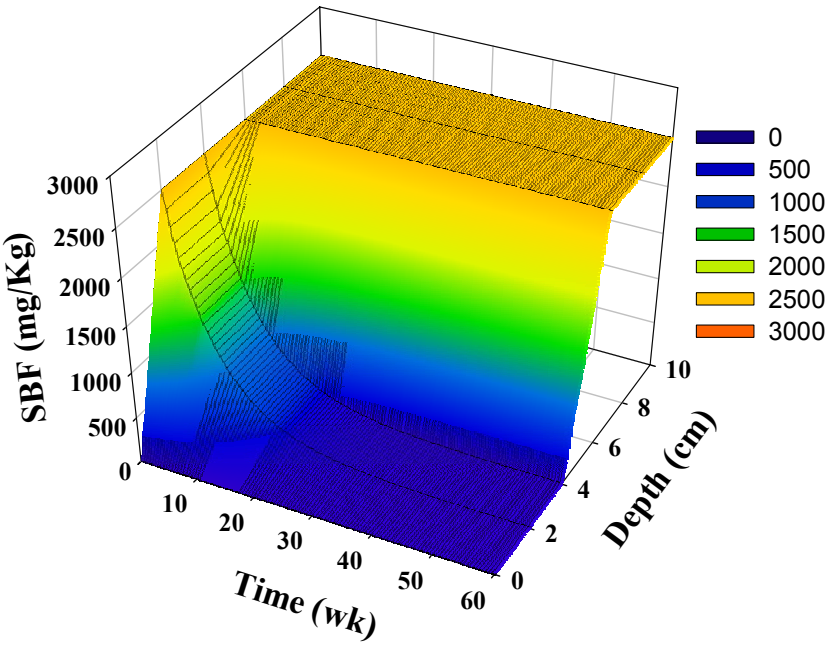
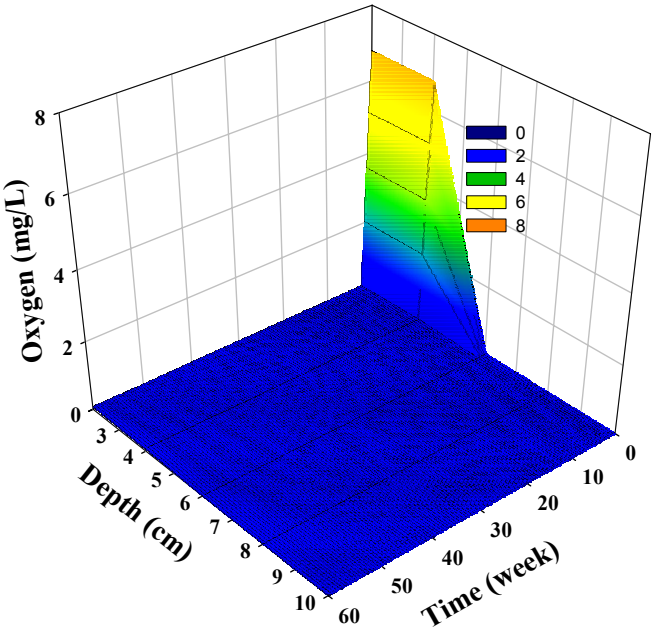


Figure 7.3. SBF profile when a) only aerobic decay (k=0.15) is active and b) when oxygen diffusion is added using a diffusion coefficient of 0.955.

a



b

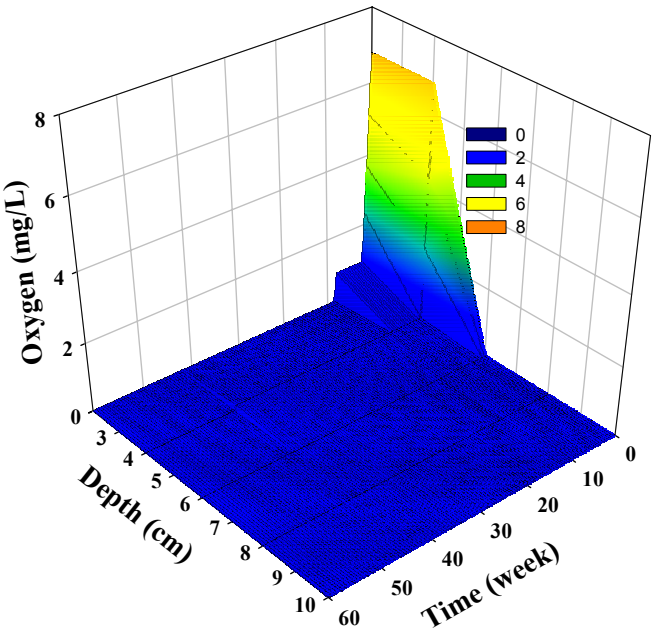
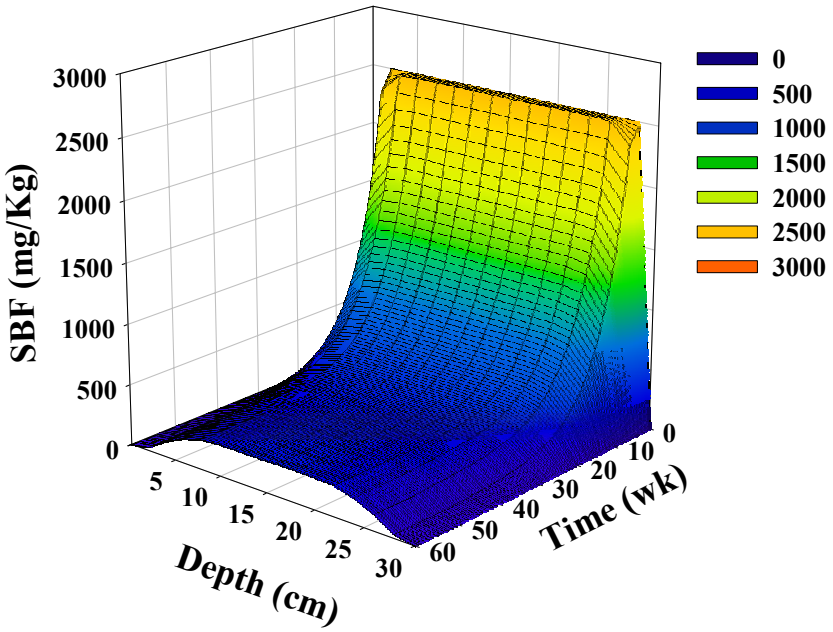


