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Quantification of Total and Oil-Degrading Bacteria in Louisiana Marsh Sediment Following the Deepwater Horizon Oil Spill using Cultivation and Real-Time PCR Assays

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QUANTIFICATION OF TOTAL AND OIL-DEGRADING BACTERIA IN LOUISIANA MARSH SEDIMENT FOLLOWING THE DEEPWATER HORIZON OIL SPILL USING CULTIVATION AND REAL-TIME PCR ASSAYS

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ABSTRACT

Although the impacts of the *Deepwater Horizon* (*DWH*) oil spill on the bacterial communities in several Gulf of Mexico habitats have been described, little if any research has been published evaluating the impacts of the spill on bacterial populations in the most severely oiled salt marsh soils of Barataria Bay, Louisiana. The aim of this study was to define the effects of the spill on the total and oil-degrading bacterial communities in these marshes. Using quantitative real-time PCR (qPCR) and traditional cultivation methods bacterial communities from contaminated marsh soils were examined thirteen, twenty-two, and twenty-nine months after the initial oiling event. Overall, the relative abundance of the n-alkane degrading bacteria decreased over time while that of the polycyclic aromatic hydrocarbon (PAH) degrading bacteria increased. The oil-degrading communities were increasingly dominated by gram-positive PAH-degrading bacteria over time and in the last sampling more than 99% of the oil degrading genes detected were from gram-positive PAH-degraders. Supplemental 16S rRNA sequencing of oil-degrading bacterial strains isolated from samples taken thirty months after the spill revealed that 66% of the isolates were gram-positive with nearly half of the gram-positives belonged to the genus *Bacillus*. The observed shift in microbial populations over time and the dominance of gram-positive PAH-degraders is indicative of a later stage of the biologically-driven attenuation of hydrocarbon-contaminated soils. Negative correlations between populations (total and oil-degrading) and TPH levels were observed on some occasions (i.e. the 16S rRNA gene count at thirteen months and PAH-degrading genes at twenty two months), indicating adverse effects of high TPH levels on bacteria. The increased abundance of oil-degraders at the lightly oiled reference sites was likely due to increased vegetative cover and lower levels of toxic hydrocarbons. Differences in the culture-based and molecular genetic data suggest that some of

the oil-degraders in the marsh sediments may be nonculturable in a laboratory setting. Based on this study, twenty-nine months after the *DWH* oil spill the oiled Louisiana salt marsh soil has come into its latter stages of naturally microbial attenuation. Also, extremely high levels of oil residuals in the marsh soil possibly have inhibited proliferation of certain microbes including oil-degrading bacteria, potentially impairing the marsh's efficiency of oil degradation and other biogeochemical processes.

INTRODUCTION

1.1 Potential Effects of the Deepwater Horizon (*DWH*) Event on Marsh Bacteria

On April 20, 2010 the British Petroleum *DWH* drilling rig experienced a blowout and began releasing crude oil into the Gulf of Mexico south of the Louisiana coast. Over the following three months nearly five million barrels of oil were introduced into the Gulf and eventually approximately 650 miles of the Gulf coast were contaminated with weathered crude oil, with 129 miles of Louisiana salt marsh being moderately to heavily oiled (Oil Spill Commission, 2011). The impacts of the spilled oil on the microbial communities in several Gulf coastal habitats have been documented (Beazley et al., 2012 and Kotska et al., 2011), but little if any research has been published evaluating the impacts of the spill on oil degrading bacterial populations in the oil-inundated salt marsh soils of southeastern Louisiana. However no work has been published evaluating the impacts of the spill on oil-degrading bacterial populations in the oil-inundated salt marsh soils of southeastern Louisiana.

A wealth of information is available which describes common effects of hydrocarbon contamination on sedimentary bacteria. It has been widely reported that hydrocarbon contamination decreases the diversity of soil microbial communities 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). Bacteria facilitate many vital processes in plant-soil systems including nutrient cycling and decomposition of organic matter and reducing microbial diversity has been shown to reduce ability of soil systems to recover from perturbations (Griffiths et al., 2001). John et al. (2009) reported 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. 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 (Andrade 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 populations 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) 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. By assessing gene abundance it was demonstrated that relative abundances of hydrocarbon degraders, especially those belonging to the classes *Gammaproteobacteria* (genera *Alcanivorax* and *Marinobacter*) and *Alphaproteobacteria* (genera *Rhodobacteraceae*) were higher in oiled sands as was the total number of bacterial gene sequences. However results indicated a dynamic population over the duration of the study, as percent abundance of the alkane-degrading genera *Alcanivorax* fell from approximately 10% of the total bacteria detected three months after the spill to less than 1% six months after the spill (Kotska et al., 2011). Similarly, work done by Alsonso Guitierrez et al. (2009) investigated the impacts of an oil spill on beach sands and rocks two years after an oil spill and concluded that community structure had been significantly altered though overall abundance and diversity did not appear inhibited. Bacterial populations were dominated by members of classes gram-positive *Actinobacteria*, which are rarely present immediately following an oil spill, and *Alphaproteobacteria*. These

findings suggest that bacterial communities will eventually evolve to cope with hydrocarbon induced stresses and new community structures will form which are more stable in the altered environments. This hypothesis is consistent with previous observations (Atlas, 1981; Macnaughton, 1999; Paisse et al., 2008). However, decreased microbial abundance in wetland sediments has been reported as long as two years after an oil-spill. El-Tarabily et al. (2002) found reduced total bacterial abundance and reduced metabolic activity in mangrove sediments two years after initial contamination. Researchers used culture based techniques along with measurement of hydrolysis of fluorescein diacetate to describe microbial community structures and metabolic activity. Every group of organisms monitored (including various bacteria, filamentous fungi, and yeasts) was significantly less abundant and less metabolically active in the polluted soils than unpolluted soils except for the aerobic and anaerobic hydrocarbon degrading bacteria, which demonstrated the opposite response. The authors of this paper note that level of contamination may be considered relatively high and suggest the decreased abundances and activities may be due to toxic effects of polycyclic-aromatic hydrocarbons (PAHs), specifically possible inhibition of microbial enzymes (El-Tarabily et al., 2002). This decrease of general microbiological activity is particularly concerning as bacteria and other microbes play a primary role in the elimination of many of the toxic and persistent components of crude oil.

The purpose of this work was to increase our knowledge of the impacts of the *DWH* spill on coastal bacterial communities. The study selected the most heavily oiled salt marshes of Barataria Bay, Louisiana to quantify the shifts in soil bacterial populations over time following the oiling by measuring 16S rRNA, alkane monooxygenase, and ring hydroxylating dioxygenase genes with realtime PCR supplemented by the conventional plating.

1.1.2 Plant/Bacteria Systems and Hydrocarbon Contamination

The impact of crude oil contamination on bacterial communities is not limited to the direct effects of physical contact with oil such as toxicity and selection of oil-degraders. Damage to native plant species due to crude oil contamination has the potential to diminish the ability of a microbial community to break down hydrocarbons. It has been reported that bacterial degradation of crude oil compounds is increased when a healthy plant community is present when compared to unvegetated soil (Ho and Banks, 2006; Ho, Applegate, and Banks, 2007; Joner et al., 2001).

