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# Determination of predominant species of oil-degrading bacteria in the oiled sediment in Barataria Bay, Louisiana

Lauren Nicole Navarre
*Louisiana State University and Agricultural and Mechanical College*

# DETERMINATION OF PREDOMINANT SPECIES OF OIL-DEGRADING BACTERIA IN THE OILED MARSH SEDIMENT IN BARATARIA BAY, LOUISIANA

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## ABSTRACT

The effects of the Deepwater Horizon (*DWH*) oil spill on the bacterial communities in coastal sediments are relatively known but few studies have been published evaluating the impacts of the spill on oil-degrading bacterial populations in the salt marsh sediments of Barataria Bay, Louisiana. The aims of this study were to determine the effects of the oil spill on the microbial community in Barataria Bay, Louisiana through the determination of predominate species of oil-degrading bacteria in marsh sediments, and to determine how well certain bacterial isolates can degrade crude oil in the region by taking samples from three areas of different oiling categories in April 2012, October 2012 and April 2013, approximately 24, 30, and 36 months after the initial oiling event. Hydrocarbon-degrading bacteria were isolated from the sediment samples using Bushnell Haas (BH) agar enriched with 1% Light Louisiana Sweet (LLS) crude oil. The isolates were subsequently purified on LB agar plates, their genomic DNAs extracted, and 16S rRNA genes amplified by polymerase chain reaction (PCR). The PCR products of 16S rRNA were then sequenced and taxonomically classified based on similarity to known sequences in BLAST database. A total of 460 isolates were obtained, representing 84 species from 45 genera in 6 phyla. Oil degradability of 10 bacterial isolates, designated to *Staphylococcus xylosus*, *Acinetobacter calcoaetious*, *Pseudomonas stutzeri*, *Bacillus pumilus*, *Micrococcus luteus*, *Rhodococcus equi*, *Microbacterium esteratomaticum*, *Vibrio* sp., *Pseudomonas mendocina*, and *Agromyces aurantiacus*, respectively, was tested in LB broth with 1% LLS crude oil. Their growth and metabolic properties in terms of optical density at 600 nm and pH in culture medium were monitored for 6 days. Among these isolates, *Micrococcus luteus*, *Vibrio*

sp., *Bacillus pumilus*, *Rhodococcus equi* and *Microbacterium esteratomaticum* appeared to have the greatest ability to degrade LLS crude oil; their growth rates were estimated to be 0.038, 0.037, 0.031, 0.029 and 0.037 per hour, respectively. All isolates showed a decrease in pH in their culture medium during their growth, with *M. esteratomaticum* having the lowest pH of 5.29 and *R. equi* having the highest pH of 6.50 at 96 hours of incubation. A decrease in pH in the cultured media is indicative of an accumulation of acid from oxidation reactions carried out by oil-degrading bacteria. The present study provided a comprehensive list of indigenous oil-degrading bacteria in Louisiana marsh sediment, which is critical in developing bioremediation strategies.

# CHAPTER ONE: INTRODUCTION

## 1.1 BACKGROUND

The coastal wetlands in the northern Gulf of Mexico account for over forty percent of coastal wetlands in the United States (Mitsch and Gosselink, 1993). These wetlands are of great importance due to a multitude of significant ecosystem services they provide, to the Gulf of Mexico and to the nation. Among these services are water quality improvement, pollutant degradation, flood control, shoreline stabilization, carbon sequestration, and many other climate regulation, and nutrient cycling, which are dependent on healthy, functioning plant-microbial-benthic systems. Microorganisms that thrive in these wetlands regulate many important processes such as nutrient cycling and decomposition of wastes. Microbial degradation of hydrocarbons has become an extremely important topic in recent years due to major oil spills such as the 1989 Exxon Valdez tanker accident and the 2010 Deepwater Horizon (DWH) event. Many species of bacteria can break down different components of oil (Hassanshahian et al., 2012). Oil hydrocarbons have been seeping naturally into the Gulf from underground reservoirs for millions of years, and over time certain bacteria have evolved enzymatic pathways that enable them to metabolize these hydrocarbons (AL-Saleh et al., 2009). Hydrocarbon-degrading bacteria have the ability to break down hydrocarbons into carbon dioxide and water and can use the energy stored in the hydrocarbon bonds as an energy source (Head et al., 2006). These microbes occur naturally in wetland environments like those of Barataria Bay. However, oil composition and concentration may affect microbial community structure both immediately and long term.

On April 20, 2010 a blowout caused the British Petroleum Deepwater Horizon rig to explode, and crude oil began gushing into the Gulf of Mexico. This oil spill released the equivalent volume of 4.9 million barrels of light crude oil from April to July 2010 (OSAT, 2010). A large amount of the oil that was released during the spill reached habitats along the northern Gulf of Mexico coastline, particularly salt marshes, including those in Barataria Bay. Salt marshes are especially vulnerable to oil contamination due to low wave action and high organic matter content of sediments which lead to greater sorption of organic contaminants (Venosa et al., 2002). Previous studies have shown that spilled oils can persist in salt marshes for years to decades (Reddy et al., 2002; Peacock et al., 2007; Slater et al., 2005; Oudot et al., 2010; Maki, 1991 and Wade, 1993).

The purpose of this study was to ascertain if the predominant species of oil-degrading bacteria in salt marshes in Barataria Bay have changed since the spill in 2010 and to determine how well the indigenous hydrocarbon-degrading bacteria are able to degrade crude oil, specifically Light Louisiana Sweet (LLS) crude oil, the same source oil released during the DWH event. It has been several years since the DWH spill, and yet there have been no works that attempted to comprehensively catalog oil degraders in the Gulf of Mexico, ascertain which degraders are the most important in a given environment or determine the abundance of these oil-degrading microorganisms. Biodegradation of hydrocarbons, in ecosystems like those in Barataria Bay, Louisiana, depend on the presence of suitable and viable microbial populations (Mills et al., 2003). Characterizing these microbial communities will provide the scientific community with valuable insights that can be used in the future to determine suitable remediation strategies after oil spills or determine microbial resilience in a given ecosystem.

## 1.2 BARATARIA BAY SALT MARSH ECOSYSTEM

Barataria Bay is located between Bayou Lafourche to the west and the Mississippi River delta to the east. The system is separated from the Gulf of Mexico by the Grande Terre Islands with Grand Isle on the western boundary. Quatre Bayoux Pass and another pass just east of Grand Isle serve as the primary connections to water from the Gulf of Mexico (USEPA, 1999). Barataria Bay is a shallow water system, the average depth of water is 2 meters and the tidal range is approximately 1 meter (LCWCRTF, 1993). Water volumes and water levels in Barataria Bay are highly influenced by tides, winds, and precipitation. The salinity average in the bay is 13 ppt, but is variable. Within the salt marsh ecosystems of Barataria Bay, the tidal range averages 0.3 m in height and salinity ranges from 6 to 22 ppt (Chabreck, 1972). Out of all ecosystems within Barataria Bay, salt marsh has the lowest land to water ratios. The major plant species in Barataria Bay is *Spartina alterniflora*, which covers over 60% of the area (Conner et al., 1987). These salt marshes provide habitats for water fowls and migrating wildlife, act as nurseries for fish and shellfish and distribute nutrients to coastal ecosystems. They also protect the coast from erosion and help dissipate tidal and storm surges. Once the oil from the *DWH* spill reached the marsh of Barataria Bay it coated the stems and leaves of plants. Oil coating the leaves can obstruct or prevent gas exchange. Some shoreline plants, like *Salicornia bigelovii*, find crude oil extremely toxic and died soon after the oil made landfall. Other plants, like *Spartina alternifora*, are extremely hearty and survived stem and leaf oiling. However, oil from the spill began to leach into marsh sediments, oiling belowground plant organs, which has resulted in the death of many plant species and increased coastal erosion (Mendelssohn et al., 2012).

Oil contamination in salt marsh ecosystems has been shown to persist for decades due to the same characteristics that make these ecosystems so productive (Natter et al., 2012). Once oil

enters a salt marsh ecosystem the oil will stick to sediment and plant surfaces. Wave action in salt marsh environments is low and cannot disperse oil. In contrast, oil contamination in beach sands is more quickly dispersed due to high-energy wave action. Components of oil associate more readily with organic matter than minerals in sediments. The high organic carbon content found in salt marsh ecosystems is conducive to conservation of hydrocarbons in sediments. A study conducted by Oudot and Chaillan (2010) found that high molecular weight components of crude oil, such as polycyclic aromatics, were conserved and persisted in coastal wetlands decades after the Amoco-Cadiz oil spill (Oudot and Chaillan, 2010).

## 1.3 HYDROCARBONS

Petroleum, or crude oil, is a naturally occurring mixture of hydrocarbons that are produced by the thermal decay of buried organic matter over geologic time. Petroleum made up of thousands of hydrocarbon compounds that vary with respect to their chemical and physical characteristics; they range from light, volatile, short-chain compounds to heavy, long-chain, branched-chain compounds (Kadali et al., 2012; Alonso-Guitierrez et al., 2009). Petroleum hydrocarbons range in carbon number from C1 to >C60 (EPA 2011). Saturates, aromatics, asphaltenes, and resins are the constituents that make up petroleum (Martinez-Gomez et. al 2010). Saturates include linear alkanes, branched alkanes, and cycloalkanes. Linear alkanes make up a large portion of crude oil, except where biodegradation has occurred (Ollivier and Magot 2005). Branched-chain alkanes are composed of carbon atoms in branched chains; the chains may occur in many different configurations. Cycloalkanes are molecules in which the carbon atoms are arranged in one or more rings, but they do not contain aromatic rings (Figure 1.1). Resins and asphaltenes, in contrast to the saturated and aromatic fractions, contain

non-hydrocarbon polar compounds that often form complexes with heavy metals (Harayama et al., 1999). Most types of petroleum also contain small amounts of hydrogen sulfide (EPA 2011). Aromatics are characterized by the presence of at least one benzene ring. The well-known examples of one-ring (or mononuclear) aromatics are benzene, toluene, ethylbenzene, and xylene (BTEX), and they are important components of gasoline. Polycyclic aromatic hydrocarbons (PAHs) consist of multiple, fused aromatic rings. Examples of these are naphthalene, anthracene, pyrene, and many more.

Petroleum that has been extracted from different reservoirs can vary both chemically and physically. The composition of refined petroleum depends on the source of the crude oil and the refining practices used (Dandie et al., 2010). The physical appearance of crude oil can range from a light, mobile, straw-colored liquid to a highly viscous, semi-solid black substance. The lower-molecular-weight components of petroleum possess moderate-to-high water solubility, whereas higher molecular weight fractions tend to form emulsions in water. The lighter weight aliphatic and aromatic components of petroleum have high mobility in soils, whereas the heavier molecular weight constituents possess low mobility (EPA 2011).

Light Louisiana Sweet crude oil has a low sulfur content and is rich in saturates and aromatics. In comparison to other crude oils, it also has a low nitrogen, nickel and Vanadium content. The LLS also has a relatively low specific gravity, 0.875 g/ml, and contains fewer higher molecular weight hydrocarbons (Atlas, 1975). This oil has a relatively low density, and is predominately composed of low molecular weight compounds, which makes it better suited to biodegradation than other crude oils such as those from Kuwait, Dubai and Saudi Arabia. Biodegradability of oil was found to be related to the content of n-alkane, asphaltene, nitrogen, sulfur and oxygen containing components of oil (Westlake et al., 1974).

## 1.4 MICROORGANISMS

Microbial ecologists have identified 79 genera of aerobic bacteria that are effective degraders of hydrocarbons in natural environments and exist in amounts capable of noteworthy oil biodegradation. (Head et al., 2006; Prince, 2005). Within those 79 genera, there are over 500 microbial species that have been recognized to be capable of degrading hydrocarbons (Head et al., 2006; Yakimov et al., 2007). Although, hydrogen-degrading bacteria exist naturally in most environments, the fraction of the total heterotrophic community represented by hydrogen-degrading bacteria is quite variable (Pinholt et al., 1979). Microorganism populations must be diverse to metabolize the range of hydrocarbon substrates found in crude (Bossert and Bartha, 1984). These diverse populations occur in natural ecosystems and either independently or in combination metabolize various hydrocarbons. The metabolic pathways that hydrocarbon degrading microorganisms use can be either aerobic (i.e. they utilize oxygen as the primary electron acceptor) or anaerobic (i.e. they utilize an alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic degradation. The most important genera of hydrocarbon degraders in both marine and soil environments are *Achromobacter, Actinobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia*, and *Pseudomonas* sp. (Leahy and Colwell, 1990). In a study of oil-degrading bacteria in the Chesapeake Bay, Austin et al. (1977) found that *Pseudomonas, Micrococcus,* and *Norcardia* sp., members of the family *Enterobacteriaceae*, actinomycetes, and coryneforms made up 95% of isolates (Austin et al., 1977). However, many other genera have been found to degrade different hydrocarbons (Coulon et al., 2007; Procopio et al., 2012).