Figure 7.4. Oxygen profile when a) only aerobic decay (k=0.15) is active and b) when oxygen diffusion is added using a diffusion coefficient of 0.955.

a



b

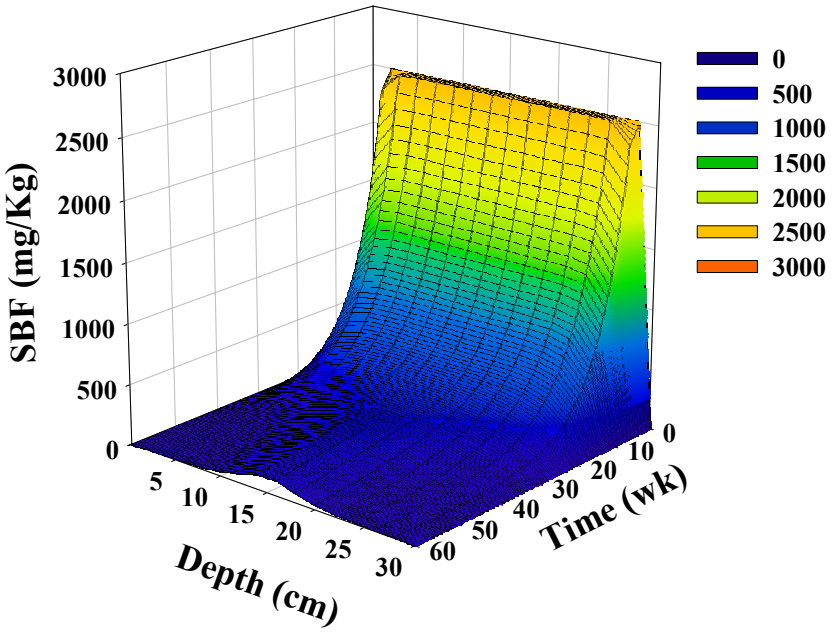
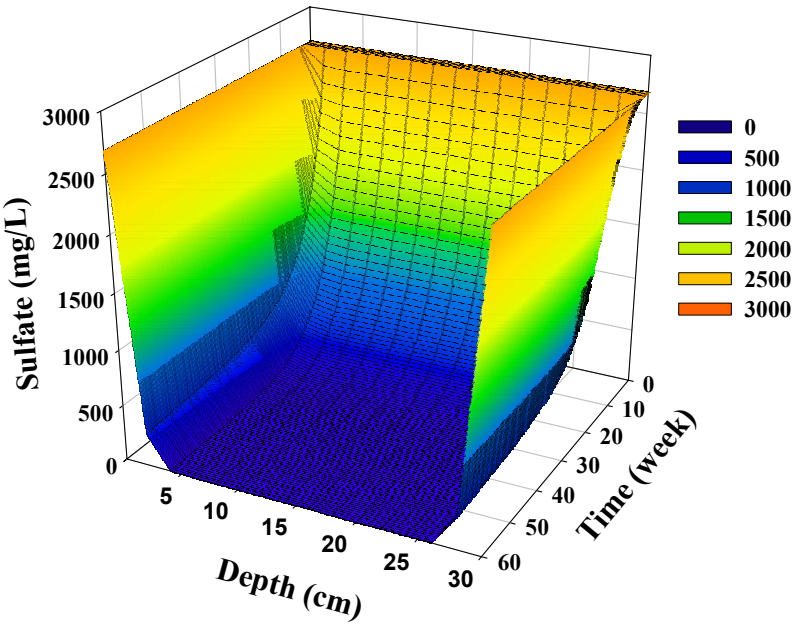


Figure 7.5. SBF profile with anaerobic decay of SBF occurs with a) no sulfate diffusion, and b) sulfate diffusion using a coefficient of 0.522.