The benefits of healthy vegetation in oil contaminated soils are numerous and varied. For example, a healthy plant community can act as a physical barrier and help prevent the spread of oil and thus helps keep pristine areas of soil clean, thus preventing perturbations in the bacterial community (Joner et al., 2001). An oft-studied and well described benefit is that of a healthy rhizosophere, which is the area of soil in which the plant roots interact and is located just beneath the surface (Daane et al., 2001). The rhizosphere is often cited as an important facilitator of bacterial degradation of hydrocarbons (Banks and Ho, 2001; Daane et al., 2001; Joner et al., 2001). The reasons for the increased degradation capacity are not completely defined but are known to include a strength of the whole bacterial population via provision of an accessible carbon source (Joner at al., 2001) and secretion of degradation-facilitating oxidative enzymes (Ho, Applegate, Banks, 2007; Joner et al,. 2001). Thus the microbe/plant communities in vegetated soils are often reported as robust and dynamic systems more capable of withstanding hydrocarbon contamination when compared to those microbe systems of unvegetated soils (Ho, Applegate, and Banks, 2007; Ho and Banks, 2006; Joner et al., 2001).

1.2 Methods for Analysis of Bacterial Populations in Oiled Soil

Modern analysis of microbial community composition benefits from the use of culture-independent methods in addition to culture-based methods as it has been shown that uncultured techniques are capable of presenting a much more accurate and detailed account of community profiles and population counts (Habi and Omori, 2003; Torvik et al., 1996; Ward et. al., 1990). Analysis of the microbial communities in the salt marshes bordering the Gulf of Mexico, specifically hydrocarbon degrading bacteria, should include use of culture-independent methods in order to guarantee the most informative data can be collected. Of particular use is analysis of the common but differentiated bacterial 16S rRNA gene. Isolating pure strains and comparing strain 16S rRNA sequences with database sequences 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 coastal sediment microbial populations following an oil spill (Alonso-Guitierrez et al., 2009). Culture independent methods are also imperative to accurately assess bacterial enzymatic activity in polluted soils. Polymerase chain reaction techniques (PCR) use DNA polymerase to amplify selected genes in order to observe their presence. The method does not require any culturing as genetic material can be extracted directly from a variety of samples, including agar cultures and soil, for use in PCR assays.

1.2.1 Use of Real Time PCR to Evaluate Bacterial Population

A relatively new and informative use of PCR is the real-time PCR method, or quantitative PCR (qPCR), which enables researchers to quantify a selected gene per a given volume of sample in real time. Real-time PCR has been used in order to quantify bacterial

hydrocarbon degrading genes. For example, Baldwin et al. (2003) developed and successfully used primer sets and protocols for quantification of numerous bacterial aromatic oxygenase genes by qPCR and Powell et al. (2006) developed and successfully used primer sets and protocols for quantification of the well described bacterial alkane monooxygenase (*alkB*) genes which they found to be positively correlated with alkane concentration in environmental samples.

Previously published work has developed a single set of primers that can be used to indicate the ability to degrade an array of PAH components (Cebron et al., 2007). The first step in bacterial degradation of PAHs is facilitated by a ring-hydroxylating dioxygenase (RHD) enzyme which contains the RHDα subunit which is encoded by a gene that is considered conserved. Polycyclic aromatic hydrocarbon RHDα genes have been reported frequently, but are usually identified by substrates tested in the research and are not inclusive of all the components for which the designated gene enables degradation. Relatedness of these genes lead to a development of a single PCR primer set for a wide range of *PAH-RHDα* genes in aerobic gram-positive bacteria as well as a primer set for aerobic gram-negative bacteria (Cebron et al., 2008). Observing the PAH degrading genes in both groups is particularly informative as it has been shown that population dynamics of gram-negative groups have been shown to differ with the dynamics of gram-positive groups following oil contamination (Cebron et al., 2008). This is likely due to the groups having different degradation capacities, as gram positive bacteria may be more resilient and more capable of degrading high molecular weight oil components including fluoranthene, pyrene and benzo[a]pyrene (Cebron et al,. 2007, Cebron et al., 2008, Kanaly and Harayama, 2000). It has been reported that gram-positive oil degraders have maintained more

robust populations for longer periods of time following contamination when compared to gram-negative bacteria (Cebron et al., 2008 and Beazely et al., 2012).

Accurate indicators of total bacterial populations are essential to acquiring useful information from quantification of genes of interest. The *PAH-RHDα* primers were indeed used by the aforementioned authors to quantify the PAH-degrading bacteria in relation to a whole population in a contaminated environment by way of comparison of *PAH-RHDα* gene copy quantification to *16SrRNA* gene copy quantification (Cebron et al., 2008). It must be noted that it is unacceptable to state the copy number of 16S rRNA obtained via qPCR as the exact bacterial population, as the number of 16S rRNA copies per bacteria varies (Nadkarni et al., 2002, Pei et al., 2010). Number of 16S rRNA copies per bacterial genome varies and can range from two to fifteen copies per genome (Pei et al., 2010) and a gene which occurs in a single copy per genome may be more indicative of the actual bacterial population (Dahillof, Baillie, and Kjelleburg, 2000). For the aforementioned reason Dahillof et al. (2000) employed PCR denaturing gradient gel electrophoresis (PCR-DGGE) and evaluated the feasibility of using the gene for the ribosomal polymerase beta subunit (*rpoB)* in bacterial community analysis. The authors found that unlike the 16S rRNA, *rpoB* exists in a single copy in all bacterial genomes and thus may be a better indicator of total bacterial population. Work has been done in which researchers used the *rpoB* primer set for quantification of total bacteria via qPCR in their environmental samples. In one such study the gene *alkB* that encodes alkane monooxygenase was quantified (using the same primer sets used in the present work) and compared to the total gene numbers acquired via *rpoB* quantifications (Powell et al., 2006).

1.3 Metabolic Pathways of Oil-Component Degradation by Bacteria

The rate at which bacteria can metabolize crude oil depends in part on the constituents that make up the contaminating oil (Atlas, 1991). For this reason this study has examined genes which indicate the ability to break down different types of crude oil components (n-alkanes and PAHs). Different molecular structures are broken down by different enzymes produced in different species and lighter weight elements such as short alkanes, naphthalene, or phenanthrene, constituents are degraded more expediently (Atlas, 1991; Atlas, 1981). In some cases cometabolism allows for the break-down of certain recalcitrant hydrocarbons only if oil-degraders are also acting on another particular substrate; in other cases metabolism of certain substrates is inhibited when others are present (Atlas, 1981). As stated above, it is common for bacterial populations to fluctuate following an oil spill as a function of the contamination (Atlas, 1981; Macnaughton, 1999; Paisse et al., 2008). Thus information on temporal pattern of respective bacterial populations will serve our understanding of bacteria-driven oil-attenuation over time after a spill.