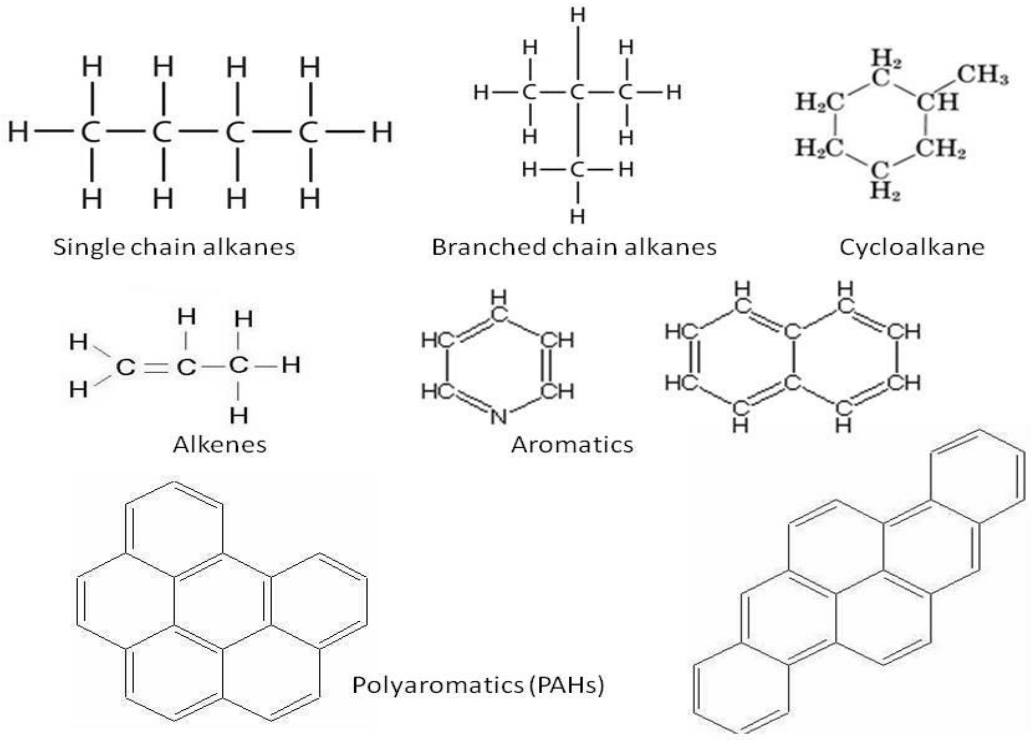


Figure 1.1: Chemical structures of alkanes, alkenes, and aromatic hydrocarbons. Adapted from Phang (1990).

Table 1.1 summarizes some major bacterial genera that have been identified to be involved in the oil degrading process. Microbial communities can adapt to hydrocarbon contamination in three ways: the induction or repression of specific enzymes, genetic changes resulting in new metabolic capabilities and the increase of a sub-population with the ability to utilize hydrocarbons endemic to the contamination (Martin, 2011).

## 1.5 AEROBIC DEGRADATION OF HYDROCARBONS BY MICROBES

A hydrocarbon's chemical structure affects its biodegradation in two ways. First, a molecule may contain functional groups that cannot react with available or inducible enzymes. Second, the structure may cause the compound to be in a physical state where microbial degradation does not easily occur. Usually, the larger and more complex the structure of a

hydrocarbon, the more slowly it is oxidized. The degree of substitution also affects degradation. Compounds that contain amine, methoxy and sulfonate groups, ether linkages, halogens, and branched-carbon chains are generally persistent. Adding aliphatic side-chains increases the susceptibility of cyclic hydrocarbons to microbial attack (Riser-Roberts, 1992) (Figure 1.2).

Table 1.1: Genera of hydrocarbon-degrading bacteria

| **Genus** | **Function** | **Reference** |
|---|---|---|
| *Aeromonas* | PAH degrader | Jacques et. al., 2007 |
| *Alcanivorax* | Alkane degrader | Kasai et al, 2002 |
| *Bacillus* | Diesel degrader, PAH degrader | Singh and Lin, 2008<br>Kotska et al., 2011 |
| *Beijerinckia* | PAH degrader | Jacques et al., 2007 |
| *Burkholderia* | PAH degrader | Jacques et al., 2007 |
| *Corynebacterium* | PAH degrader | Jacques et al., 2007 |
| *Cycloclasticus* | Aromatic degrader | Kasai et al., 2002 |
| *Flavobacterium* | PAH degrader | Jacques et al., 2007 |
| *Pseudomonas* | PAH, alkane degrader | Hemalatha and Veeramanikandan, 2011<br>Tanase et al. 2012 |
| *Dietzia* | Aromatic, alkane degrader | Procópio et al., 2013 |
| *Rhodococcus* | Alkane degrader | Tanase et al., 2012 |
| *Acinetobacter* | Alkane degrader | Throne-Holst et al., 2007 |
| *Gordonia* | Alkane degrader | Kato et al., 2009 |
| *Moraxella* | PAH degrader | Kasai et al., 2002 |
| *Mycobcterium* | PAH degrader | Jacques et al., 2007; Haritash and Kaushik, 2009 |
| *Nocardia* | PAH degrader | Jacques et al., 2007 |
| *Paenibacillus* | PAH degrader | Haritash and Kaushik, 2009 |
| *Paracoccus* | PAH degrader | Jacques et al., 2007 |
| *Sphingomonas* | PAH degrader | Jacques et al., 2007 |

Table 1.1: Continued

| Genus | Function | Reference |
|---|---|---|
| *Stenotrophomonas* | PAH degrader | Jacques et al., 2007 |
| *Vibrio* | PAH degrader | Kasai et al., 2007 |
| *Achromobacter* | PAH degrader | Gupta, 2012 |
| *Brevibacterium* | PAH degrader | Jain et al., 2005 |
| *Staphylococcus* | PAH degrader | Gupta, 2012 |

Under aerobic conditions, aliphatic compounds are easily degraded because of their structural similarity to fatty acids and plant paraffins. The general degradation pathway for an aliphatic involves sequential formation of an alcohol, an aldehyde, and a fatty acid. The fatty acid is cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation(Figure 1.3).

Aliphatics may be broken down when a monooxygenase enzyme catalyzes the replacement of a terminal carbon atom by an oxygen atom. The second pathway utilizes a dioxygenase enzyme to incorporate two oxygen atoms into an aliphatic to form a hydroperoxide. Saturated aliphatics and alkenes are degraded in a very similar manner (Britton, 1984). Although microorganisms exist that can completely degrade alicyclics, degradation is believed to mostly occur through commensalistic behavior between two microorganisms. Through a series of reactions, one organism converts cyclohexane to cyclohexanone; however, the organism is unable to lactonize and open the ring. A second organism performs lactonization, ring opening and mineralization of the remaining aliphatic compound (Perry, 1984). The general pathway for aromatic hydrocarbons involves cis-hydroxylation of the ring structure with dioxygenase to form a diol. The ring is cleaved by dioxygenases to form a dicarboxylic acid. Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the side chain, followed by

cleavage of the ring structure. The degradative pathway for a highly branched compound, such as pristane or phytane, may proceed by omega oxidation to form a dicarboxylic acid instead of a monocarboxylic acid (Hamme et al., 2003).

The ability of a microorganism to degrade hydrocarbons is inherently influenced by the composition of the hydrocarbons. Kerosene, for example, which consists almost exclusively of medium chain alkenes under certain conditions, is totally biodegradable. Similarly, crude oil is biodegradable, but for heavy, asphaltic-naphthenic compounds, only about 11% may be biodegradable within a reasonable time period, even if the conditions are favorable (Bartha, 1986; Okoh et al., 2001; Ghazali et al., 2004). Okoh (2002) reported that heavier crude oils are generally much more difficult to biodegrade than lighter ones. Rahman et al. (2002), have reported that the percentage of degradation by a mixed bacterial consortium decreased from 78% to 52% as the concentration of crude oil was increased from 1 to 10% in soil. Prior exposure of a microbial community to hydrocarbons, either from anthropogenic sources or natural seeps, is important in determining how quickly hydrocarbon inputs can be biodegraded (Leahy and Colwell, 1990).

## 1.6 HYDROCARBON TOXICITY TO THE MICROBIAL COMMUNITY

The effects of hydrocarbon contamination on microbial communities in salt marsh sediments along the northern coast of the Gulf of Mexico are potentially far reaching and must continue to be assessed. It has been widely reported that hydrocarbon contamination decreases the diversity of soil microbial populations immediately following exposure (Alonso-Gutierrez et al. 2009; Atlas, 1991; Hood et al., 1975; John et al., 2009; Mcnaughton et al., 1999; Nyman et al., 1998).

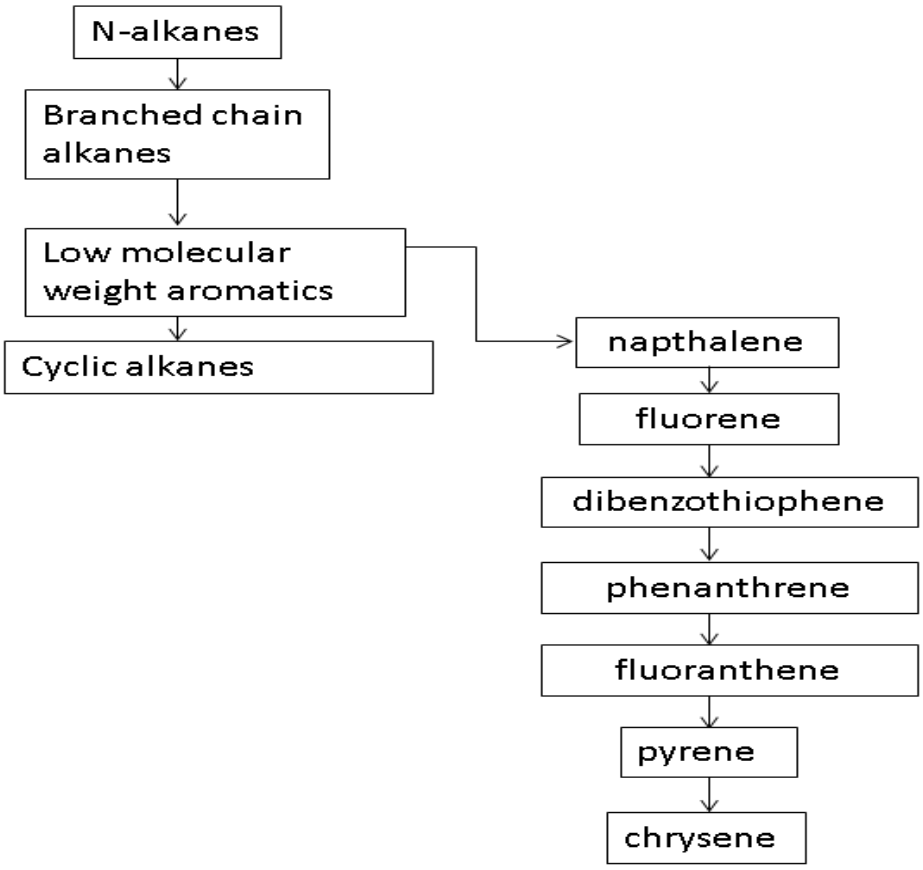


Figure 1.2: Hydrocarbon order of susceptibility to microbial attack. Adapted from Coulon et al. (2007) and Jain et al. (2011).

Microbes facilitate many vital processes in soil systems including nutrient cycling and decomposition of organic matter, and decreased microbial diversity has been shown to reduce the ability of soil systems to recover from perturbations (Griffiths et al., 2001). John et al. (2009) reported that the nitrogen-fixing bacterial populations of a wetland environment decreased as hydrocarbon concentrations increased. This population decrease was correlated with decreased levels of ammonium and nitrate, both vital forms of assimilable nitrogen for plant communities.