a



b

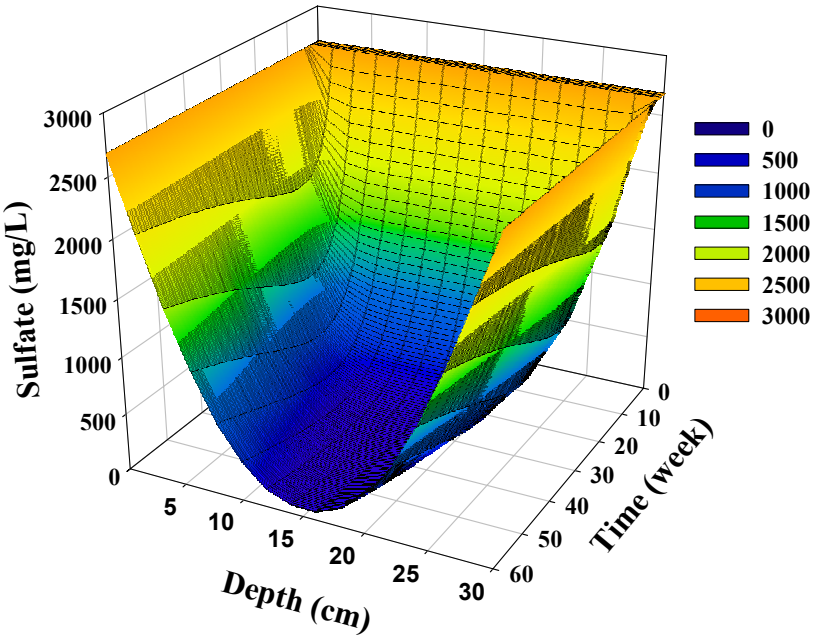


Figure 7.6. Sulfate profile with a) anaerobic decay (k=0.12) only and b) anaerobic decay plus sulfate diffusion (D=0.522).

7.4.4 Simulation of Aerobic-Anaerobic Degradation with Diffusion of SO_4 and O_2

All of the model parameters were set to the experimentally determined or estimated values (Table 7.2) and the model was run to generate SBF, oxygen, and sulfate profiles. The output is presented graphically in Figure 7.7, and Figure 7.9

Table 7.2

Input Parameters Used for Full Model Run

Parameter		SBF		O_2	SO_4	NOUR
Depth		30		3	3	NA
Concentration	Initial	2500		6.8	2688	
	Top	0		6.8	2688	
	Bottom	0		0	2688	
Diffusion Coefficient		0		0.955	0.522	
Decay Coefficient		Aerobic	Anaerobic			0.3
		0.15	0.12			
Lag Time		0	5			

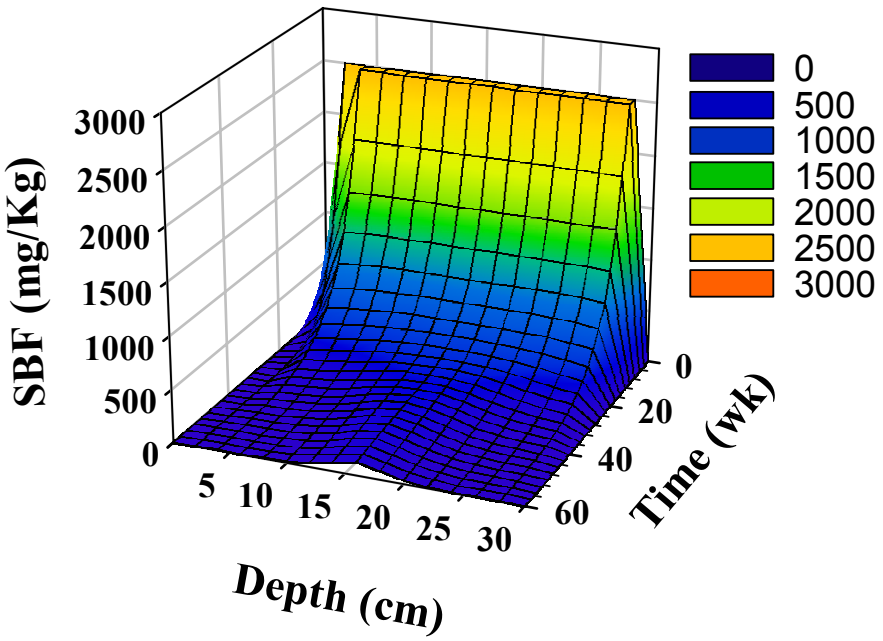


Figure 7.7. Predicted SBF profile with all model parameters set.

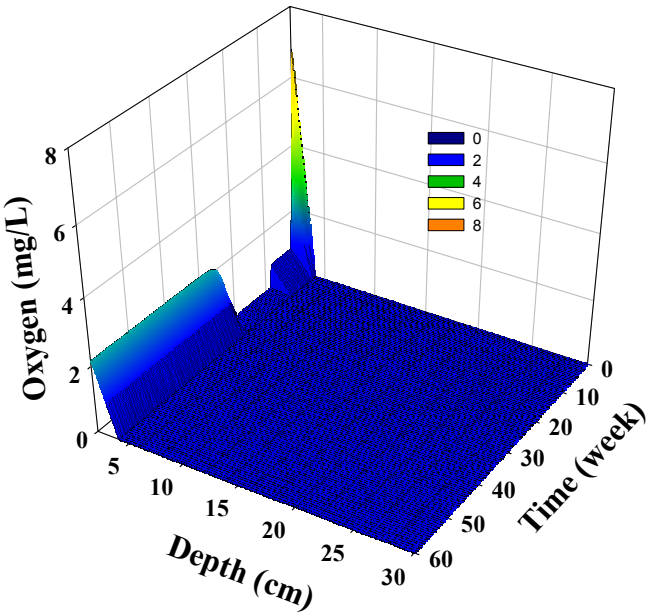


Figure 7.8. Predicted oxygen profile with all model parameters set.