1.3.1 Metabolism of n-alkanes

The present work attempts to estimate alkane-degradation potential by way of quantification of the *alkB* gene, thus it is primarily focused on aerobic degradation of the linear n-alkanes. However, anaerobic degradation of alkanes and aerobic degradation of branched alkanes have been observed. Anaerobic pathways are highly varied and specific to their substrate, but require nitrate or sulfate to act as the terminal electron acceptor and therefore occur at a much slower pace than aerobic metabolic pathways (Rojo, 2009; Spormann and Widdel,

2000). Branched alkanes are generally more difficult to metabolize, but their pathways are still not completely understood (Rojo, 2009).

Rojo (2009) compiled a comprehensive review of research of alkane metabolism and describes multiple pathways in great detail. With the exception of methane the aerobic metabolism of n-alkanes generally begins with the oxidation of a terminal methyl group, though it has been reported that oxidation can occur at both ends of an alkane or at a sub-terminal methyl group. In the usual case of terminal methyl group oxidation the methyl group is oxidized to a primary alcohol, then to an aldehyde, and then to a fatty acid. The fatty acid joins with coenzyme A (CoA) and eventually becomes part of acetyl-CoA via β-oxidation. If both ends of the alkane are oxidized it is done by ω-hydroxylation and results in a ω-hydroxy fatty acid which eventually forms a dicarboxylic acid that can be acted upon by β-oxidation. Sub-terminal oxidation results in a secondary alcohol being oxidized into a corresponding ketone which is oxidized into an ester. The ester is hydrolysed and forms an alcohol and a fatty acid (Rojo, 2009).

There are a variety of enzymes responsible for the initiation of the breakdown of alkanes and it is generally informative to consider enzymes on a basis of length of the carbon chain they act upon (Rojo, 2009). Short alkanes ranging in length from two to four carbons (C_2-C_4) are generally broken down via enzymes similar to methane monooxygenase, while many alkanes >C_5 are acted upon by enzymes similar to the GPo1 *alkB* enzyme of *Pseudomonas putida*, which is a thoroughly described alkane-degrading enzyme (Powell et al., 2006, Rojo, 2009). The actual GPo1 *alkB* enzyme has been shown to metabolize alkanes ranging from C_3-C_{13} (van Beilen et al., 2005, Rojo, 2009). There are many exceptions to this two-category system, as many C_5-C_{11}

alkanes are hydroxylated by a cytochrome p450 family and many long chain alkanes (>C_{18}) are acted upon by enzymes altogether unrelated to those mentioned here (Rojo, 2009).

1.3.2 Metabolism of Polycyclic Aromatic Hydrocarbons

The bacterial degradation of PAHs by bacteria is commonly initiated by a ring-hydroxylating dioxygenase (RHD) which is, in part, coded by the *PAH-RHDα* genes targeted by the PCR primers that are being used in this study (Cebron et al., 2007). The primers indicate capacity to degrade PAHs by targeting a gene which codes for one of the three components of the ring-hydroxylating dioxygenase enzymes, the iron sulfate protein α-subunit (Cebron et al., 2007; Habe and Omori, 2003). Though these α-subunits are not present in every metabolic pathway of PAH degradation they do act in a range of pathways and thus can act as indicators of ability to metabolize PAHs (Cebron et al., 2007; Kanalay and Harayama, 2003). The genes targeted by the cited *PAH-RHDα* primer sets for the gram negative bacteria in the work published by Cebron et al. (2007) are nahAc, nahA3, nagAc, ndoB, ndoC2, pahAc, pahA3, phnAc, phnA1, bphAc, bphA1, dntAc and arhA1. The gram positive *PAH-RHDα* alleles can be classified into four major groupings: the narA-like genes, the nidA/pdoA1-like genes the phdA/pdoA2-like genes and the nidA3/fadA1-like genes. The primer sets developed for gram positive bacteria were made to target all four groups. The end result was a pair of primer sets that, when used with qPCR techniques, provide a more comprehensive method for analysis of a bacterial population's capacity to break down PAH components when compared to the common method of using several highly specific primer sets (such as those published by Baldwin et al.) to establish characteristics of a diverse bacterial population (Cebron et al., 2008). Figure 1.1 was constructed by the Cebron et al. (2007) as they developed their primers and indicated specific genes covered by the use of their primer sets.