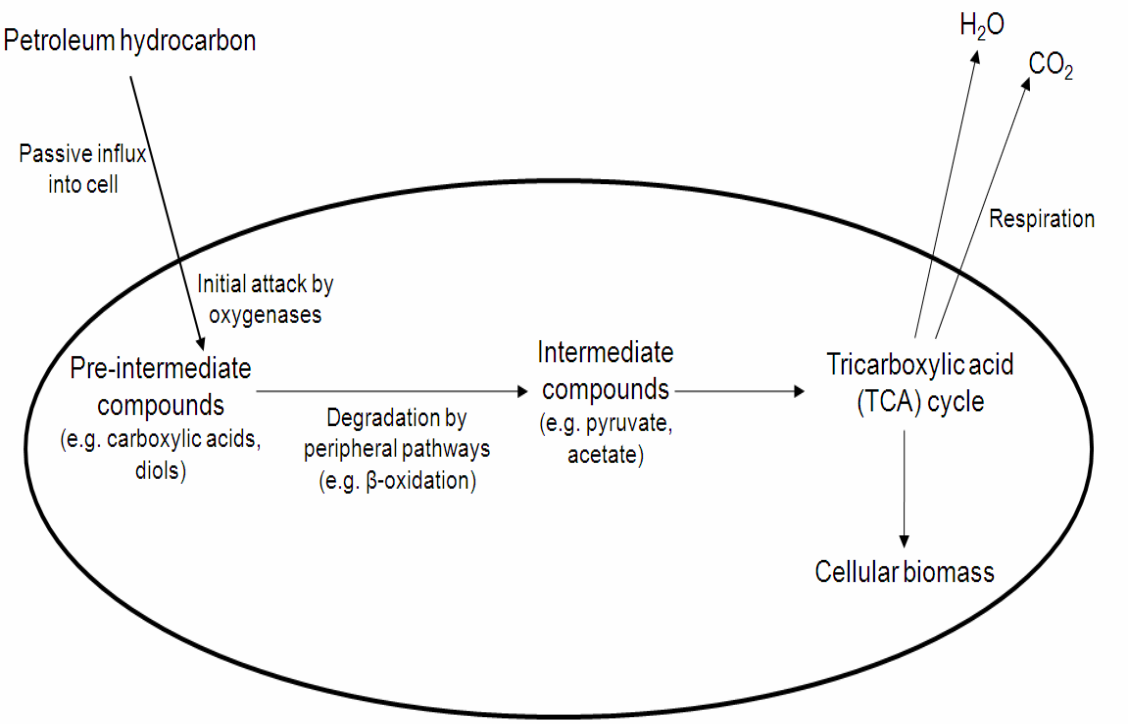


Figure 1.3: Overview of aerobic hydrocarbon degradation pathways in microorganisms. Adapted from Das and Chandran (2011).

In addition, some heavy metals, including chromium, nickel, copper, iron, lead, and vanadium, have been reported in elevated concentrations in a salt marsh following an oil spill (Andrande et al., 2004). Heavy metals have been shown to be toxic to microbes (Baath, 1989), and researchers have reported a range of effects on soil microbial communities, including decreased population numbers and decreased diversity of native species (Anyanwu et al., 2011; Iqbal et al., 2005).

The decreased diversity of microbial communities immediately following hydrocarbon contamination is generally accompanied by a significant increase in specialized hydrocarbon-utilizing bacteria in the soil. Kotska et al. (2011) have reported a significant response of bacterial communities to the *DWH* spill in beach sands in the northern Gulf of Mexico three to six months after the spill.

## 1.7 METHODS FOR ANALYZING BACTERIAL POPULATIONS IN HYDROCARBON-CONTAMINATED SOILS

Bacterial communities have traditionally been analyzed through culture-based techniques. Culture-based techniques begin with choosing a medium that will support bacterial growth. Optimal bacterial growth on media is dependent on the nutritional requirements of the bacteria. Therefore it is essential to understand the physiology of the bacteria being studied. Undefined media such as, nutrient broth, Luria-Bertani (LB) broth and tryptic broth are commonly used for the isolation, cultivation and maintenance of many wetland heterotrophic bacteria. Once a medium has been selected, environmental samples may be diluted and spread onto plates. Plates are then incubated, and after incubation colonies that grew on the plates are counted. Once colonies are counted, individual colonies isolated and purified. A number of phenotypic and genotypic methods can be employed to identify and classify isolated bacterial strains (Hou and Williams, 2013). The most precise method of determining the phylogenetic position of an isolate is through the analysis of nucleic acids. The 16S and 23S rRNA gene sequences are highly conserved between different species of bacteria, which makes these nucleic acid sequences useful as a measure of phylogenetic relationships of environmental isolates (Weisburg et al., 1991; Lane, 1991).

Isolating pure strains and comparing their PCR amplified 16S rRNA gene sequences with existing sequences in database can present a culture based account of the composition of a bacterial community (Torvik et al., 1996; Tuan et al., 2011; Ward et al., 1990). This method is widely used and has been successfully implemented in order to identify members of microbial populations in marine, estuarine, and riverine sediment following an oil spill (Alonso-Guitierrez et al., 2009; Kostka et al., 2011; Malik et al., 2008; Head et al., 2006; Greer, 2010).

### 1.7.1 Use of the Polymerase Chain Reaction (PCR) to Evaluate Microbial Populations

Molecular methods rely on the characterization of cellular components such as nucleic acids, proteins, fatty acids, and other taxon-specific compounds (Rossello-Mora and Amann, 2011). These molecules can be extracted directly from environmental samples, and analysis of the molecular composition can be used to understand the composition of the microbial community (Amann et al., 1995; Green and Voordouw, 2003). Culture independent methods should be employed to ascertain the microbial community structure, in areas like salt marshes, in order to guarantee that the most informative data are collected. The polymerase chain reaction has the ability to produce millions of copies of a portion of a desired gene, an entire gene, or gene clusters with high accuracy within 3 to 4 hours. PCR-based methods are also extremely helpful in the detection and quantification of microorganisms found in soil and water (Wilson et al., 1999). This technique, in recent years, has been employed for the analysis of catabolic genes involved in the biodegradation of organic pollutants (Wilson et al., 1999).

For the detection of organisms or genes from polluted environments, two variants of the PCR technique are often used: simple PCR and multiplex PCR. Simple PCR uses a pair of primers in a single amplification reaction, whereas multiplex PCR uses multiple pairs simultaneously to amplify several genes in a single reaction (Markoulatos et al., 2002).

PCR amplification is dependent on the extraction and purification of nucleic acids in sufficient amounts and of satisfactory quality from environmental samples. Insufficient lyses of cells could result in preferential extraction of DNA from Gram-negative bacteria, whereas very harsh treatments may result in shearing of DNA from readily lysed cells (Wintzingerode et al., 1997). Additionally, PCR amplification efficiency can be greatly hindered by the presence of inhibitory substances such as humic acids, organic matter, and clay particles that may be extracted with DNA in environmental samples.

# CHAPTER TWO: MATERIALS AND METHODS

A flowchart is shown in Figure 2.1 to illustrate the sequence of work for the current study.

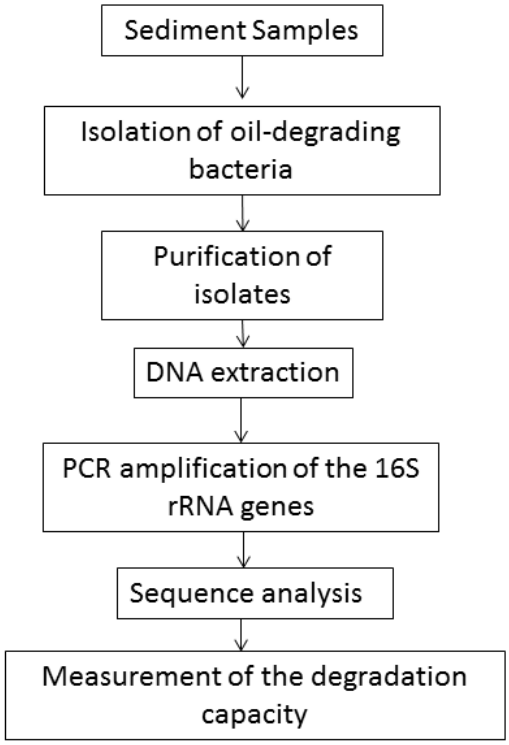


Figure 2.1: Flowchart of the current study

## 2.1 SAMPLING

### 2.1.1 Sampling sites

For the isolation of oil-degrading bacteria, marsh sediment samples were collected from Barataria Bay, Louisiana (Table 2.1). Sites were chosen based on their relative concentrations of oil. Reference sites were those with little visible oil. Moderately oiled marshes were those with

oil initially coating plant shoots partially or entirely, but oil concentrations in the soil of which were generally less than 200 mg $g^{-1}$. Heavily oiled marshes generally were those with oil coating shoots entirely. The shoots were in a horizontal position because the extra weight of the viscous, weathered oil prevented the shoots from returning to their vertical position. These heavily oiled marshes generally had soil oil concentrations greater than 300 mg $g^{-1}$, and there was little evidence of recovery 6-12 months after the landfall of the oil. Seven sites per oiling category were chosen within these three designated areas for a total of twenty-one sampling stations that covered an approximate area of 40 $km^2$ (Figure 2.3).

### 2.1.2 Sample Collection

Sediment samples were taken from the surface to a depth of two to three centimeters in April and November of 2012 and April 2013. The samples were placed in pre-autoclaved jars and stored in an ice chest while being transported back to the laboratory for microbial analysis. Upon arrival at the laboratory, they were stored at 4 °C for no more than twelve hours before being processed for bacterial isolation, and the remainder of the samples were stored at –20 °C or –80 °C for future DNA extraction or total petroleum hydrocarbons (TPH) analysis.

Total petroleum hydrocarbon (TPH) analysis of the sediment samples performed by the Louisiana State University Wetland Plant Ecology Laboratory quantified the extent of oil contamination and verified our visual observations of oil contamination. In April 2012, the average TPH concentrations at the RF, MD, and HV sites were 0.7 mg/g, 15.2 mg/g, and 161.8 mg/g, respectively. However, the concentrations within each sampling site were extremely variable. Within the seven reference sites, the values ranged from 0.1 mg/g to 1.6 mg/g. The medium-oiled site values ranged from 0.7 mg/g to 66.8 mg/g, and the heavily oiled sites ranged

in concentration from 6.2 mg/g to 296.3 mg/g. In November 2012, average TPH concentrations at the RF, MD, and HV sites were 0.2 mg/g, 4.1 mg/g, and 62.0 mg/g, respectively. The reference sites had TPH concentrations that ranged in value from 0.0 to 0.3 mg/g. The medium-oiled sites contained TPH concentrations in the range of 0.5 mg/g to 11.7 mg/g, and the heavily oiled sites ranged in concentration from 0.7 mg/g to 196.8 mg/g. Lastly, the April 2013 average TPH concentrations were 0.3 mg/g, 20.8 mg/g, and 151.8 for the RF, MD, and HV sites, respectively. TPH concentrations ranged from 0.0 to 0.6 mg/g at the reference sites, from 0.2 to 73.4 mg/g at the medium-oiled sites, and from 0.6 to 421.6 mg/g at the heavily oiled sites. Generally, there was a two-fold increase in the TPH concentration from reference sites to heavily oiled sites during all sampling dates.

## 2.2 ISOLATION OF OIL-DEGRADING BACTERIA

Sediment samples were homogenized thoroughly after removing roots, shells, and other debris in it with a sterile forcep. Then, one gram of sediment sample was suspended and vortexed in 10 mL of phosphate buffer solution. One hundred microliters of the solution was pipetted into Petri dishes containing Bushnell Haas (BH) agar (magnesium sulfate, 0.20 g/1000 mL; calcium chloride, 0.02 g/1000 mL; monopotassium phosphate 1.00 g/1000 mL; dipotassium phosphate 1.00 g/100 mL; ammounium nitrate 1.00 g/1000 mL; ferric chloride, 0.05 g/1000 mL; agar-agar, 15 g/1000 mL; pH 6.98-7.02) and sterilized 1% crude oil (v/v), which was spread onto the medium with a sterile, L-shaped rod. The BH agar plates were incubated at 37 °C for seven days. After seven days, bacterial colonies that had grown on the BH plates were subcultured at least twice in an effort to purify the isolates. Specifically, individual isolates on the BH plates were streaked onto Luria-Bertani (LB) agar (tryptone, 10 g/1000 mL, yeast extract, 5 g/1000 mL;

NaCl, 10 g/1000 mL; agar-agar, 20 g/1000 mL; pH 7–7.5) for purification. Purified bacterial isolates were then stored in a liquid medium stock consisting of liquid LB broth (tryptone 10 g; yeast extract 5.0 g and 10.0 g NaCl/1000 mL) and 20% (v/v) glycerol.