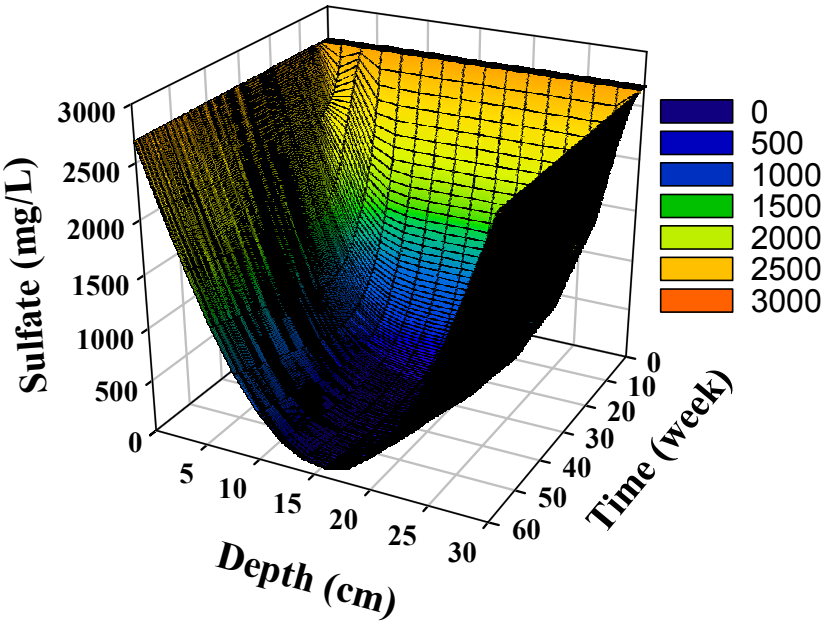


Figure 7.9. Predicted sulfate profile with all model parameters set.

CHAPTER 8 CONCLUSIONS

The literature review conducted as part of this research revealed that there are some initial findings concerning the anaerobic degradation of SBF and similar compounds under anaerobic (specifically sulfate reducing) conditions. The information available is enough to predict that the degradation of SBF could occur in deep anaerobic sediments, but the extent of degradation and the time required to achieve a significant degradation could not be predicted from the literature.

An initial mathematical framework to model SBF fate in marine sediments was developed using existing microbial growth theory and with the input parameters obtained from a review of existing information from related articles and research. With these initial parameters, the model as developed in a spreadsheet solution performed well, in other words the predicted results compared reasonably with existing research and articles. A sensitivity analysis using assumed parameters revealed that when all other parameters were assumed to be in expectable ranges the biodegradation of the SBF would be controlled by sulfate reducing organisms. The predictions showed that unless diffusion of sulfate were impeded to a great extent sulfate would never become limiting, thus it would be the dominant electron acceptor present in the sediments. Aerobic organisms did contribute to the depletion of the SBF in the first few centimeters of sediment.

Two techniques were used (growth based and molecular) to characterize the microbial populations and the examination of four nutritional types of organisms as well as four phylogenetic groups of organisms were performed. Straightforward conclusions can be drawn concerning the applicability of the methods used to these types of environmental samples, but conclusions as to the meaning of the results from the samples themselves are only preliminary and should be verified by further testing and repetition.

The most obvious conclusion concerning the methods used is that the use of the FISH technique and phylogenetic probes is not recommended for these sediments. Although FISH has been successfully used by other researchers in sandy marine sediments, sediments from the Gulf of Mexico have little sand and up to 74 percent clay. It was not possible to successfully dislodge or extract cells from the Gulf of Mexico sediments by sonication, slow centrifugation, and supernatant removal, or dissolution of material in acid or base before fast centrifugation. The sediments had to be analyzed as a whole since too many organisms were present in each fraction in all of the attempts to separate organisms from the sediment particles. The use of DAPI as a general DNA stain on whole sediment samples resulted in a yellow fluorescence of the sediment particles, which masked the fluorescence of the DAPI stained organisms and caused an underestimation of the total microbial populations.

The degradation of surrogate SBF tetradecene and ethyl oleate under conditions designed to mimic the floor of the Gulf of Mexico were examined. Sub-samples of NF and FF samples from three locations were incubated at 4°C at atmospheric pressure and at pressures similar to those they would experience at the depth of sampling. The results showed that the incubation pressure had no effect on the degradation of the substrates. The organisms in all of the sediments could degrade the ethyl oleate spiked into the sub samples in a reasonably short time. The degradation

of tetradecene typically required a longer lag period before degradation began and proceeded at a slower rate.

A summary of the degradation kinetics as determined from the experimental data for ethyl oleate degradation revealed that there was a significant difference in the kinetic coefficients in sediments with a detectable pre-exposure to SBF. This supports the theory that pre-exposure to SBF (even though the exposure was to IO's not to esters) caused an increase in the capable microbial population and thus an increased degradation rate. The removal of ethyl oleate from a contaminated site could be described using a first order k value of -0.22 ± 0.02 week^{-1}. The removal of ethyl oleate from an uncontaminated site can be predicted using a k value of -0.11 ± 0.02 week^{-1}. The lag time is another important factor in modeling the time required to remove a substrate from the sediment. Ethyl oleate degradation at most sites occurred with at most 11 weeks lag. The presence of SBF in the sediment sample also had an effect on the lag time. The lag times for ethyl oleate degradation in sediments that contained SBF (GC112 and VK916 NF) were significantly ($p=0.05$) shorter (average of 3 weeks) than those that did not contain detectable amounts of SBF (average of 4 weeks).