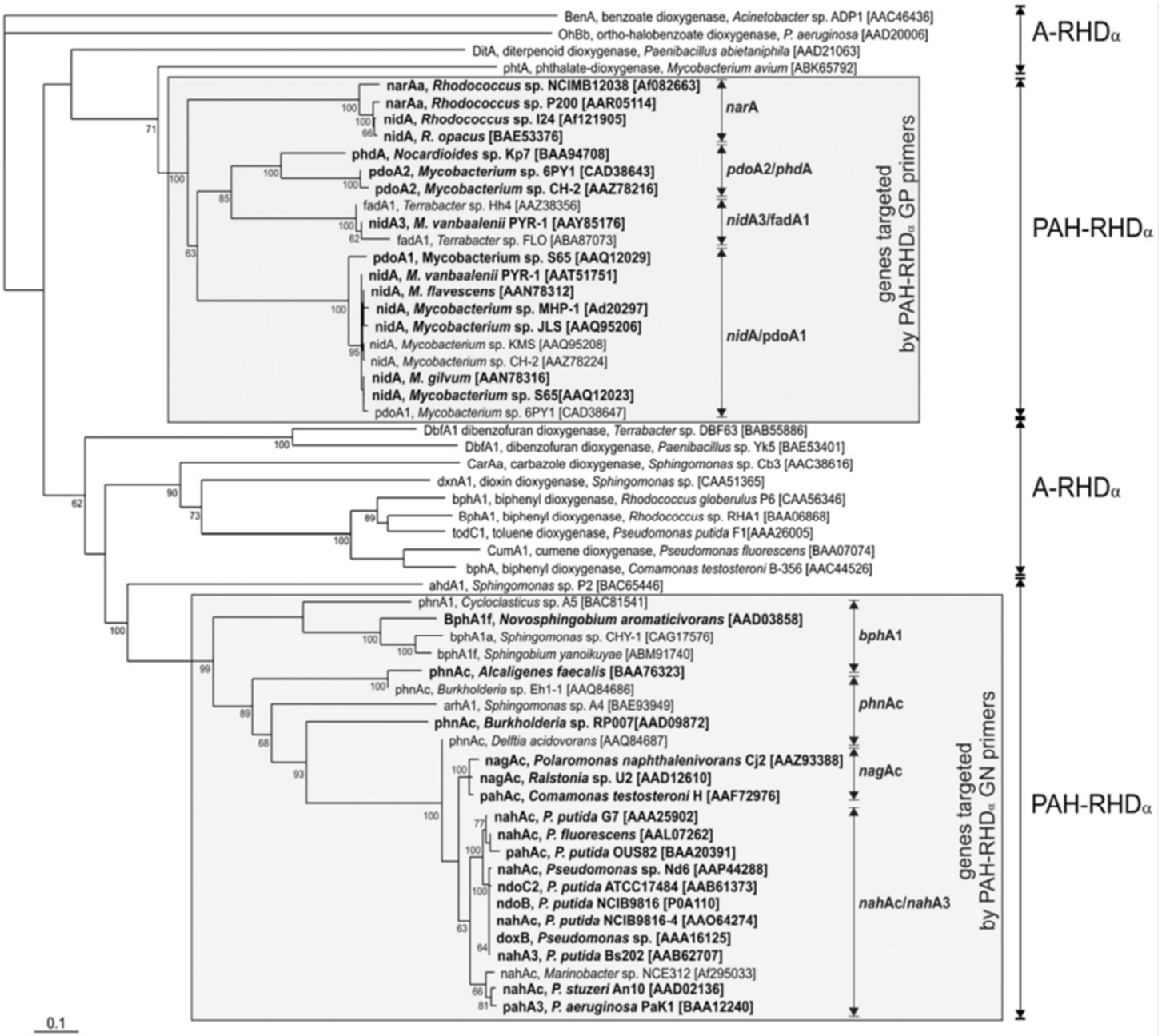

Figure 1.1**:** Genes confirmed to be targeted by RHDα primers (Cebron et al. 2008).

A model pathway of PAH degradation involving the α-subunit can be considered in the case of naphthalene. In the first step *cis*-naphthalene dihyrdodiol is produced after oxidation of the aromatic nucleus at the 1,2 position by naphthalene dioxygenase (Habe and Omori, 2003). It is this initial oxygenase enzyme, along with many very closely related oxygenase enzymes, that contains the aforementioned iron sulfate protein α-subunit (Cebron et al., 2007; Habe and Omori, 2003). 1,2 Dihydroxynapthalene is then formed by the dehydrogenation of *cis*-naphthalene and is *meta*-cleaved into precursors of salicyladehyde which transform into salicylaldehyde. Salicylate is formed and further metabolized into TCA cycle intermediates (Habe and Omori, 2003).

METHODS

2.1 Test Area Description

This study consisted of data collected from seventeen 0.5m^2 plots in May 2011, February 2012, and September 2012 (corresponding to thirteen, twenty-two, and twenty-nine months after the initial oiling event, respectively). The plots were subsections of larger 50m^2 plots which had been established by the National Oceanographic and Atmospheric Administration (NOAA) along a marsh in northern Barataria Bay, Louisiana which was heavily contaminated with oil following the *DWH* oil spill (Fig 2.1, Fig 2.2). The larger sites had been assessed and categorized as "highly oiled" or "reference" in October of 2010 using Shoreline Cleanup Assessment Techniques (SCAT). The SCAT observations and data indicated that oiling conditions including oil presence, thickness, and distribution were similar for all of the experimental sites (Fig. 2.3, Zengel and Michel, 2013).

Two distinct zones of oiling were observed and delineated- one along the coast and one further inland. The1-3m span along the coast of the marsh was referred to as "zone A" (Fig. 2.4). This area was generally barren and consisted of a thin crusty layer of oil. "Zone B" was the area of the plot extending from zone A into the marsh over the extent of the oiling and included oil covered soil and rooted vegetation (Zengel and Michel, 2013). The majority of the small plots used for this study were near the shoreline in zone A; however, by the sampling done in February 2012 some sites' zone A had completely eroded. In these cases, the edge of zone B was used for sample collection. This is significant because the sites were used to test methods including various types of vegetation raking and oil-dispersant treatment. However, treatments were only applied inland in zone B due to fear of accelerating the erosion of the coast, therefore

we could not consider our plots to have been treated by the same methods (Zengel and Michel, 2013).

Figure 2.1: Location of sampling sites in relation to the *DWH* oil spill (Google Maps, 2013)

Figure 2.2: Satellite view of test area showing orientations of the highly oiled and reference areas (Google Maps, 2013)

Figure 2.3 Aerial view of some highly oiled sites depicting site set-up and size (Zengel and Michel, 2013)

Figure 2.4: Ground level of NOAA test plot depicting “zone A” (Zengel and Michel, 2013)

2.2 Collection and Transportation of Samples

Sampling was conducted by hand using nitrile gloves. Several small samples of the top 2-3cm were collected from within the $0.5m^2$ plot in order to obtain accurate representation of the entire plot. The soil was placed in sterile jars or bags and stored in an ice chest for the duration of the trip. Samples were brought to the lab and stored at 4° C for no more than twelve hours before being processed using culture based techniques and then stored at -20° C or -80° C until the DNA extraction process or TPH analysis.

2.3 Growth Media Description

Total bacterial counts were estimated following plating on Difco™ marine agar. Bushnell Haas (BH) agar was used to culture bacteria capable of degrading crude oil. The composition of the BH agar (grams per liter) is as follows: magnesium sulfate 0.20, calcium chloride 0.02, monopotassium phosphate 1.00, dipotassium phosphate 1.00, ammonium nitrate 1.00, ferric chloride 0.05, agar 15.00. Final pH was adjusted to 7.00 ± 0.02 with sodium hydroxide and hydrochloric acid (Atlas, 2005). Source oil was put into the agar at a concentration of 0.05% (v/v). The oil used was Macondo source oil (MC252) which was relatively light and had an API of about 37 (Platts Oilgram, 2010) All growth media and unsterile culture containers were autoclaved at 121°C and 15 psi for thirty minutes before processing.

2.4 Soil Sample Processing

All soil samples were mixed by using nitrile glove-covered hands in order to form representative soil samples which were used for all assays. A small sample of fresh sediment was weighed during initial processing and then dehydrated in an oven for two to three days. Weight loss at the end of this period used to calculate for water content of the soil samples. A

conversion factor (dw) was derived from this data for population calculations by simply dividing the dry weight by the fresh weight. All metrics of bacterial population in this work were reported per gram of dry soil weight. Total petroleum hydrocarbons (TPH) were extracted using dichloromethane and analyzed gravimetrically (Lin and Mendelssohn, 1996).

2.5 Estimation of Bacterial Populations using Culture-based Techniques

Fresh soil samples were mixed into slurry which was one part soil and nine parts phosphate buffer solution (PBS). Total aerobic and aerobic oil-degrading CFU counts were estimated by plating two or three ten-fold serial dilutions of the slurry. Plates were incubated at 29-30° C in an incubator. Total aerobic counts were estimated at thirty six hours and all oil degrading counts were made one week after plating. Negative control plates or tubes were included in all assays. Observed colonies (C_o), dry weight conversion factor (dw) and dilution factors (*d*) were used to calculate the colony forming units (CFU) per gram of dry soil using the following equation:

$$CFU = C_o / d / dw$$

2.6 Estimation of Bacterial Populations using Molecular Genetic Techniques

Genetic template DNA from 0.25 grams of each soil sample was extracted using MoBio PowerSoil® DNA Isolation kit. The kit uses a bead beating method in which samples are mechanically homogenized in addition to traditional chemical lysis and buffering. The kit also provides two different solutions of patented Inhibitor Removal Technology® which removed inhibitors including humic acids. After precipitation of contaminants, silica spin filters are used in concert with high salt solutions in order to bind the sample DNA to the silica spin filter. The DNA on the silica filter was also rinsed with ethanol in order to remove residual salts, humic

acids, and contaminants. DNA was rinsed from the spin filter and stored in 10 mM Tris elution buffer.