Table 2.1: Sample collection dates, site descriptors and site coordinates.

| SITE DATA | | |
|---|---|---|
| Sampling Date | Sample Sites | GPS coordinates |
| | RF1 | N29.46220 W89.92577 |
| April 2012 | RF2 | N29.46202 W89.92854 |
| October 2012 | RF3 | N29.46624 W89.93355 |
| April 2013 | RF4 | N29.46869 W89.93079 |
| | RF5 | N29.46926 W89.92708 |
| | RF6 | N29.47227 W89.92883 |
| | RF7 | N29.47495 W89.93376 |
| | MD1 | N29.46045 W89.92133 |
| | MD2 | N29.45439 W89.89770 |
| | MD3 | N29.45914 W89.94647 |
| | MD4 | N29.47042 W89.89387 |
| | MD5 | N29.46539 W89.91216 |
| | MD6 | N29.46095 W89.88492 |
| | MD7 | N29.44812 W89.92171 |
| | HV1 | N29.44060 W89.90432 |
| | HV2 | N29.45843 W89.90920 |
| | HV3 | N29.45563 W89.88975 |
| | HV4 | N29.45673 W89.88630 |
| | HV5 | N29.44893 W89.93904 |
| | HV6 | N29.44105 W89.93337 |
| | HV7 | N29.45087 W89.89541 |

## 2.3 DNA EXTRACTION, PCR OF 16S rRNA, AND SEQUENCING

Genomic DNAs of the isolates were extracted from bacterial cells via lysis of the cells by heating at 98 °C for ten minutes in a thermocycler (Bio-Rad iCycler™ MyiQ™ optical module,

Bio-Rad Laboratories, Hercules, CA, USA). The 16S rRNA genes were PCR-amplified with the 27F forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R reverse primer (5'-ACG GCT ACC TTG TTA CGA CTT-3' ) (Integrated DNA Technologies) (Weisburg et al. 1991). The PCR mixture contained 1μL of template DNA, 12.5 μL of GoTaq Green Master Mix (Promega), 2 μL of each 10 μM primers, and 7.5 μL of nucleus-free water. The DNA amplification was performed on a Bio-Rad iCycler iQ™ Real time PCR detection system using the following protocol: an initial denaturation step at 94 °C for five minutes, followed by 30 cycles of 95 °C for 45 seconds, 55 °C for one minute, and 72 °C for one minute, and a final annealing step of 72 °C for one minute. The quality of the DNA was confirmed by spectrophotometric analysis and gel electrophoresis. The A260/A280 method uses a spectrophotometer to measure the light absorbance of the DNA at 260 and 280 nm. This ratio is an accurate indicator of DNA purity, with pure DNA having an A260/A280 ratio of 1.8. The amplified DNA was verified with 1.5% agarose gel electrophoresis.

PCR products were then purified using ethanol precipitation. The purified samples were sent for sequencing to Louisiana State University's Genomics Facility (https://biosci-batzerlab.biology.lsu.edu/ Genomics). Sequences ranging between 850 and 1200 base pairs were blasted using the Standard Nucleotide NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE= Nucleotides).

## 2.4 MEASUREMENT OF BACTERIAL GROWTH

Ten isolates that had been stored in glycerol were revived and grown on slants of LB agar. Freshly prepared LB broth was autoclaved and placed in 30 autoclaved test tubes, each

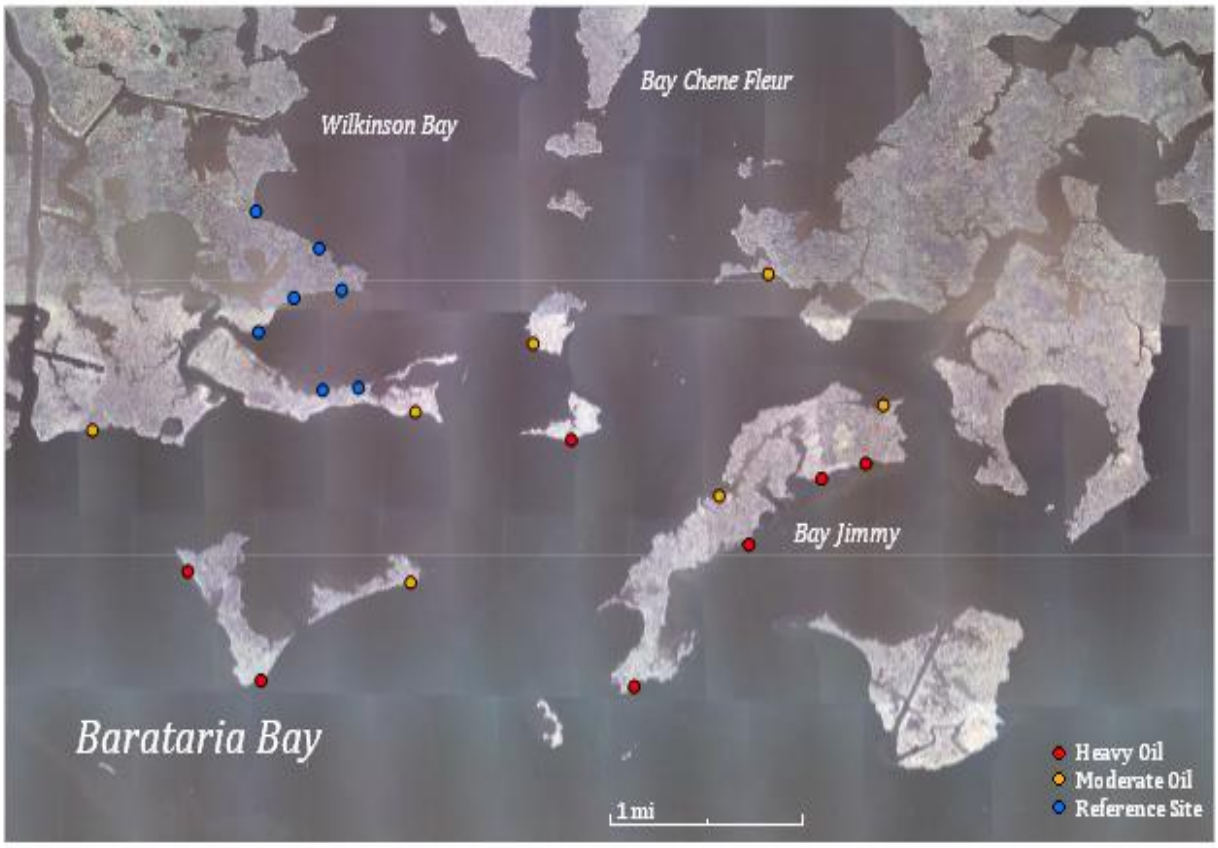


Figure 2.2: Map of sampling sites

containing 10 mL of LB broth and 1% (v/v) crude oil, which was added after autoclaving. The crude oil was sterilized using a syringe filter with a 0.2 μm pore size. Isolates were allowed to grow on the LB slants for three days, and then a loopful of isolated bacteria was inoculated into the 30 test tubes containing the LB broth and crude oil mixture. Three replicates were performed for each isolate. The samples were incubated at 36 °C with no shaking and no light. Samples were collected daily over a period of six days; pH and optical density at 600 nm were measured using a pH probe and a Thermo Scientific 2000 Nanodrop, respectively. A test tube containing only autoclaved LB broth and crude oil served as a control.

# CHAPTER THREE: RESULTS

## 3.1 PREDOMINANT OIL-DEGRADING BACTERIA IN MARSH SEDIMENT

### 3.1.1 Temporal patterns

A total of 460 bacterial colonies were isolated and sequenced. Taxonomic classification showed that these isolates belonged to 84 species in 45 genera under classes *Bacilli, Actinobacteridae, Gammaproteobacteria*, A*lphaproteobacteria*, *Betaproteobacteria* and *Flavobacteriia* in 4 phyla *Firmicutes, Actinobacteria, Bacteriodetes* and *Proteobacteria*. The isolates were then grouped by sampling dates to determine if time played a role in the composition of the community structure (Table 3).

In the April 2012 sampling, *Firmicutes* was the most abundant phylum at the RF, MD, and HV sites. It accounted for 67%, 37% and 51%, respectively, of the bacterial isolates at each oiling category. The second most abundant phylum was *Actinobacteria* at RF (23%), but *Proteobacteria* at MD (29%) and HV (31%). Within the phylum *Proteobacteria*, only two classes were identified, *Alphaproteobacteria* and *Gammaproteobacteria*. The relative abundance of *Alphaproteobacteria* at each site was 4% of reference samples, 10% of medium-oiled samples, and 12% of heavily oiled samples. *Gammaproteobacteria* represented 6% of reference samples, 19% of medium-oiled samples and 19% of heavily oiled samples. *Bacteriodetes*, class *Flavobacteriia*, were identified only in medium-oiled sites and accounted for 13% of the bacterial isolates at those sites (Table 3.1).

From April 2012 to October 2012, relative abundance of *Firmicutes* decreased from 67% to 39% at reference sites and from 51% to 41% in heavily oiled sites. However, relative abundance of *Firmicutes* in medium-oiled sites increased from 37% to 41%. *Proteobacteria* saw

an increase in relative abundance at all sites in October. At reference sites, relative abundance of *Proteobacteria* was 47%; in medium-oiled and heavily oiled sites relative abundance was 49% and 41%, respectively. *Gammaproteobacteria* relative abundance increased from April 2012 to October 2012. *Gammaproteobacteria* made up at least 40% of the isolates collected at all sites in October. The relative abundance of *Actinobacteria* at all sampling sites was between 10 % and 14%, a decrease in relative abundance from April 2012, which averaged 20%. October was the first sampling date when *Bacteriodetes* was present at reference and heavily oiled sites. However, the relative abundance of *Bacteriodetes* decreased from 13% to 5% in October.

During the April 2013 sampling, all classes of bacteria identified were present at every site, except for *Flavobacteriia* in heavily oiled samples. The relative abundance of *Firmicutes* decreased from 39% to 25% at reference sites and from 41% to 38% at heavily oiled sites after October 2012. However, the relative abundance of *Firmicutes* in medium-oiled sites increased from 41% to 48% after October 2012. The relative abundance of *Actinobacteria* increased from 10% to 27% at reference sites, but decreased from 14% to 8% at medium-oiled sites and from 14% to 13% at heavily oiled sites. *Gammaproteobacteria* continued to be the largest class of *Proteobacteria* in abundance. Relative abundance of *Gammaproteobacteria* did not change much from October 2012 to April 2013 at reference and heavily oiled sites, even though medium-oiled sites saw a drop—from 40% in October to 28% in April 2013. Overall relative abundance of *Alphaproteobacteria*, *Betaproteobacteria*, and *Bacteriodetes* increased from October 2012 to April 2013 (Table 3.1).

3.2.2. Effects of oiling

Once temporal patterns had been observed, the bacterial isolates were then pooled according to oiling category to determine the effects that different levels of oiling may have had on the oil-degrading bacterial communities. In a comparison of phyla, all sites had very similar abundances of *Firmicutes, Actinobacteria, Proteobacteria and Bacteriodetes*. At reference sites the relative abundance of phyla was 43% *Firmicutes*, 21% *Actinobacteria*, 34% *Proteobacteria*, and 3% *Bacteriodetes*.

Table 3.1: Relative abundance of phyla and classes at each sampling date and oiling category

| Sampling Date | Oiling category | Phyla | *Firmicutes* | *Actinobacteria* | *Proteobacteria* | | | Bacteriodetes |
|---|---|---|---|---|---|---|---|---|
| | | Class | *Bacilli* | *Actinobacteridae* | *γ-Proteo* | *α-Proteo* | *β-Proteo* | Flavobacteriia |
| Apr. 2012 | RF | | 67 | 23 | 6 | 4 | 0 | 0 |
| | MD | | 37 | 21 | 19 | 10 | 0 | 13 |
| | HV | | 51 | 18 | 19 | 12 | 0 | 0 |
| | | | | | | | | |
| Oct. 2012 | RF | | 39 | 10 | 47 | 0 | 0 | 4 |
| | MD | | 41 | 14 | 40 | 9 | 0 | 5 |
| | HV | | 41 | 14 | 41 | 0 | 0 | 2 |
| | | | | | | | | |
| Apr. 2013 | RF | | 25 | 27 | 41 | 2 | 2 | 3 |
| | MD | | 46 | 8 | 28 | 8 | 4 | 6 |
| | HV | | 38 | 13 | 45 | 2 | 2 | 0 |

*Numbers indicate relative abundance.