The degradation of tetradecene from the sediments could also be described using a first order kinetic model. The average k value for the removal of tetradecene linked to sulfate reduction was -0.05 ± 0.01 week^{-1}. It appears that the rate of tetradecene removal was not linked to sulfate reduction in the far field sediments. The lag time for sulfate reduction was longer than for tetradecene removal and sulfate reduction was slower than tetradecene removal if sulfate was removed. It could be possible that tetradecene was inhibiting the SRB (and thus sulfate reduction) because in VK916 NF and VK916 FF sulfate removal either did not occur or occurred after a very long lag.

The information obtained in the study was used to compile a final version of a visual basic spreadsheet based model. The model worked as predicted, in other words the predicted results compared reasonably with existing research and articles.

CHAPTER 9 LITERATURE CITED

Aeckersberg, F., F. Bak, and F. Widdel, 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch. Microbiol. 156:5-14.

Aeckersberg, F., F.A. Rainey, and F. Widdel. 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Arch. Microbiol. 170:361-369.

Alexander, M., 1994. Biodegradation and bioremediation. San Diego, CA: Academic Press, Inc. 302 pp.

Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, and D.A. Stahl**.** 1990a. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919-1925.

Amann, R.I., L. Krumholz, and D.A. Stahl. 1990b. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172:762-770.

Amann, R.I., W. Ludwig, and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Micobiol Rev. 59:143-169.

Atlas, R.M. 1997. Handbook of microbiological media. Florida: CRC Press. 1364 pp.

Berner, R.A., 1980. Early diagenesis: A theoretical approach. Princeton, NJ: Princeton University Press.

Berte-Corti and Bruns. 1999. The impact of oxygen tension on cell density and metabolic diversity of microbial communities in alkane degrading continuous flow cultures. Microbial Ecol. 37:70-77.

Bothner, M.H., C.M. Parmenter, D.C. Twichell, C.F. Polloni, and H.J. Knebel. 1992. A Geologic map of the sea floor in western Massachusetts Bay, constructed from digital sidescan sonar images, photography, and sediment samples, U.S. Geological Survey digital data series DDS-3.

Boudreau, B.P. and J.T. Westrich. 1984. The dependence of bacterial sulfate reduction on sulfate concentration in marine sediments. Geochimica et Cosmochimica Acta 48: 2503-2516.

Boudreau, B.P. 1997. Diagenetic models and their implementation: Modelling transport and reactions in aquatic sediments. Heidelberg: Springer-Verlag. 414 pp.

Brandsma, M.G. 1996. Computer simulations of oil-based mud cuttings discharges in the North Sea. In: The Physical and Biological Effects of Processed Oily Drill Cuttings (Summary Report). London: E&P Forum. Pp. 25-40.

Britton, L.N. 1984. Microbial degradation of aliphatic hydrocarbons. In Gibson, D.T., ed. Microbial degradation of organic compounds. New York: Marcel Dekker. Pp 89-129.

Burke, C.J. and J.A. Veil., 1995. Synthetic-based drilling fluids have many environmental plusses. Oil Gas J. 93:59-64

Caldwell, M.E., R.M. Garrett, R.C. Prince, and J.M. Suflita. 1998. Anaerobic biodegradation of long-chain n-alkanes under sulfate-reducing conditions. Environ. Sci. Technol. 32:2191-2195.

Chakravarty, M., H.D Singh, and J.N. Barnah, 1975. A kinetic model for microbial growth on liquid hydrocarbons. Biotechnol. Bioeng. 17:399-412.

Cochran, W.G. 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics. 6:105-116.

Continental Shelf Associates. 2004. Gulf of Mexico Comprehensive Synthetic Based Muds Monitoring Program Final Report. U.S. Dept. of the Interior, Minerals Management Service. http://www.gomr.mms.gov/homepg/regulate/environ/techsumm/rec_pubs.html#2004.

Cornelissen, G. and D.T.H.M. Sijm. 1996. An energy budget model for biodegradation and cometabolism of organic substances. Chemosphere 33:817-830.

Cripps, S.J., S. Westerlund, T.G. Jacobsen, J. Hovda, G. Kjeilen, S.D. Olsen, V. Eriksen, and J.P. Aabel. 1999. Ekofisk 1 Drill Cuttings piles characterization and management plan. Report RF-1999/041.

Darley, H.C.H. and G.G. Gray. 1988. Composition and properties of drilling and completion fluids. Fifth Ed. Houston, TX: Gulf Publishing Co. 643 pp.

Delvigne, G.A.L. 1996. Laboratory investigations on the fate and physicochemical properties of drill cuttings after discharge into the sea. pp 16-24 In: The physical and biological effects of processed oil drill cuttings (summary report). London: E&P Forum.

Dicks, B., T. Bakke, and I.M.T. Dixon. 1986/87. Oil exploration and production: Impact on the North Sea. Oil.Chem. Pollut. 3:289-306.