The quality of the DNA was confirmed by spectrophotometric analysis and gel electrophoresis. The A260/A280 method uses a spectrophotometer in order 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. Acceptable DNA was run on 1.5% agrose gel to confirm quality and stored at -80ºC or -20ºC in elution buffer provided by MoBio until needed for further application. Once it was time to begin qPCR analysis DNA was removed from freezer, diluted by a factor of 10 or 100 in nuclease free water and placed into 4ºC for the duration of the qPCR experiments (less than eight weeks). All extracted DNA represented DNA per 0.25 grams of wet soil for consistency.

2.6.2 Real-Time PCR Standard Curve Template

Integrated DNA Technology® (IDT) gBlocks™ gene fragments were used in order to establish the standard curves for real-time PCR assays. Gene fragments were double stranded, linear, and had unphosphorylated ends. Sequences for the necessary gene fragments were acquired from the Genbank database from the website for the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/genbank (n.d.) using accession numbers provided in previously published works. Primers were tested via the sequence extractor at bioinformatics.org and projected amplicon sequences were submitted to IDT in order that gene fragments could be synthesized. The sequences and source organisms are listed in Table 2.2.

Table 2.1: Gene fragments and corresponding accession number used to establish the real-time PCR standard curve

STANDARD CURVE SEQUENCES		
Gene	**Organism**	**Genbank Accession Number**
16S rRNA	***Pseudomonas putida***	**KC207085**
AACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTG CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCAC GTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG GCCTGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGT AGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTT CCCGGGCCTTGTACACACCG		
GN PAH-RHD	***Pseudomonas putida***	**M83949**
GGGATGCATACCACGTGGGTTGGACGCACGCGTCTTCGCTTCGCTCGGGAGAGTCTATCTTCGCGTCGCTCGCTGGCAACGCA GTGCTGCCCCCTGAAGGTGCAGGCTTGCAAATGACCTCCAAATACGGCAGCGGCATGGGTGTGTTGTGGGACGGATATTCAGG CGTGCATAGCGCAGACTTGGTTCCGGAGTTGATGGCATTCGGCGGCTCTAAGCAGGAAAGGCTGAACAAAGAAATTGGCGAT GTTCGCGCCCGGATTTATCGCAGCCACCTCAACTGCACCGTTTTCCCGAACAACAGCA		
GP PAH-RHD	***Rhodococus opacus***	**DQ846881**
CGGTGCCGACAACTTTGTCGGCGACGCGTATCACACCATGATGACCCACCGCTCGATGGTCGAGCTGGGGCTCGCCCCGCCCG ACCCGCAGTTCGCGCTCTATGGCGAACACATCCACACCGGGCACGGGCACGGCCTGGGTATCATTGGTCCGCCGCCGGGTATG CCGTTGCCGGAGTTCATGGGCCTTCCGGAGAACATCGTTGAAGAGTTGGAACGTCGGCTCACGCCGGAGCAGGTCGAAATCTT CCGGCCCACTGCCTTCATCCATG GCACCGTGTTCCCG		
alkB	***Pseudomonas putida***	**AJ233397**
GATGGCTTTCGGGTGGTGGCAGTTAACAAGCGCGAACTACATTGAACACTACGGTTTGCTGCGTGAAAAAATGGCCGATGGAC GATATGAACATCAAAAGCCACATCACTCGTGGAACAGTAACCACATCGTGT		

2.6.3 qPCR Conditions

Conditions for qPCR using the primers shown in table 2.2 were as follows: All reactions were 10µl in volume and consisted of 5.0µl of Bio-Rad SsoAdvanced™ SYBR Green Supermix (containing necessary buffer, hot start Sso7d-fusion polymerase, SYBR® green dye, dNTPs, $MgCl_2$, enhancers, and stabilizers), 500 nM of each forward and reverse primer, 4.0 µl of DNA template diluted to 10^{-1} or 10^{-2} concentration of the original DNA extraction in order to minimize PCR inhibition and acquire a signal within the standard curve. Well volumes were adjusted to a final volume of 10 µl using PCR grade nuclease free water.

The qPCR conditions were all derived from the published research. The 16S rRNA, *GN PAH-RHDα*, and *GP PAH-RHDα* used conditions published by Cebron et al.(2008) with the

following exceptions: (1) Each assay's extension step was lengthened by 10 seconds and (2) the annealing temperature for the *GP PAH-RHDα* primer set was changed from 54º C to 56º C. The changes were made after optimizing the assays using our own thermocycler. Efficiencies consistently fell between 85% to 100% following optimization.

For the 16S rRNA primers: (1) 95º C for 5 minutes; (2) Forty cycles of a 30 seconds of denaturing at 95º C, 30 seconds at an annealing temperature of 56º C, and 40 seconds of 72º C; (3) Seven minutes at 72º C. For the *alkB* assay degenerative versions of the primers not matching the strain referenced in table 2.2 were removed. This optimization decreased the inclusiveness of the primers but greatly increased the assay's consistency and efficiency. Assay parameters were as follows: (1) 94º C for 15 min; (2) fourty cycles of 20 second of denaturing at 84º C, 30 seconds of annealing at 50º C, and 45 seconds at 72º C; (3) One minute at 45º C. For *GN PAH-RHDα* primers: (1) 95º C for 5 minutes; (2) Forty cycles of a 30 seconds of denaturing at 95º C, 30 seconds at an annealing temperature of 57º C, and 40 seconds of 72º C; (3) Seven minutes at 72º C. For *GP PAH-RHDα* primers: (1) 95º C for 5 minutes; (2) Forty cycles of a 30 seconds of denaturing at 95º C, 30 seconds at an annealing temperature of 56º C, and 40 seconds of 72º C; (3) Seven minutes at 72º C. Primers and conditions are summarized in table 2.2.