The phyla relative abundance in medium-oiled sites was 43% *Firmicutes*, 13% *Actinobacteria*, 37% *Proteobacteria*, and 7% *Bacteriodetes*. In heavily oiled sites the composition was 45% *Firmicutes*, 14% *Actinobacteria*, 40% *Proteobacteria*, and 1% *Bacteriodetes* (Figure 3.1).

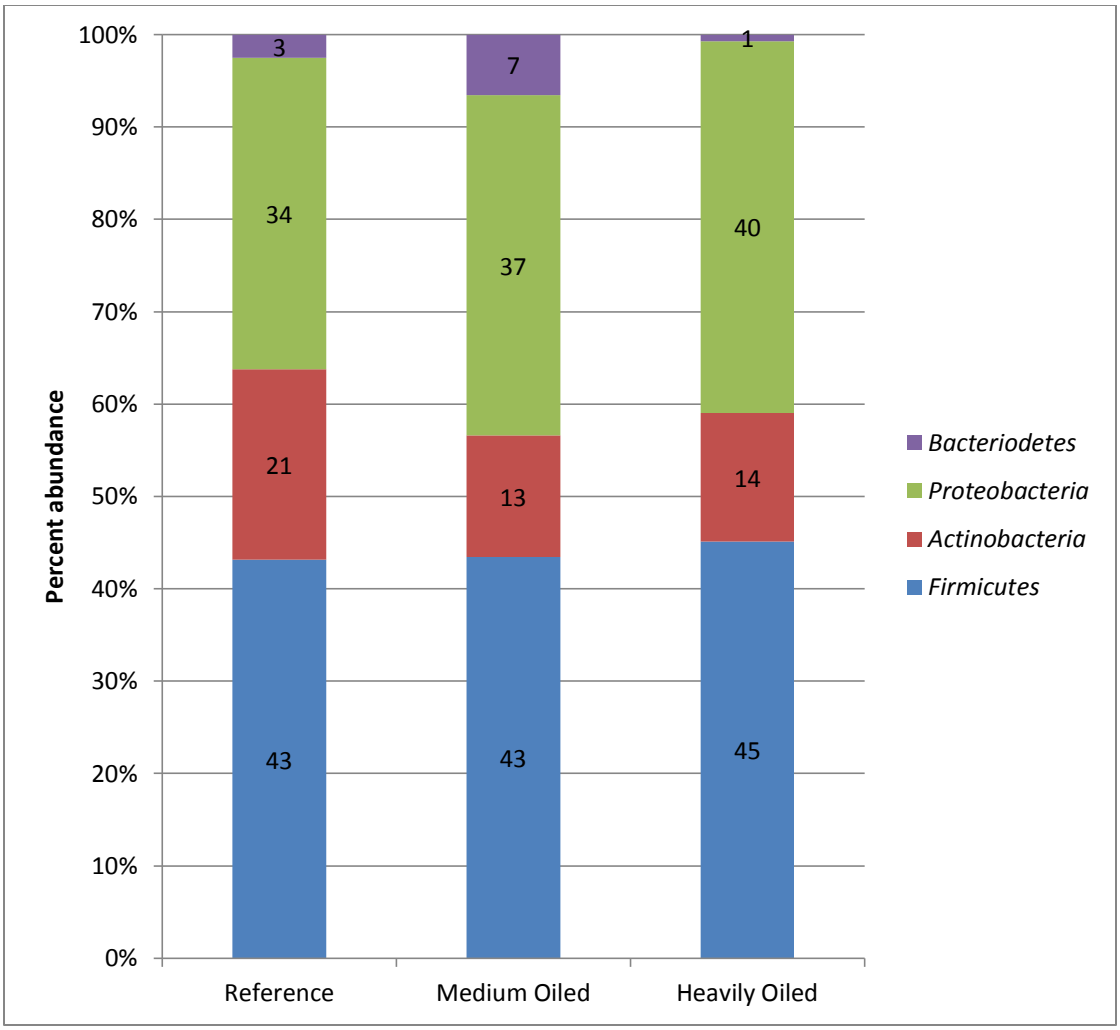


Figure 3.1: Relative abundance of bacterial phyla at each oiling category

Taking a more in-depth look at the bacterial community structure, relative abundance of the orders of the bacteria at each oiling category is illustrated in Figure 3.2. *Bacillales* was the most abundant order observed at all three oiling categories, making up 43% at reference sites, 42% at medium-oiled sites, and 45% at heavily oiled sites. The most abundant family within the order *Bacillales* was *Bacillaceae*. *Bacillus sp*., a well-known gram-positive aromatic degrader, lies within the family *Bacillace*ae. The next most abundant orders were *Actinomycetales* and *Pseudomonadales*. The relative abundance of *Actinomycetales* was 21% at reference sites, 13% at medium-oiled sites, and 14% at heavily oiled sites. The most abundant families within

*Actinomycetales* were *Microbacteriaceae* and *Nocardiaceae* (Table 3.2). *Micrococcus sp.* and *Mycobacterium sp.* are two examples of gram-positive aromatic degraders found within the order *Actinomycetales*. The relative abundance of *Pseudomonadales* was 18% at reference sites, 16% at medium-oiled sites, and 28% at heavily oiled sites. Within the order *Pseudomonadales*, the most abundant family was *Pseudomonadaceae*, and the most abundant genus was *Pseudomonas* (Table 3.2). *Pseudomonas sp.* is a gram-negative, oil-degrading bacterium that can degrade alkanes and aromatics. *Bacillales*, *Actinomycetales*, and *Pseudomonas* together make up 82% of the isolates at reference sites, 71% of the isolates at medium-oiled sites, and 87% of the isolates at heavily oiled sites. *Enterobacteriales*, which consists of genera such as *Shigella* and *Cronobacter*, constituted 9% of isolates in reference site samples and 8% of isolates from medium-oiled site samples. Relative abundance of *Flavobacteriales* was 3% at reference sites and 7% at medium-oiled sites. Reference sites did not have the following orders: *Vibrionales*, *Rhodobacterales*, and *Caulobacterales*. The order *Oceanospirillales* was not detected at medium-oiled sites. The orders *Lactobacillales*, *Aermonadales*, *Sphingomonadales*, *Rhodobacterales*, and *Caulobacterales* were not detected at heavily oiled samples.

## 3.2 CRUDE OIL UTILIZATION BY ISOLATES

### 3.2.1 Optical density values and TPH

Differing turbidities and oil dispersion patterns were observed in the test tube cultures over the course of six days (Figure 3.5). This fact further confirms that the isolated bacterial strains were able to grow in the presence of crude oil. At no point during the six days of experimentation did the control ever appear turbid.

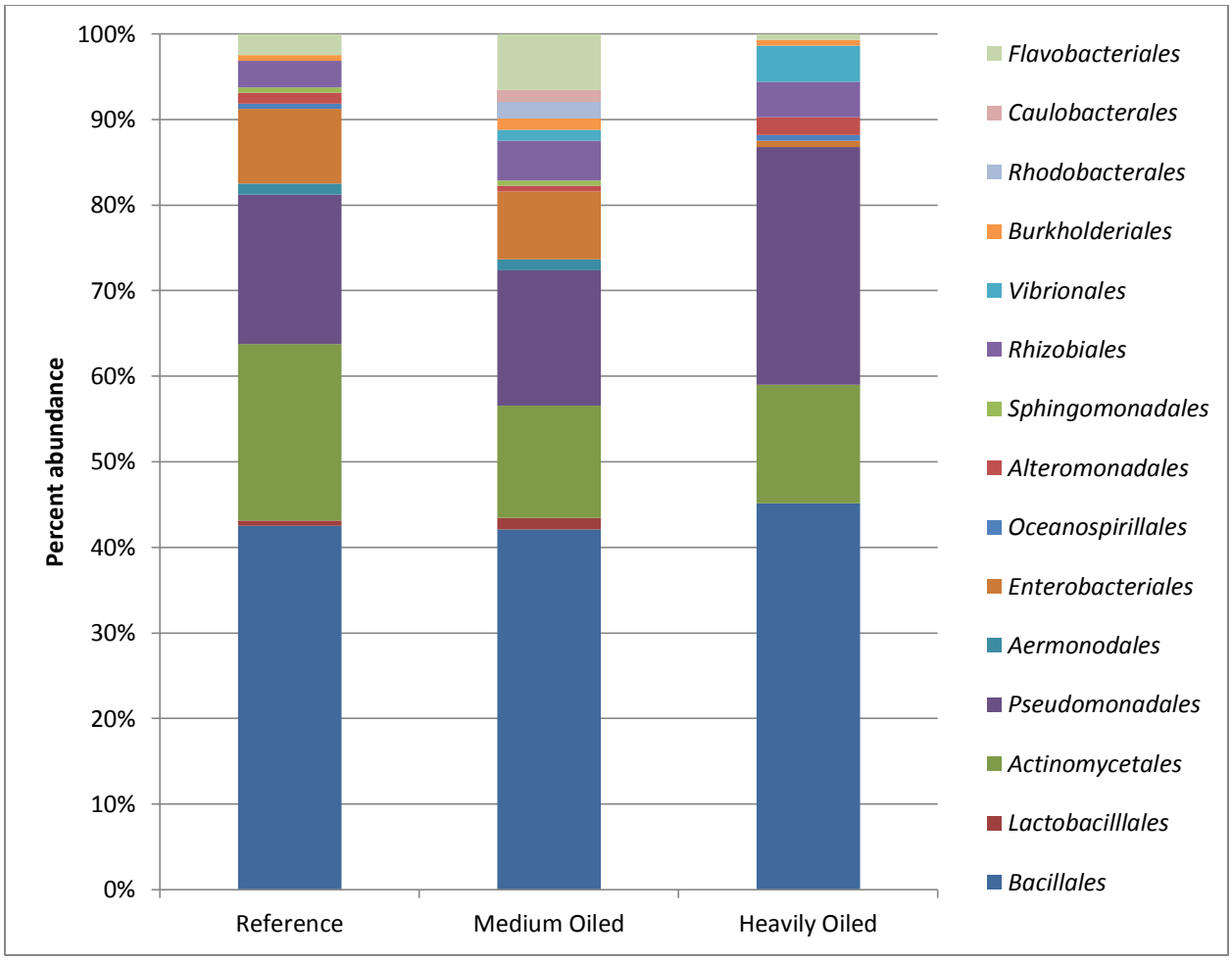


Figure 3.2: Relative abundance of bacterial orders at each oiling category

In the control test tubes, there was a distinct layer between the oil and water interfaces, the suggestion being that little to no oil dispersion had taken place (Figure 3.4). The changes in optical density (Figure 3.3) and pH (Figure 3.5) suggest that for most of the isolates tested, bacterial growth entered the exponential phase quickly after the incubation started and reached its peak by day three or four, after which the growth became slower and decreased after 96 hours. The peak values for the ten isolates were 0.101±0.016 for *Staphylococcus xylosus*, 0.099±0.001 for *Acinetobacter calcoaeticus*, 0.071±0.019 for *Pseudomonas stutzeri*, 0.125±0.016 for *Bacillus pumilus*, 0.187±0.008 for *Micrococcus luteus*, 0.126±0.011 for *Rhodococcus equi*, 0.144±0.007 for *Microbacterium esteratomaticum*, 0.148±0.011 for *Vibrio* sp., 0.068±0.005 for *Pseudomonas*