Dyksterhouse, S.E., J.P. Graym, R.P. Herwig, J.C. Lara, and J.T. Staley. 1995. *Cycloclasticus pugetii* gen. nov., s. nov., an aromatic hydrocarbon degrading bacterium from marine sediments. Int. J. Syst. Bacteriol. 45:116-123.

Ellery, W.N. and M.H. Schleyer. 1984. Comparison of homogenization and ultrasonication as techniques in extracting attached sedimentary bacteria. Mar. Ecol. Prog. Ser. 15:247-250.

Epstein, S.S. and J. Rossel. 1995. Enumeration of sandy sediment bacteria: search for optimal protocol. Mar. Ecol. Prog. Ser. 117:289-298.

Fedorak, P.M. and S.E. Hrudey. 1986. Nutrient requirements for the methanogenic degradation of phenol and p-cresol in anaerobic draw and feed cultures. Water Res. 20:113-122.

Federal Register. 2004. Final NPDES Permit for New and Existing Sources and New Dischargers in the Offshore Subcategory of the Oil and Gas Extraction Category for the Western Portion of the OCS of the Gulf of Mexico. Pp 60150-60151.

Friedheim, J. 1997. Second-generation synthetic drilling fluids. SPE Distinguished author series, Journal Petroleum Tech.

Friedheim, J.E. and H.L. Conn. 1996. Second generation synthetic fluids in the North Sea: are they better? IADC/SPE 35061. In: IADC/SPE drilling conference. New Orleans, 12-15 March 1996. Richardson, TX: Society of Petroleum Engineers, Inc. Pp 215-228.

Frischer, M.E., J.M. Danforth, M.A. Newton Healy, and F.M. Saunders. 2000. Whole-cell versus total RNA extraction for analysis of microbial community structure with 16S rRNA-targeted oligonucleotide probes in salt marsh sediments. Appl. Environ. Microbiol. 66:3037-3043.

Gallaway, B.J., R.G. Fechhelm, G.F. Hubbard and S.A. MacLean. 1998. Opportunistic sampling at a synthetic drilling fluid discharge site on the continental slope of the northern Gulf of Mexico: The Pompano Development, 13-14 March 1998. Report to BP Exploration, Inc. Houston, TX. LGL Ecological Research Associates, Inc. Bryan, TX. Various pages.

Garcia-Valdes, E., E. Cozar, R. Rotger, J. Lalucat, and J. Ursing. 1988. New naphthalene degrading marine *Pseudomonas* strains. Appl. Environ. Microbiol. 54:2478-2485.

Gauthier, M.J., B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, and J.C. Betrand. 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. Nov., a new, extremely halotolerant, hydrocarbon degrading marine bacterium. Int. J. Syst. Bacteriol. 43:568-576.

Geig, L.M. and J.M. Suflita. 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum contaminated aquifers. Environ. Sci. Technology. 36:3755-3762.

Geiselbrecht, A.D., R.P. Herwig, J.D. Deming, and J.T. Staley. 1996. Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon- degrading marine bacteria from Puget Sound sediments. Appl. Environ. Microbiol. 62:3344-3349.

Geiselbrecht, A.D., B.P. Hedlund, M.A. Tichi, and J.T. Staley. 1998. Isolation of marine polycyclic aromatic hydrocarbon (PAH) degrading *Cytoclasticus* strains from Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cytoclasticus* strains. Appl. Environ. Microbiol. 64:4703-4710.

Getliff, J., A. Roach, J. Toyo, and J. Carpenter. 1997. An overview of the environmental benefits of LAO based drilling fluids for offshore drilling. Report from Schlumberger Dowell. 10 pp.

Greenberg A.E., L.S. Clesceri, and A.D. Eaton (eds). 1992. Standard methods for the examination of water and wastewater. American Public Health Association/American Water Works Association/Water Environment Federation. Washington, DC.

Growcock, F.B., S.L. Andrews, and T.P. Frederick. 1994. Physicochemical properties of synthetic drilling fluids. IADC/SPE 27450. In: 1994 IADC/SPE Drilling Conference. Dallas, TX, 15-18 February 1994. Richardson, TX: International Association of Drilling Contractors/Society of Petroleum Engineers, Inc. (IADC/SPE). Pp 181-190.

Herman, D. and D.J. Roberts. 2005. A Marine Anaerobic Biodegradation Test Applied to the Biodegradation of Synthetic Drilling Mud Base Fluids. Soil and Sediment Contamination 14:433-447.

Halvorson, H.O. and N.R. Ziegler. 1933. Applications of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. J. Bacteriol. 25:101-121.

Kleikemper, J., M.H. Schroth, W.V. Sigler, M. Schmucki, S. M. Bernasconi, and J. Zeyer. 2002. Activity and diversivity of sulfate reducing bacteria in a petroleum hydrocarbon contaminated aquifer. Appl. Environ. Microbiol. 68:1516-1523.

Koizumi, Y., J.J. Kelly, T. Nakagawa, H. Urakawa, S. El-Fantroussi, S. Al-Muzaini, M. Fakui, Y. Urushigawa, and D.A. Stahl. 2002. Parallel characterization of anaerobic toluene- and ethylbenzene- degrading microbial consortia by PCR-denaturing gradient gel electrophoresis, RNA-DNA Membrane hybridization, and DNA microarray technology. Appl. Environ. Microbiol. 68:3215-3225.