Table 2.2: Primers and conditions used for real-time PCR assays

PRIMERS USED IN FOR REAL-TIME PCR

Primers	Target Gene	Sequence: 5' to 3'	Amplicon Size	Annealing Temperature	Reference
1401 F	16S rRNA	CGGTGTGTACAAGACCC	435	56	Cebron et al. (2008)
968 R		AACGCGAAGAACCTTAC			
AlkBFd	Alkane Monooxygenase (*alkB)*	AACTACMTCGARCAYTACGG	100	50	Powell et al. (2006)
AlkBRd		TGAAGATGTGGTTGCTGTTCC			
RHDα GN F	Gram Negative PAH-RHDα	GAGATGCATACCACGTKGGTTGGA	306	57	Cebron et al. (2008)
RHDα GN R		AGCTGTTGTTCGGGAAGAYWGTGCMGTT			
RHDα GP F	Gram Positive PAH-RHDα	CGGCGCCGACAAYTTYGTNGG	292	56	Cebron et al. (2008)
RHDα GP R		GGGGAACACGGTGCCRTGDATRAA			

2.6.4 Calculation of Gene Quantities

In order to quantify gene copies in soil, qPCR standard curves had to be accurately established. Gene fragments for the standard curves were ordered and shipped from IDT in 200ng quantities and then rehydrated with TE buffer. The number of genes in an aliquot was calculated using the mass (m) of the fragments in a given volume and the molecular weight (W_m) using the following equation:

$$\text{copies } \mu l^{-1} = (m \times 10^{-9} \times 6.02 \text{ X } 10^{23})/W_m.$$

Stock solution was used to create a serial dilution consisting of five to eight ten-fold dilutions and gene copy concentration was calculated for each dilution and entered into Bio-Rad CFX Manager 2.1. The program used these standard concentrations to automatically generate starting concentration data for DNA from samples in each well. A composite for these readings

from each site was used to generate an estimated quantity per gram of dry soil for each site (Q_c). Sample DNA was diluted to a concentration "D" (D= 10^{-1} or 10^{-2}) to minimize PCR inhibitors from the environmental samples. The following equation was used in order to calculate gene copy per dry gram of soil

$$Q_d=(Q_c/D)(200\mu l/g)/dw.$$

Note that initial DNA concentration extractions of 0.250 g of soil were stored in 50µl of 10 mM Tris elution buffer, thus 1 gram of soil was equated to 200µl DNA dilution. In order to more accurately assess the changes in the abundance of oil-degrading genes in the bacterial community by accounting for overall variations in bacterial populations a previously used established method was used in which gene copy number of these oil degrading genes were presented relative to the number of 16S rRNA per dry gram of soil (Cebron et al. 2008). These numbers were calculated after gene abundance per dry gram of soil was determined.

2.6.5 Validation of qPCR output

The qPCR assays from which data were collected were all based on an established standard curve with a calculated efficiency of 85-105%, and r^2>0.99, and replicates of individual standards having a threshold cycle standard deviation less than 0.5 cycles. No template negative controls (NTCs) were also included and lead to no products other than primer dimer. Melt curves and derived melt curves were used to validate the amplification of the desired product and to confirm that primer dimers or some form of inhibition invalidated our quantification. One *alkB* assay resulted in a small peak consistent with the published description of the same assay's peak for primer dimer. The dimer did not affect the quantification of the gene (Powell et al. 2006). Standard template gBlocks® were considered to provide a melting temperature consistent with

the desired product and melted in concordance with their respective assays' published melting point. According to the melt curves no NTCs produced a product. Below is an example of a melt curve that was considered indicative of acceptable qPCR product along with an example of a primer dimer, which manifested itself below the established melting temperature.

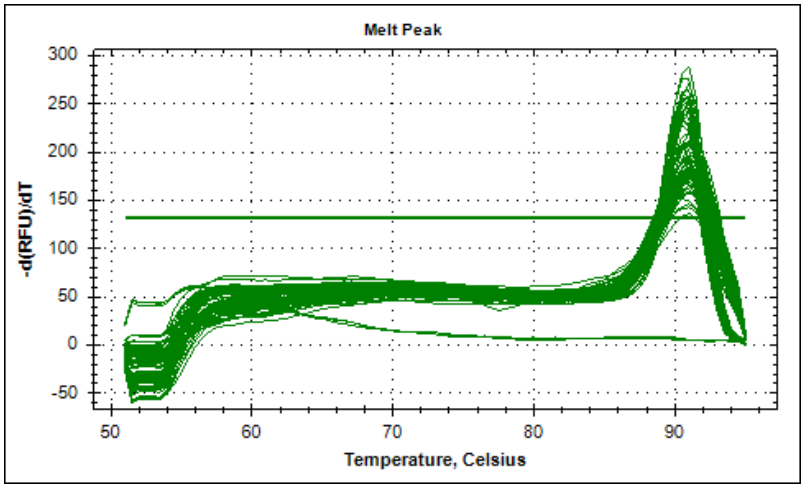

Figure 2.5: Validated 16S rRNA assay with no dimers or NTC contamination

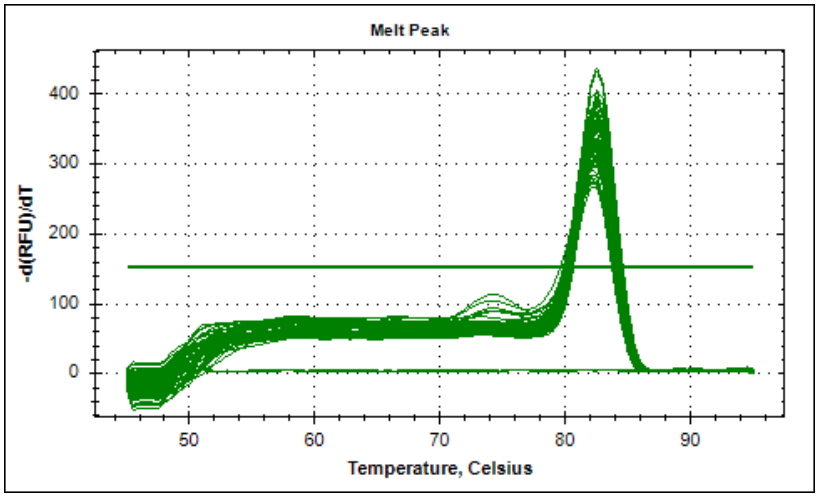

Figure 2.6: Validated *alkB* assay with primer dimer at temperature consistent with that reported by Powell et al. (2006).

2.7 Statistical Analysis

Analysis of data was carried out in SAS version 9.4 for Windows, IBM SPSS 18 for Windows, and Microsoft Excel 2010 for Windows. In the SAS program data sets for qPCR-based gene quantities and TPH concentrations were tested for normality by conducting Shapiro-Wilk tests using PROC UNIVARIATE. Because many of the gene quantity data sets and all of the TPH data sets failed to satisfy the tests for normality, even after arcsine and log transformation, analysis of genetic data was conducted using nonparametric tests. Functional gene ratios and quantities were analyzed in SPSS using nonparametric univariate Kruskal-Wallis for group comparisons and subsequent pairwise comparisons. Spearman's rank correlation tests (PROC CORR) were used to describe the relationship between gene data and TPH data. Culture based data was log-transformed prior to statistical analysis. Group comparisons of culture-based ratio data sets were also conducted using ANOVA in SPSS and regression analysis in Microsoft Excel. Data is reported with standard deviation and uses a significance level of $\alpha=0.05$.