Table 3.2: Taxonomic classification of bacterial isolates

| Site | Phyla | Class | Order | Family | No. of isolates |
|---|---|---|---|---|---|
| | *Firmicutes* | *Bacilli* | *Bacillales* | *Bacillaceae* | 64 |
| RF | | | | *Staphylococcaceae* | 1 |
| | | | | *Paenibacillacea* | 2 |
| | | | | *Bacillales Family XII Incertae Sedis* | 1 |
| | | | *Lactobacilllales* | *Streptococcaceae* | 1 |
| | *Actinobacteria* | *Actinobacteridae* | *Actinomycetales* | *Microbacteriaceae* | 20 |
| | | | | *Streptomycetaceae* | 8 |
| | | | | *Mycobacteriaceae* | 2 |
| | | | | *Nocardiaceae* | 2 |
| | | | | *Micromonosporaceae* | 1 |
| | *Proteobacteria* | *Gammaproteo* | *Pseudomonadales* | *Pseudomonadaceae* | 25 |
| | | | | *Moraxellaceae* | 3 |
| | | | *Enterobacteriales* | *Enteriobacteriaceae* | 14 |
| | | | *Oceanospirillales* | *Halomonadaceae* | 1 |
| | | | *Alteromonadales* | *Alteromonadaceae* | 1 |
| | | | | *Shewanellaceae* | 1 |
| | | | *Aermonodales* | *Aeromonadaceae* | 2 |
| | | *Alphaproteo* | *Rhizobiales* | *Hyphomicrobiaceae* | 1 |
| | | | | *Rhizobiaceae* | 1 |
| | | | | *Xanthomonadaceae* | 3 |
| | | | *Sphingomonadales* | *Sphingomonadaceae* | 1 |
| | | *Betaproteo* | *Burkholderiales* | *Alcaligenaceae* | 1 |
| | *Bacteriodetes* | *Flavobacteriia* | *Flavobacteriales* | *Flavobacteriaceae* | 4 |
| MD | *Fimicutes* | *Bacilli* | *Bacillales* | *Bacillaceae* | 59 |
| | | | | *Paenibacillaceae* | 2 |
| | | | | *Bacillales Family XII Incertae Sedis* | 2 |
| | | | | *Staphylococcaceae* | 1 |
| | | | *Lactobacillales* | *Lactobacillaceae* | 1 |
| | | | | *Streptococcaceae* | 1 |
| | *Actinobacteria* | *Actinobacteridae* | *Actinomycetales* | *Microbacteriaceae* | 10 |
| | | | | *Nocardiaceae* | 3 |
| | | | | *Mycobacteriaceae* | 1 |
| | | | | *Streptomycetaceae* | 4 |
| | | | | *Micrococcaceae* | 2 |
| | *Proteobacteria* | *Gammaproteo* | *Pseudomonadales* | *Pseudomonadaceae* | 22 |
| | | | | *Moraxellaceae* | 2 |
| | | | *Aeromonadales* | *Aeromonadaceae* | 2 |
| | | | *Enterobacteriales* | *Enterobacteriaceae* | 12 |
| | | | *Alteromonadales* | *Pseudoalteromonadaceae* | 1 |
| | | | *Vibrionales* | *Vibrionaceae* | 2 |
| | | *Alphaproteo* | *Rhizobiales* | *Xanthobacteraceae* | 5 |
| | | | | *Hyphomicrobiaceae* | 1 |
| | | | | *Phyllobacteriaceae* | 1 |

Table 3.2: Continued

| Site | Phyla | Class | Order | Family | No. of isolates |
|---|---|---|---|---|---|
| MD | | | *Sphingomonadales* | *Sphingomonadaceae* | 1 |
| | | | *Rhodobacterales* | *Rhodobacteraceae* | 3 |
| | | | *Caulobacterales* | *Caulobacteraceae* | 2 |
| | | *Betaproteo* | *Burkholderiales* | *Burkholderiaceae* | 1 |
| | | | | *Alcaligenaceae* | 1 |
| | *Bacteriodetes* | *Flavobacteriia* | *Flavobacteriales* | *Flavobacteriaceae* | 10 |
| HV | *Firmicutes* | *Bacilli* | *Bacillales* | *Bacillaceae* | 53 |
| | | | | *Paenibacillaceae* | 4 |
| | | | | *Bacillales Family XII Incertae Sedis* | 7 |
| | | | | *Staphylococcaceae* | 1 |
| | *Actionbacteria* | *Actinobacteridae* | *Actinomycetales* | *Microbacteriaceae* | 9 |
| | | | | *Streptomycetaceae* | 1 |
| | | | | *Norcardiaceae* | 10 |
| | *Proteobacteria* | *Gammaproteo* | *Pseudomonadales* | *Pseudomonadaceae* | 36 |
| | | | | *Moraxellaceae* | 4 |
| | | | *Vibrionales* | *Vibrionaceae* | 6 |
| | | | *Enterobacteriales* | *Enterobacteriaceae* | 1 |
| | | | *Oceanospirillales* | *Alcanivoracaceae* | 1 |
| | | | *Alteromonadales* | *Pseudoalteromonadaceae* | 3 |
| | | *Alphaproteo* | *Rhizobiales* | *Rhizobiaceae* | 3 |
| | | | | *Xanthobacteraceae* | 1 |
| | | | | *Hyphomicrobiaceae* | 1 |
| | | | | *Rhodobiaceae* | 1 |
| | | *Betaproteo* | *Burkholderiales* | *Comamonadaceae* | 1 |
| | *Bacteriodetes* | *Flavobacteriia* | *Flavobacteriales* | *Flavobacteriaceae* | 1 |

*mendocina*, and 0.068±0.008 for *Agromyces aurantiacus*. Among all tested strains, *M. luteus, Vibrio* sp., *B. pumilus, R. equi, and M. esteratomaticum* grew the fastest (Figure 3.3).

*A. aurantiacus* and *A. calcoaeticus* were isolated from reference sites in April 2012. *P. stutzeri, R. equi*, and *Vibrio* sp. were isolated from heavily oiled sites in April 2012. *B. pumilus* was isolated from a reference site in November 2012. *M. luteus* was isolated from a medium-oiled site in November 2012. *S. xylosus, M. esteratomaticum* and *P. mendocina* were all isolated from heavily oiled sites in November. It appeared that in general the isolates that were

able to degrade crude oil at elevated rates were typically obtained from sites that contained higher levels of TPH (medium and heavily oiled sites), whereas the slowest degraders were typically from reference sites. For example, the fastest degraders, *Micrococcus luteus*, *Rhodococcus equi*, *Microbacterium esteratomaticum*, and *Vibrio* sp., were isolated from MD and HV sites, whereas the slowest degrader, *Agromyces aurantiacus*, was isolated from an RF site (Table 3.3).

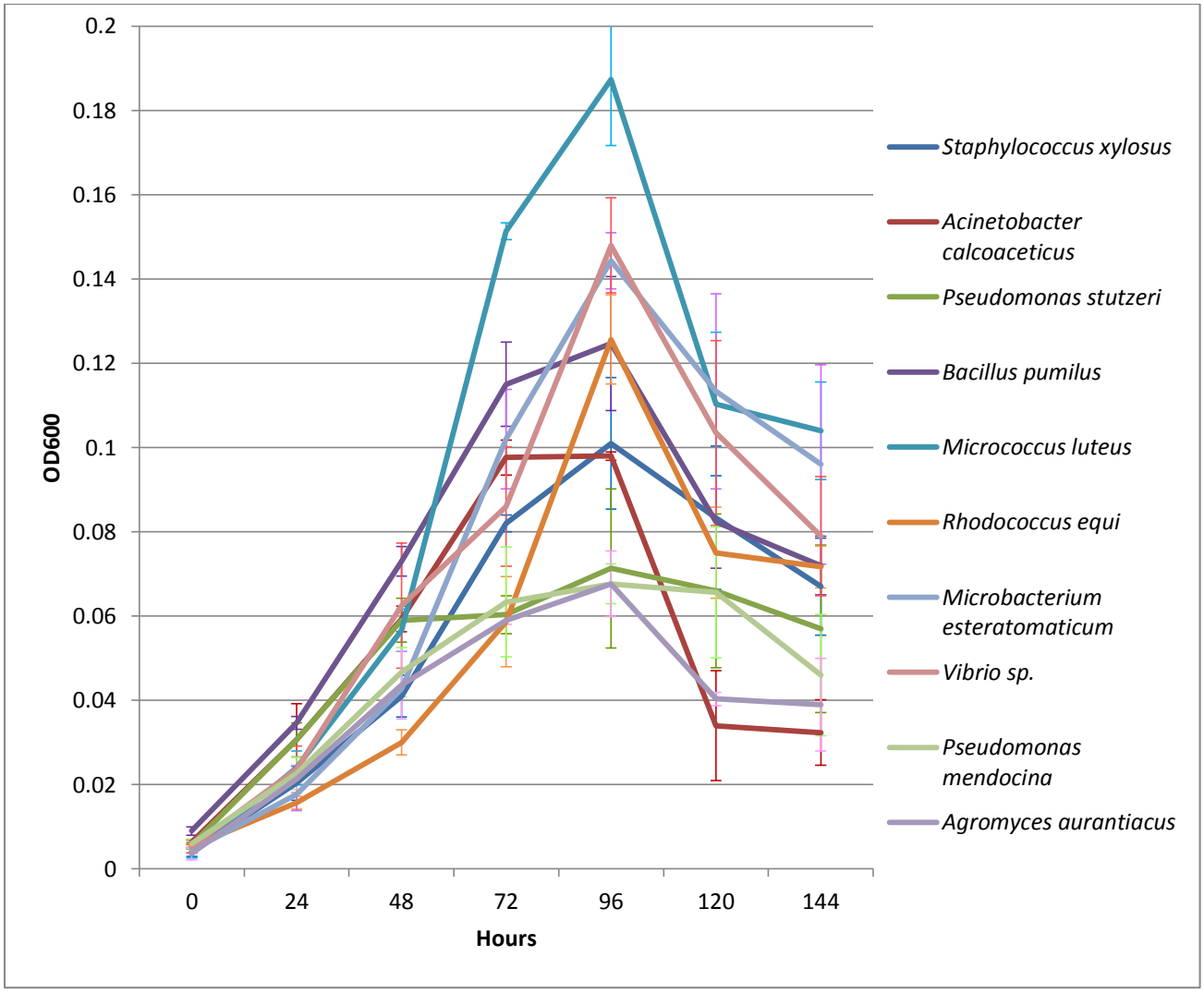


Figure 3.3: Growth curves of pure cultures

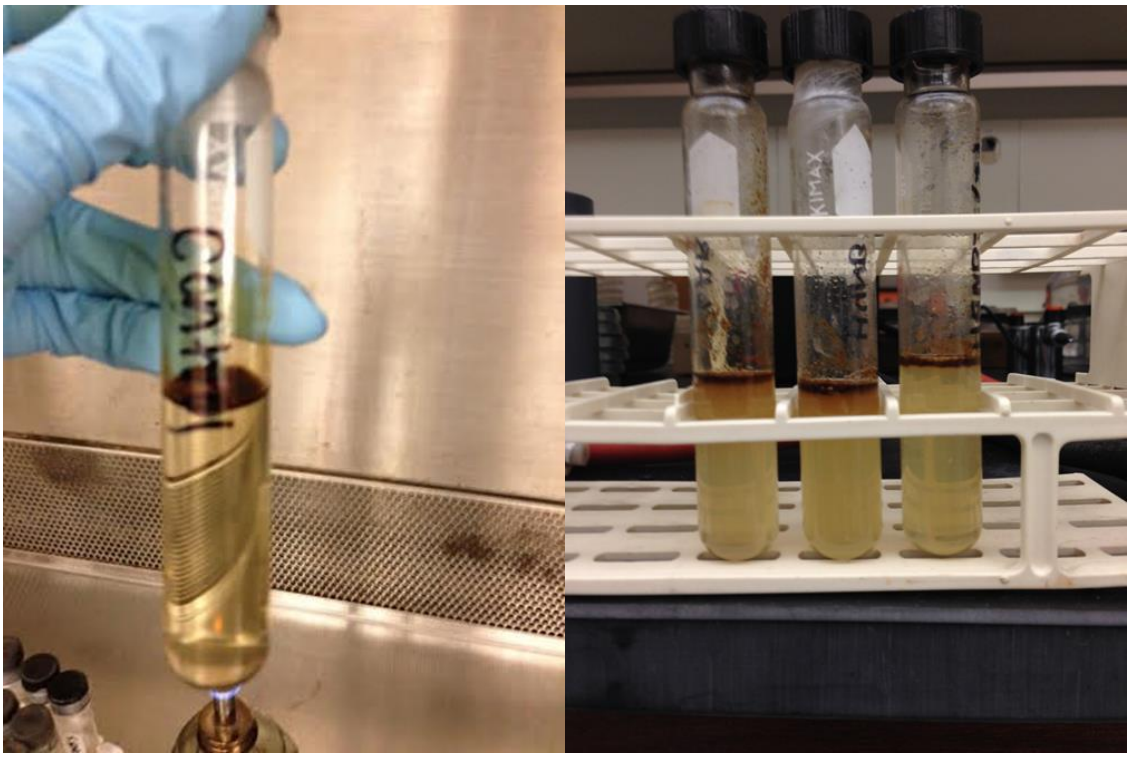

Figure 3.4: Appearance of culture medium after six days of incubation. Differences in the oil dispersion patterns and turbidity between the control and bacterial cultures were apparent.