Kropp, K.G., I.A Davidova, and J.M. Suflita. 2000. Anaerobic oxidation of n-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. Appl. Environ. Microbiol. 66:5393-5398.

Kubista, M., B. Aakerman, and B. Norden. 1987. Characterization of interaction between DNA and 4',6-diamidino-2-phenylindole by optical spectroscopy. Biochemistry. 26:4545-4553.

Kusel, K., H.C. Pinkart, H.L. Drake, and R. Devereaux. 1999. Acetogenic and sulfate reducing bacteria inhabiting the rhizoplane and deep cortex cells of the sea grass *Halodule wrightii*. Appl. Environ. Microbiol. 65:5117-5123.

Lapidus, L. and G.F. Pinder. 1982. Numerical Solution of Partial Differential Equations in Science and Engineering. New York, NY: John Wiley and Sons.

Li, Y.H. and S. Gregory. 1974. Diffusion of ions in seawater and in deep-sea sediments. Geochim. et Cosmochim. Acta. 11:703-714.

McCarty, P.L. 1972. Energetics of organic matter decomposition. In: Mitchell, R., ed. Water Pollution Microbiology. Hoboken, NJ: Wiley Publ. Pp 91-118.

McKee, J.D.A., K. Dowrick, and S.J. Astleford. 1995. A new development towards improved synthetic based mud performance. SPE/IADC 29405. In: 1995 SPE/IADC drilling conference. Amsterdam, 28 February-2 March 1995. Richardson, TX: Society of Petroleum Engineers. Pp 613-621.

Meckenstock, R.U., E. Annweiler, W. Michaelis, H.H. Richnow, and B. Schink. 2000. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66:2743-2747.

Molecular Probes. 2001. Product information: DAPI nucleic acid stain. www.probes.com.

Murray, J.W., V. Grundmanis, and W.M. Smethie. 1978. Interstitial water chemistry in the sediments of Saanich Inlet. Geochim. Cosmochim. Akta, 42:1011-1026.

Neff, J.M. 1987. Biological effects of drilling fluids, drill cuttings and produced waters. In: D.F.Boesch and N.N. Rabalais, eds., Long-term effects of offshore oil and gas development. London: Elsevier Applied Science Publishers 469-538.

Neff, J.M., S. McKelvie, and R.C. Ayers. 2000. Environmental impacts of synthetic based drilling fluids. Published by U.S. Dept. of the Interior, Minerals Management Service Gulf of Mexico OCS Region, New Orleans. LA. OCS Study MMS 2000-064. 145 pp.

Okpokwasili, G.C., C.C. Somerville, D.J. Grimes, and R.R. Colwell. 1984. Plasmid- associated phenanthrene degradation by Chesapeake Bay sediment bacteria. Colloq. Inst. Francaise Rech. Exploit. Mer. 3:601-610.

Pineda de Castro, L.F. and M. Zacharias. 2000 (poster). Ligand docking using implicit continuum salvation models: Binding of DAPI to minor groove of DNA. 18th International MGMS Meeting: Modelling Biomolecular Mechanism:From States to Processes at the Atomic Level, 5th - 8th April, 2000 at the University of York, UK.

Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25: 943-948.

Ravenschlag, K., K. Sahm, C. Knoblauch, B.B. Jorgensen, and R. Amann. 2000. Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine arctic sediments. Appl. Environ. Microbiol. 66:3592-3602.

Rublee, P. and B.E. Dornseif. 1978. Direct counts of bacteria in the sediments of a North Carolina salt marsh. Estuaries. 1:188-191.

Schallenberg, M., J. Kalff, and J.B. Rasmussen. 1989. Solutions to problems in enumerating sediment bacteria by direct counts. Appl. Environ. Microbiol. 55:1214-1219.

Scheraga, M.M., M. Meskill, and C.D. Litchfield. 1979. Analysis of methods for the quantitative recovery of bacteria sorbed onto marine sediments. In C. D. Litchfield and P. L. Seyfried, eds. Methodology of biomass determinations and microbial activities in sediments. Amer. Soc. Test. Mater. ASTM STP 637:21-39.

Scherrer, P. and G. Mille. 1989. Biodegradation of crude oil in an experimentally polluted peaty mangrove soil. Mar. Pollut. Bull. 20:430-432

Schink, B. 1985. Degradation of unsaturated hydrocarbons by methanogenic enrichment cultures. FEMS. Microbiol. Ecol. 31:69-77.

Schink, B. 1989. Anaerober abbau von Kohlenwasserstoffen. Erdol Kohle Erdgas. 42:116-118.

Shen, Y., L.G. Stehmeier, and G. Voordouw. 1998. Identification of hydrocarbon-degrading bacteria in soil by reverse sample genome probing. Appl. Environ. Microbiol. 64:637-645.

So, M.C. and L.Y. Young. 1999a. Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl. Environ. Microbiol. 65:2969-2976.

So M.C. and L.Y. Young. 1999b. Initial reactions for anaerobic alkane degradation by the sulfate reducer strain AK-01. Appl. Environ. Microbiol. 65:5532-5540.

So, M.C. and L.Y. Young. 2001. Anaerobic Biodegradation of alkanes by enriched consortia under four different reducing conditions. Environ. Toxicol. Chem. 20:473-478.