RESULTS

3.1 qPCR Results

The qPCR analysis of the 16S rRNA genes revealed differences between the total bacterial populations at the sites from each sampling date ($p<0.05$; $n=39$). Though bacterial abundance in the highly oiled sites decreased by more than half between thirteen and twenty-two months and then more than doubled by twenty-nine months no significant differences were detected in the highly oiled sites. A population decrease at twenty-two months and subsequent increase at twenty-nine months was also observed in the reference sites. There was a six-fold decrease from thirteen to twenty-two months in the references sites ($p<0.05$; $n=8$) and a roughly ten-fold increase observed from February to September ($p<0.05$; $n=8$). The use of ratios (functional gene quantity per 16S rRNA quantity) accounted for this variation (Fig. 3.1).

Table 3.1: Real-time PCR generated gene quantities per gram of dry soil. Values are means ± standard deviations.

	REAL-TIME PCR QUANTIFICATION OF GENES			
	Site Type	**Estimated Quantity at 13 months (10^6 copies)**	**Estimated Quantity at 22 months (10^6 copies)**	**Estimated Quantity at 29 months (10^6 copies)**
16S rRNA	High Oil	9700 ± 8500	4000 ± 3000	9300 ± 4900
	Low Oil/Ref	12000 ± 5400	1800 ±680	18000 ± 8400
alkB	High Oil	6.2 ± 3.0	3.2 ± 2.9	2.5± 1.8
	Low Oil/Ref	57 ± 34	3.0 ± 1.2	5.0 ± 3.5
GN PAH-RHDα	High Oil	2.1 ± 2.0	2.3 ± 1.3	2.9 ± 1.3
	Low Oil/Ref	2.1± 0.87	1.3 ± 1.5	3.5 ± 1.6
GP PAH-RHDα	High Oil	87 ± 62	90 ± 40	970 ± 520
	Low Oil/Ref	82 ± 50	87 ± 34	1800 ± 1300

Real-time PCR results for the highly oiled sites were as follows: Significant differences were observed in *alkB* ($p<0.05$; n=39), *GN PAH-RHDα* ($p<0.05$; n=39), and *GP PAH-RHDα* ($p<0.05$; n=38) gene ratios over the course of the three samplings. Relative abundance of *alkB* was relatively unchanged from the first to second samplings but by the third sampling at twenty-nine moths the ratio was roughly one third of what it had been at twenty-two ($p<0.05$; n=26) and thirteen months ($p<0.05$; n=26). Both *RHDα* gene ratios tended to increase over the course of the experiment. Between the thirteen and twenty-two month samplings there was a four-fold increase in *GN PAH-RHDα* abundance ($p<0.05$; n=26) and a roughly three-fold increase in *GP PAH-RHDα* abundance ($p<0.05$; n=25). The relative abundance of the *GN PAH-RHDα* at twenty-nine months was relatively similar to that at twenty-two months and about five-fold greater than at thirteen months ($p<0.05$; n=26). The *GP PAH-RHDα* abundance at twenty-nine months was about three-fold greater than at twenty-two months ($p<0.05$; n=25) and roughly ten-fold greater than it had been at thirteen months ($p<0.05$; n=0.05).

The overall trends observed in the reference sites closely resembled the results from the highly oiled soils and were as follows: Significant differences were observed in *alkB* ($p<0.05$; n=12), *GN PAH-RHDα* ($p<0.05$; n=12), and *GP PAH-RHDα* ($p<0.05$; n=12) gene ratios over the course of the three samplings. The abundance of *alkB* decreased between thirteen months and twenty-two months and again at twenty-nine months but neither decrease was significant. However the relative abundance of *alkB* in the final sampling were nearly ten-times smaller than it had been in the first sampling ($p<0.05$; n=12). Between samplings at thirteen and twenty-two months there was a more than ten-fold increase in the relative abundance of *GN PAH-RHDα* ($p<0.05$; n=12) and about a six fold increase of *GP PAH-RHDα* abundance ($p<0.05$; n=12). Both *RHDα* abundances more than doubled from twenty-two and twenty nine months but neither

increase was significant. A roughly ten-fold increase occurred between the first and final samplings for the *GN PAH-RHDα* ($p<0.05$; n=8) and the *GP PAH-RHDα* ($p<0.05$; n=8). On two occasions reference sites provided relative abundances of functional genes that were significantly greater than the highly oiled samples from the same sampling trip. At thirteen months the relative abundance of *alkB* was about five times greater in the reference sites than in the highly oiled sites ($p<0.05$; n=17) and at twenty-two months the *GN PAH-RHDα* abundance was about four-fold greater in the reference sites ($p<0.05$; n=17).

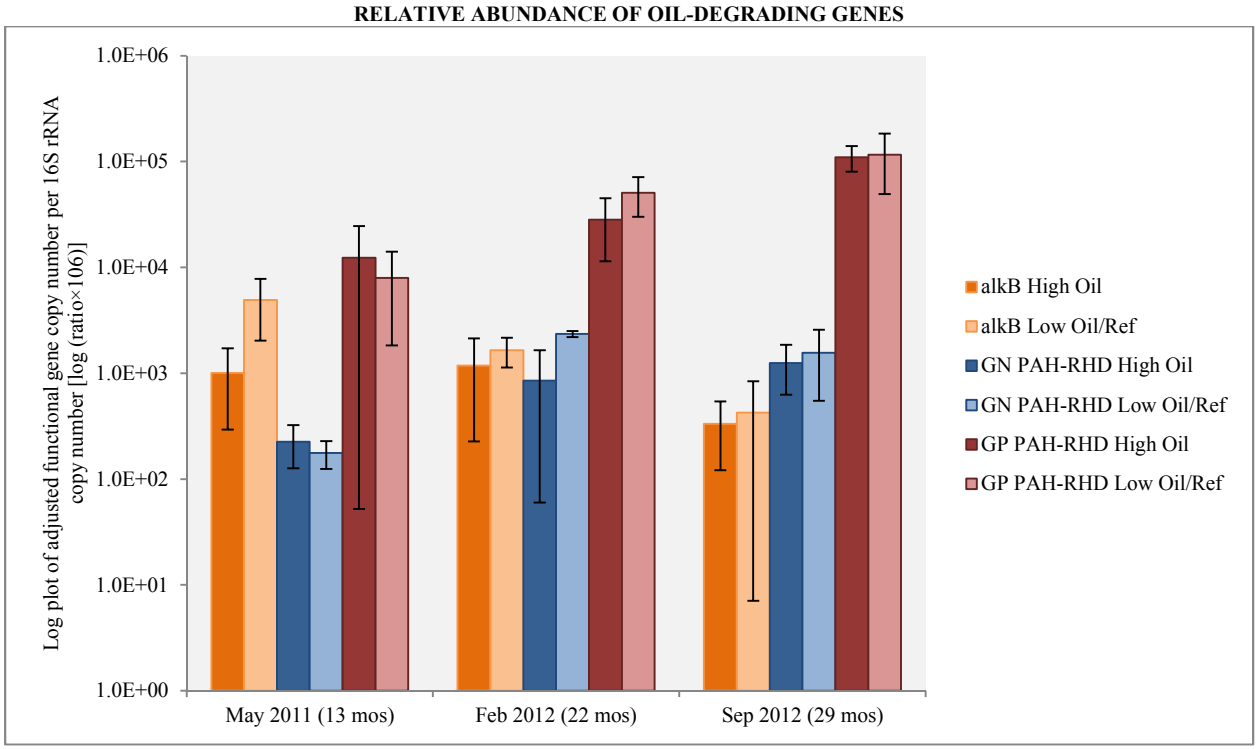

Figure 3.1: Log plot of adjusted real-time PCR determined oil-degrading genes per 10^6 copies of 16S rRNA