Table 3.3: Comparison of OD600 values of tube cultures and TPH concentrations of sampling sites among tested strains.

| Isolate ID* | Strain | peak OD600 value | TPH concentration (mg/g) |
|---|---|---|---|
| R3A | *A. calcoaeticus* | 0.099±0.001 | 1.6 |
| R6A | *A. aurantiacus* | 0.068±0.008 | 0.5 |
| H2A | *Vibrio sp.* | 0.148±0.011 | 106.3 |
| H4A | *R. equi* | 0.126±0.011 | 267.6 |
| H5A | *P. stutzeri* | 0.071±0.019 | 6.2 |
| R7O | *B. pumilus* | 0.125±0.016 | 0.3 |
| M6O | *M. luteus* | 0.187±0.008 | 11.7 |
| H4O | *S. xylosus* | 0.101±0.016 | 76.8 |
| H4O | *M. esteratomaticum* | 0.144±0.007 | 76.8 |
| H5O | *P. mendocina* | 0.068±0.005 | 16.8 |

*The first letter represents the site type (RF, MD, or HV), the number corresponds to the given site within an oiling category, and the second letter represents the month in which the tested strains were isolated (April 2012 and October 2012)

3.2.2 Optical density and growth rates

Growth rates of all isolates ranged from 0.021 $h^{-1}$ and 0.038 $h^{-1}$. *M. luteus* had the highest growth rate of 0.038 $h^{-1}$. The species *P. mendocina* and *A. aurantiacus* had the lowest growth rate of 0.021 $h^{-1}$. Table 3.4 shows the growth rates for all tested bacterial isolates as well as their optical density values from 0 to 144 hours of incubation.

Table 3.4: Bacterial growth rates and optical density values in tube culture

| Isolate strain | OD at 600 nm | | | | | | | Growth rate ($h^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| | 0 hr | 24 hr | 48 hr | 72 hr | 96 hr | 120 hr | 144 hr | |
| *S. xylosus* | 0.004±0.001 | 0.02±0.004 | 0.041±0.005 | 0.082±0.002 | 0.091±0.029 | 0.083±0.017 | 0.067±0.012 | 0.029 |
| *A. calcoaetious* | 0.006±0.001 | 0.031±0.009 | 0.059±0.003 | 0.098±0.004 | 0.098±0.001 | 0.034±0.013 | 0.032±0.008 | 0.028 |
| *P. stutzeri* | 0.006±0.001 | 0.031±0.004 | 0.059±0.005 | 0.060±0.005 | 0.071±0.019 | 0.064±0.029 | 0.050±0.026 | 0.027 |
| *B. pumilus* | 0.009±0.001 | 0.059±0.004 | 0.111±0.018 | 0.115±0.010 | 0.125±0.016 | 0.082±0.011 | 0.072±0.007 | 0.031 |
| *M. luteus* | 0.004±0.001 | 0.024±0.004 | 0.057±0.016 | 0.145±0.028 | 0.187±0.061 | 0.110±0.008 | 0.104±0.018 | 0.038 |
| *R. equi* | 0.005±0.001 | 0.016±0.002 | 0.030±0.003 | 0.059±0.011 | 0.119±0.021 | 0.075±0.011 | 0.072±0.007 | 0.029 |
| *M. esteratomaticum* | 0.004±0.001 | 0.018±0.004 | 0.043±0.001 | 0.102±0.012 | 0.177±0.044 | 0.113±0.023 | 0.083±0.039 | 0.037 |
| *Vibrio sp.* | 0.005±0.002 | 0.024±0.006 | 0.068±0.022 | 0.086±0.014 | 0.148±0.088 | 0.099±0.029 | 0.081±0.026 | 0.037 |
| *P. mendocina* | 0.006±0.001 | 0.023±0.004 | 0.047±0.006 | 0.063±0.013 | 0.064±0.009 | 0.059±0.022 | 0.046±0.014 | 0.021 |
| *A. aurantiacus* | 0.004±0.002 | 0.021±0.003 | 0.044±0.008 | 0.059±0.001 | 0.068±0.008 | 0.040±0.002 | 0.039±0.011 | 0.021 |

3.2.3 pH of bacterial culture in test tubes

The pH values of all ten bacterial cultures differed greatly during the course of the six-day testing period, while the control stayed at about the same pH over 6 days. This was to be expected because no biodegradation should have occurred in the control tubes. All isolates in the LB broth amended with 1% crude oil at time zero were near a neutral pH. They reached their lowest pH around day four. *S. xylosus*, *P. stutzeri*, and *M. esteratomaticum* achieved the lowest pH values, 5.40, 5.90, and 5.29, respectively. *A. calcoaetiacus*, *B. pumilus*, *M. luteus*, and *R. equi* had the highest pH values, 6.35, 6.35, 6.45 and 6.50, on the fourth day. Six bacterial isolates saw a slight increase in pH values after day 4, and the other four isolates saw a slight decrease in pH values. However, pH values after day 4 only increased or decreased by an average of 0.06 (Figure 3.5).

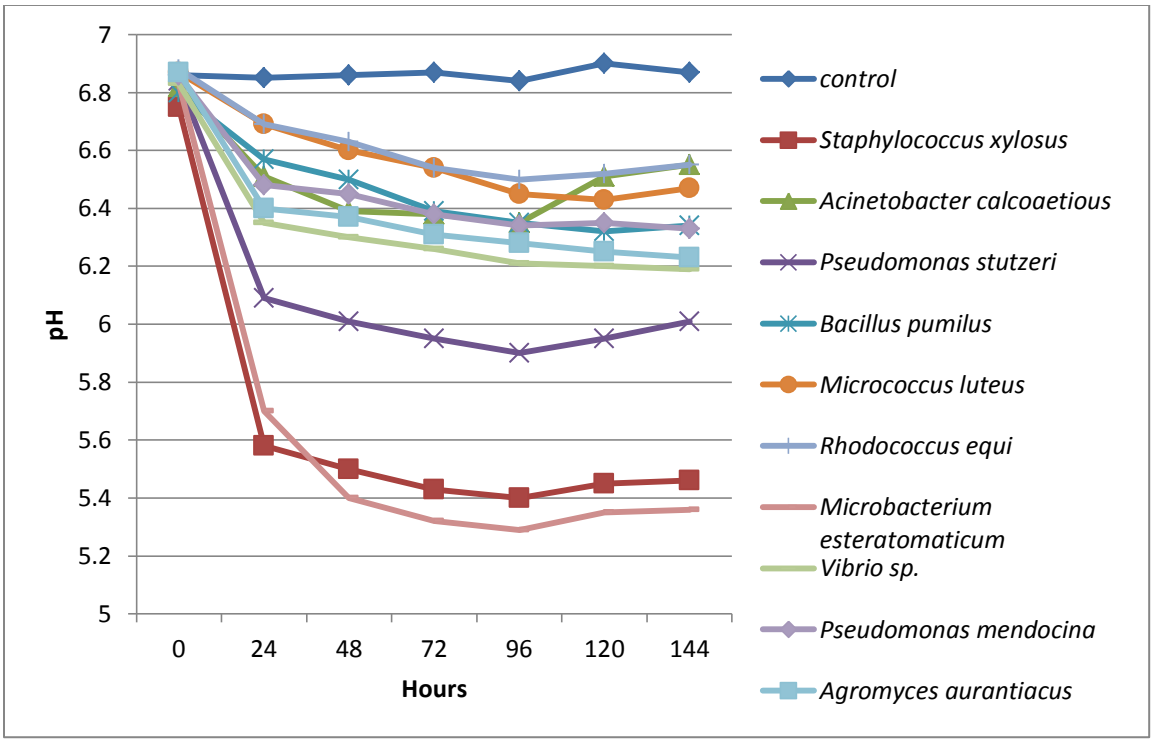


Figure 3.5: Changes in pH of culture medium by bacterial isolates during their utilization of crude oil

# CHAPTER FOUR: DISCUSSION

## 4.1 ISOLATION AND SEQUENCING OF HYDROCARBON-DEGRADING BACTERIA

A total of 460 bacterial isolates were isolated from crude-oil-enriched Bushnell Haas (BH) agar plates that were incubated for one week. The isolates were ruled as hydrocarbon degraders because they were able to grow on BH agar in the absence of any other carbon substrates except for crude oil.

It has been established that the compositions of bacterial populations following contamination are dynamic and that changes are episodic (Alonso-Gutierrez et al., 2009; Atlas, 1981; Hui, 2011; Kotska et al., 2011). As bacterial communities evolve to cope with hydrocarbon-induced stresses, a new community structure will form (Macnaughton, 1999; Paisse et al., 2008.). The results of this study suggest that the population of oil-degrading bacteria two and three years after the BP oil spill contained fewer n-alkane degraders and relatively more PAH degraders.

Though gram-positive aromatic degraders were dominant throughout the study, there were increasing numbers of *Bacteriodetes* and *Proteobacteria*, especially *Gammaproteobacteria*, in October 2012 and April 2013. The occurrence of these bacteria may be indicative of later stages of hydrocarbon degradation. Polycyclic aromatics are degraded more slowly than straight or branched-chain alkanes, and they are believed to be some of the most toxic compounds within crude oil (Perry, 1984; Jain et al., 2011). Jimnez et al. (2011) observed a shift in community structure that was correlated with the chronological degradation of components of crude oil. Bacteria that were most efficient at degrading alkanes were succeeded by bacteria that were capable of degrading aromatics. Many *Proteobacteria* are known to be PAH degraders; these

bacteria include *Vibrio sp*., *Pseudomonas sp*., *Sphingomonas sp.,* and *Acinetobacteria sp*. *Flavobacteria* is a well-known hydrocarbon-degrading genus from the phylum *Bacteriodetes* (Jacques et al., 2007; Hemalatha and Veermanikandan, 2011; Tanase et al., 2012; Throne-Holst et al., 2007).

The dominance of gram-positive bacteria throughout the study, most of which are PAH degraders, also supports the idea of later degradation of crude oil. Approximately three-fourths of 150 isolates from April 2012 samples were gram-positive, with 52% of the bacterial colonies belonging to the phylum *Firmicutes* and 20% belonging to the phylum *Actinobacteria.* The genus *Bacillus* was by far the most prevalent member of the community and constituted 48% of the sequences isolated from April 2012. Fifty-four percent of 150 isolates from November 2012 were gram-positive, with 41% of the sequences belonging to the phylum *Firmicutes,* and 13% belonging to the phylum *Actinobacteria.* Fifty-two percent of 160 isolates from April 2013 were gram-positive, with 36% of the sequences belonging to the phylum *Firmicutes,* and 16% belonging to the phylum *Actinobacteria.* Kotska et al. (2011) have reported a shift from alkane-degrading bacteria of the genus *Alcanivorax* toward gram-positive oil degraders, such as *Bacillus sp.* and *Microbacterium sp,* in a study of Florida beach sands following the BP oil spill. Zhuang et al. (2003) stated that at least 69% of the population of naphthalene-degrading bacteria they observed in oil that impacted tropical coast sediments was gram-positive. These two studies suggest that the shift from n-alkanes to PAHs is indicative of later stages of biological degradation of hydrocarbon-contaminated soils. Beazely et al. (2012) suggest that high percentages of *Firmicutes* in oil-contaminated salt marshes could be used as an indicator of the later stages of hydrocarbon degradation. These studies suggest that the trend of bacteria switching from n-alkanes to PAHs as energy sources is indicative of the later stages of

biological degradation of hydrocarbon-contaminated soils. The prevalence of gram-positive aromatic degraders in this study was most likely due to the dynamic environment of the sampling sites. Hydrocarbon-degrading bacteria that live in salt marshes must be able to withstand changes in pH, salinity, temperature, moisture, and oxygen concentration in order to survive. Gram-positive bacteria have a peptidoglycan cellular envelope, which makes them less susceptible to physical disturbance in salt marsh and intertidal areas than gram-negative bacteria (Alonso-Guitiettez et al., 2009).