Soetaert, K., P.M.J. Herman, and J.J. Middelburg. 1996. A model of early diagenetic processes from the shelf to abyssal depths. Geochimica et Cosmochimica Acta 60:1019-1040.

Stahl, D.A. 1986. Molecular approaches for the measurement of density, diversity, and phylogeny. In: Hurst, C.J., G.R. Knudsen, M.J. McInerney, M.V. Walters, and L.D. Stetzenbach, eds. Manual of Environmental Microbiology. Washington, DC: ASM Press. Pp 102-114.

Tagger, S., N. Truffaunt, and J. Le Petit. 1990. Preliminary study on relationships among strains forming a bacterial community selected on naphthalene from marine sediment. Can. J. Microbiol. 36:676-681.

Tanious, F.A., J.M. Veal, H. Buczak, L.S. Ratmeyer, and W.D. Wilson. 1992. DAPI (4',6-diamidino-2-phenylindole) binds differently to DNA and RNA: minor-groove binding at AT sites and intercalation at AU sites. Biochemistry. 31:3103-3112.

Thomas, H.A., Jr. 1942. Bacterial densities from fermentation tube tests. J. American Water Works Assoc. 34:572.

Tso, S.F. and G.L. Taghon. 1997. Enumeration of protozoa and bacteria in muddy sediments. Microb. Ecol. 33:144-148.

Ullman, W.J. and R.C. Aller. 1982. Diffusion coefficients in nearshore marine sediments. Limnol. Oceanogr. 27:552-556.

U.S. EPA. 1996. Oil and gas extraction point source category; final effluent limitations guidelines and standards for the coastal subcategory; final rule. Part III. 40 CFR 435. *Federal Register*. 61:66086, 66120-66122.

Van Capellan, P., J.F. Gaillard, and C. Rabouille. 1993. Biogeochemical transformation in sediments: kinetic models of early diagenesis. In: Wollast, R., F.T. Mackenzie, and L. Chou, eds. Interactions of C, N, P and Global Change. Berlin: Springer-Verlag. Pp 401-446.

Van Capellan, P. and Y. Wang. 1995. Metal cycling in surface sediments: Modeling the interplay of transport and reaction. In: Allen, H.E., ed. Metal Contaminated Sediments. Chelsea, MI: Ann Arbor Press. Pp 21-64.

Velji, M.I. and L.J. Albright. 1985. Microscopic enumeration of attached marine bacteria of seawater, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatments. Can. J. Micobiol. 32:121-126.

Vik, E.A., S. Dempsey, and B.S. Nesgard. 1996. Evaluation of available test results from environmental studies of synthetic based drilling muds. Version 4. Aquateam Report Number: 96-010. OLF Project. Acceptance Criteria for Drilling Fluids. Aquateam-Norwegian Water Technology Centre A/S. Oslo, Norway. 127 pp.

West, P.A., G.C. Okopokwasili, P.R. Brayton. D.J. Grimes, and R.R. Colwell. 1984. Numerical taxonomy of phenanthrene-degrading bacteria isolated from Chesapeake Bay. Appl. Environ. Microbiol. 48:988-993.

Wijsman, J.W.M., P.M.J. Herman, J.J. Middelburg, and K.A Soetaert. 1997. Model for early diagenetic processes in sediments of the continental shelf of the Black Sea. Estuarine, Coastal and Shelf Science 54: 403-421.

Woomer, P.L. 1994. Most probable number counts. In: Weaver, R.W., S. Angle, P. Bottomley, D. Bezdicek, S. Smith, A. Tabatabai, and A. Wollum, eds. Methods of soil analysis, Part 2. Microbiological and biochemical properties. Soil Science Society of America. 5:59-79.

Yu, W., W.K. Dodds, M.K. Banks, J. Skalsky, and E.A. Strauss. 1995. Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes. Appl. Environ. Microbiol. 61:3367-3372.

Zevallos, M.A.L., J. Candler, J.H. Wood, and L.M. Reuter. 1996. Synthetic-based fluids enhance environmental and drilling performance in deepwater locations. SPE 35329. In: SPE International Petroleum Conference & Exhibition of Mexico. Villahermosa, Tabasco, Mexico, 5-7 March 1996. Richardson, TX: Society of Petroleum Engineers, Inc. Pp. 235-242.

The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island territories under U.S. administration.

The Minerals Management Service Mission

As a bureau of the Department of the Interior, the Minerals Management Service's (MMS) primary responsibilities are to manage the mineral resources located on the Nation's Outer Continental Shelf (OCS), collect revenue from the Federal OCS and onshore Federal and Indian lands, and distribute those revenues.

Moreover, in working to meet its responsibilities, the **Offshore Minerals Management Program** administers the OCS competitive leasing program and oversees the safe and environmentally sound exploration and production of our Nation's offshore natural gas, oil and other mineral resources. The MMS **Minerals Revenue Management** meets its responsibilities by ensuring the efficient, timely and accurate collection and disbursement of revenue from mineral leasing and production due to Indian tribes and allottees, States and the U.S. Treasury.

The MMS strives to fulfill its responsibilities through the general guiding principles of: (1) being responsive to the public's concerns and interests by maintaining a dialogue with all potentially affected parties and (2) carrying out its programs with an emphasis on working to enhance the quality of life for all Americans by lending MMS assistance and expertise to economic development and environmental protection.