Gas chromatography/mass spectrometry data were pooled from four reference sites and four heavily oiled sites in Bay Jimmy in February 2012 (Table 4.1). Bay Jimmy is close to the Barataria Bay sites in my study, and environmental conditions in Bay Jimmy are similar; GC/MS data should therefore be similar in Bay Jimmy and Barataria Bay. A little less than two years after the spill, many low-molecular-weight alkanes and aromatics had been degraded at reference and heavily oiled sites. As the lower molecular weight alkanes were exhausted, bacteria that could utilize higher weight and more complex molecular compounds began to thrive. This pattern is evident from the predominance of PAH-degraders found in Barataria Bay in April 2012 through April 2013. The latest TPH data collected from the Barataria Bay sites indicate that a large concentration of oil is still present in the soil. This is most likely due to the fact that the hydrocarbon compounds still found in the soil are extremely recalcitrant and may take a very long time to degrade.

## 4.2 HYDROCARBON UTILIZATION

Turbidity in the culture test tubes was observed (Figure 3.4), and the optical densities of the tubes were measured. Due to the fact that samples became more turbid over a given time, it

was postulated that the bacteria were able to grow in LB broth supplemented with crude oil. *M. esteratomaticum*, *Vibrio* sp., *M. luteus*, and *B. pumilus* have shown the best ability to utilize and degrade crude oil, as determined by their optical densities and growth rates. *A. auranticus* was the least efficient at utilizing crude oil as an energy source. These bacterial strains were isolated from areas with medium to heavily oiling, and because of this they may be able to degrade hydrocarbons more efficiently than isolates found at reference sites. However, reference sites in Barataria Bay still have high TPH concentrations compared to concentrations reported in other studies of hydrocarbon-contaminated environments (Table 4.2).

Table 4.1: Gas chromatography/mass spectroscopy data for pooled reference and heavily oiled sites in Bay Jimmy, February 2011.

| Analyte | | Reference site | Heavily oiled site |
|---|---|---|---|
| **Alkanes** | | **R2 (ug/g)** | **O2 (ug/g)** |
| | C5 to C10 | 0.00 | 0.00 |
| | C11 to C15 | 0.07 | 4.78 |
| | C16 to C20 | 1.77 | 939.86 |
| | C21 to C25 | 1.55 | 1287.03 |
| | C26 to C30 | 2.88 | 1002.67 |
| | C31 to C35 | 2.69 | 523.48 |
| | **Total alkanes** | 8.96 | 3757.82 |
| **Aromatics** | | **R2** | **O2** |
| | C10 to C12 | 0.00 | 5414.14 |
| | C13 to C15 | 13.50 | 19239.23 |
| | C16 to C17 | 206.09 | 25913.83 |
| | C18 to C20 | 865.68 | 37778.91 |
| | C21 to C 22 | 69.90 | 343.81 |
| | **Total aromatics** | 1155.00 | 88689.91 |
| **TPH (C5 to C35)** | | 1163.96 | 92447.74 |
| **Ratio of lighter (C5 to C15) to heavier alkanes (C16 to C35)** | | 0.008 | 0.001 |

Table 4.2: Similar studies of oil-degrading bacteria in soils and TPH concentrations.

| TPH concentrations in other microbial studies | | | | | |
|---|---|---|---|---|---|
| Study | Min (mg/kg) | Max (mg/kg) | Avg (mg/kg) | Soil Type | Sampling dates |
| This study* | 300 | 162,000 | 57, 600 | LA salt marsh | April 2012 to April 2013 |
| Kostka et al. 2011* | 3.1 | 3600 | 807.6 | Pensacola Beach sands | 2 July to 2 September 2011 |
| Beazley et al. 2012* | BD | 189 | | AL salt marsh | June, July and September 2010 |
| Horel et al. 2012* | | | 141.9±57.5 | AL salt marsh | 2011 July |
| Andrade et al. 2004 | 1,710 | 92,340 | | Spainsh Estuary | 2003 February |
| Al-Awadhi et al. 2012 | 10 | 56 | | Kuwait coastal seawater | 2010 March |
| Ribeiro et al. 2013 | 260 | 620 | | Portugese salt marsh | |
| Anderson et al. 2001 | 800 | 46,000 | | NY salt marsh | |
| El-Tarabily 2002 | | | 34,200 | Mangrove | |
| Vinas et al. 2002 | | | 8,000 | | |

*Denotes a *DWH* oil spill study. BD is below the detection capabilities of instrument used.

Growth and metabolic activity in the culture medium was also apparent from the decrease of pH. The decrease of the pH of a culture medium might be due to the production of metabolites or excretion during the growth of the cultured bacteria. Jacques et al. (2007) stated that the change in pH along with growth of bacteria revealed a significant reduction in pH due to acidification. Likewise, Sayavedra-Soto et al. (2006) have stated that biodegradation is always accompanied by a significant drop in pH, the suggestion being that there is an accumulation of acid in the medium. Both of these studies support the findings of this study. At 96 hours, *M. esteratomaticum* had the lowest pH of 5.29, and *R. equi* had the highest pH of 6.50.

# CHAPTER FIVE: SUMMARY

Environmental quality is inextricably linked to our quality of life here on Earth. Global resources are being over-utilized and degraded by anthropogenic activities that have lasting global consequences. The problems associated with hydrocarbon contamination assume an increasing prominence in many countries. Pollution problems like hydrocarbon contamination often result in disturbances of both the biotic and abiotic components of the ecosystem. Coastal environments in the Gulf of Mexico have been exposed to natural seeps of oil for thousands of years. Barataria Bay has also been exposed to anthropogenic hydrocarbons from oil spills, petroleum exploration, transportation, and waste oil disposal for many decades. The fact that the bacterial community in Barataria Bay has been exposed to hydrocarbons for so long most likely means that many bacteria have adapted to efficiently degrade hydrocarbons. However, oil from a spill is often made up of hydrocarbons in high concentrations that can exert toxic stress on oil-degrading bacteria. While these bacteria do specialize as oil-degraders, they are ubiquitous in the environments they live in and perform vital ecosystem services.

The objective of this study was to determine the predominant species of hydrocarbon-degrading bacteria in marsh sediment in Barataria Bay post the *DWH* oil spill. Reference, medium-oiled, and heavily oiled sites were sampled 24, 30, and 36 months after the initial oiling event. A total of 460 bacterial strains were isolated and sequenced, among which 84 species were identified from 45 genera in 4 phyla (Table 3.2). Taxonomic classification revealed that all bacteria belonged to the phyla *Firmicutes*, *Actinobacteria*, *Bacteriodetes*, and *Proteobacteria*.

At a class level, all bacterial isolates were from *Bacilli*, *Actinobacteridae*,

G*ammaproteobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Flavobacteriia*. Prevalent genera isolated from samples were *Bacillus*, *Microbacteria*, *Micrococcus*, *Streptomyces*, *Pseudomonas*, *Esherichia*, *Agromyces*, and *Flavobacteria*. The predominance of PAH-degraders was likely due to the depletion of alkanes and the persistence of recalcitrant PAHs in the soil. Gram-positive PAH-degraders were the dominant bacteria at all sites during every sampling event. The ability of many gram-positive bacteria to degrade recalcitrant hydrocarbons and survive in dynamic conditions reflects their peptidoglycan cellular envelop and likely has allowed gram-positive PAH-degrades to thrive in hydrocarbon-contaminated, estuarine environments. *Proteobacteria*, particularly *Pseudomonas* sp.and *Sphingomonas*, are known to degrade higher molecular weight PAHs and will most likely remain abundant in microbial communities in Barataria Bay for quite some time.

In this study 10 bacterial isolates were identified to be *Micrococcus luteus*, *Microbacterium esteratomaticum*, *Rhodococcus equi*, *Vibrio* sp., *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Staphylococcus xylosus*, *Bacillus pumilis*, *Agromyces aurantiacus*, and *Acinetobacter calcoaceticus*. Among these ten isolates, *Micrococcus luteus* was found to be the best degrader of crude oil, whereas *Agromyces aurantiacus* was least effective at degrading crude oil. This may be due to the fact that *Micrococcus luteus* was isolated from a site with a higher concentration of oil. Bacteria that are exposed to higher concentrations of crude oil, over time, become more efficient oil degraders. Throughout the study, however, all sites had TPH concentrations that would be considered "high" in other studies. Therefore, the genetic and physiological differences between *Micrococcus luteus* and *Agromyces aurantiacus* may play a larger role in degradation ability and should be examined thoroughly.

The pH values of all cultured media studied decreased over the 144-hour experiment. A

drop in pH has been shown to be indicative of growth and metabolic activity. The decrease in pH is most likely due to an accumulation of acid from oxidation reactions during biodegradation.

Overall, the *DWH* oil spill has impacted the marsh sediments in Barataria Bay, Louisiana but the full existent of the impact on microbial populations is largely unknown . The predominance alkane degraders early on in the spill and the subsequent predominance of PAH-degrading bacteria is evidence of microbial succession. The predominance of these bacteria and the decline of others may impact nutrient cycling, waste decomposition and heavy metal deposition.

# CHAPTER SIX: FUTURE STUDIES

The first oil-degrading bacterium was isolated in the 1940's, and countless scientific studies since then have been carried out to understand who these degraders are and how they function. However, there is still much we do not understand about these extremely vital bacteria.

Many bacteria have genes that encode for enzymes involved in hydrocarbon degradation; however, hydrocarbon-degrading bacteria seem to have mechanisms that help them adapt to efficiently degrade hydrocarbons in many different environments. Genomic and metabolic research about hydrocarbon-degrading bacteria is needed to identify the genetic equipment that makes a bacterium an efficient hydrocarbon degrader under varying environmental conditions and to understand how it may develop in a bacterial community.

While a terrible natural disaster, the *DWH* oil spill has provided researchers with an opportunity for both short-term and long-term monitoring of oil-degrading bacteria after a major hydrocarbon contamination event in many different ecosystems such as salt marshes, mangrove swamps, beaches, and intertidal regions. Data collected in these areas over the next several years will be invaluable to the enhancement of understanding of hydrocarbon-degrading bacteria.

Due to the fact that some molecular techniques may be biased, using a range of molecular testing should be encouraged. A biochemical testing method, phospholipids fatty acids (PLFA), can be used to determine changes in abundance of microbial groups based on changes in phosopholipid profiles. There are many nucleic acid-based techniques, such as PCR, amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA), denaturing gradient gel electrophoresis (DGGE), and terminal-restriction fragment length polymorphism (T-RFLP) that can be used to profile microbial communities or to identify a

particular microbial species. Newer molecular techniques, such as stable isotope probing (SIP) and reverse sample genome probing (RSGP), seek to link microbial identity to ecological function.

In addition to molecular techniques, it is still important to isolate and characterize pure cultures of hydrocarbon-degrading bacteria that inhabit less commonly studied environments. Even though many different culture techniques have been developed, only a fraction of the bacteria involved in biodegradation of contaminants in soil and water can be cultured in the laboratory. The development of specialized culturing techniques that are both suitable to, and representative of the many unexplored ecosystems that may contain unique bacteria, is certainly an area that requires additional research.

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# VITA

Lauren Nicole Navarre was born in Carrollton, Georgia to Robert and Kristi Navarre. Lauren has a younger brother, Brandon. Lauren attended the United States Naval Academy from 2006-2008, majoring in Oceanography. It was there in Annapolis, MD, on the banks of the Severn River, that Lauren discovered her passion for environmental sciences. After transferring home to the University of West Georgia, Lauren majored in Environmental Science with a concentration in Geology. While she pursued her education in environmental science at West Georgia, her research focused on arsenic in groundwater in rural Carroll County, Georgia. Lauren completed her bachelor's degree in August 2011 and enrolled in Louisiana State University in the spring of 2012 to pursue her Master's degree in Environmental Science. Under the guidance of her Major Professor, Dr. Aixin Hou, Lauren studied the effects of crude oil on oil-degrading bacteria populations in Barataria Bay, Louisiana. During her education at Louisiana State University, Lauren worked as a teaching assistant for two years. She was a teaching assistant for OCS1005: Introduction to Oceanography, ENVS 4035: Aquatic Pollution and ENVS 1126: Introduction to Environmental Sciences. For the 2012-2013 academic year, she was the Coast and Environment Graduate Organization's secretary and served as a student Vice President for Phi Kappa Phi honor society for the 2013-2014 academic year.