Spearman's correlation was used for comparison of TPH and genetic data. The overall abundance as indicated by the 16S rRNA gene abundance was significantly negatively correlated with TPH in May 2011 (r_s=-0.55; $p<0.05$) but positively correlated in February 2012 (r_s=0.74;

$p<0.05$). The ratios for the *GN PAH-RHDα*/16S rRNA showed a negative correlation in February (r_s=-0.89; $p<0.001$) and a similar negative correlation of *GP PAH-RHDα* for the same sampling (r_s=-0.80; $p<0.05$).

3.2 Culture-Based Results

The culture based estimation of total aerobic bacterial populations supported the supposition of shrinking populations by showing that aerobic populations in the highly oiled sites at thirteen months were about four-fold greater than they were at twenty-two months ($p<0.05$; n=22). The reference sites, however, did not reveal any changes. Ratios of aerobic oil-degrading CFU per total aerobic CFU were used to examine the oil-degrading populations for the May 2011 and February 2012 samplings. The ratios revealed that the relative abundance of oil-degraders in the highly oiled sites at twenty-two months was nearly five-fold greater than at thirteen months ($p<0.05$; n=21). The abundance of oil-degraders in the reference also increased, nearly doubling, from thirteen to twenty-two months but the difference was not significant.

At thirteen months the highly oiled sites revealed a marginally larger estimate of oil-degraders than the reference sites and at twenty-two months the highly oiled sites revealed roughly five times the oil degraders as the reference sites but neither difference was significant. Regression analysis of the relative abundance of oil degraders and TPH data revealed no significant correlation in either sampling. Regression revealed that total aerobic CFU decreased as TPH increased for the sampling at twenty two months (r^2=0.69, $p<0.05$). This is in contrast to the positive correlation indicated by the 16S rRNA qPCR data for the same sampling.

Table 3.2: Culture-based estimates of bacterial populations and oil-degrading ratios. Values are means ± standard deviations.

Quantitative-PCR Estimates for Gene Copy Number per Gram of Soil (dry weight)

	Site Type	Estimated Population (10^5 CFU) at 13 months	Estimated Population (10^5 CFU) at 22 months
Total Aerobic CFU g^{-1} (dw)	High Oil	49 ± 35	14 ±12
	Low Oil/Ref	32 ± 9.2	32 ± 5.3
Total Oil Degrading CFU g^{-1} (dw)	High Oil	2.7 ± 2.0	3.1 ± 0.98
	Low Oil/Ref	1.2 ± 4.8	3.0 ± 0.98
Oil Degrading CFU/ Total Aerobic CFU	High Oil	6.5±4.7%	35±28%
	Low Oil/Ref	5.2±4.3%	9.1±2.4%

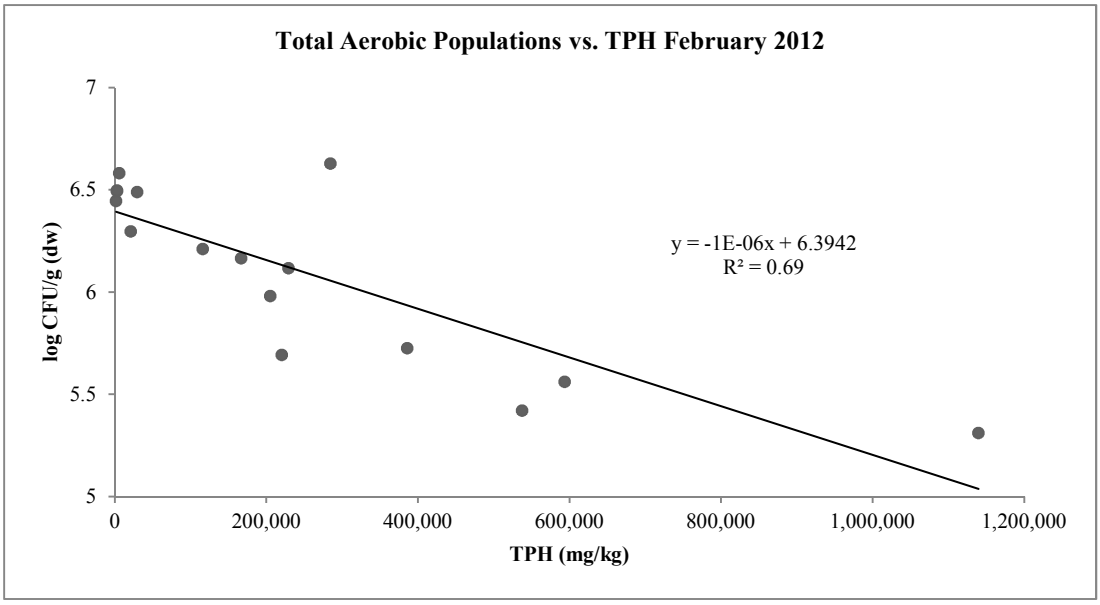

Figure 3.2: Regression analysis of aerobic CFU vs. TPH for February 2012. Significant at $p<0.05$, $r^2=0.69$, and $n=15$.

DISCUSSION

4.1 Dominance of the Gram-Positive PAH-Degraders

It has been well established that the compositions of bacterial populations following hydrocarbon contamination are dynamic and that the nature of the changes are unique to each event (Alonso-Gutierrez et al., 2009; Atlas, 1981; Beazely et al.,2012, Kotska et al., 2011). The bacterial communities evolve to cope with hydrocarbon- induced stresses and new community structures will form which are better suited to oil contamination (Atlas, 1981; Macnaughton, 1999; Paisse et al., 2008). The results of this study revealed that the population of oil-degrading bacteria in the oil-inundated soil of northern Barataria Bay was dynamic and contained relatively fewer n-alkane degraders and relatively more PAH-degraders as time went on. This is not surprising, as alkanes are generally degraded before PAHs and will be exhausted more quickly (Atlas, 1991). Though the ratios for both PAH-degrading genes increased in each successive sampling the oil degrading communities were dominated by the gram-positive PAH degraders, with more than 99% of the oil-degrading genes detected in the final sampling being *GP PAH-RHDα*. Supplemental 16S rRNA sequencing of oil-degrading isolates from October 2012 samples from the same sites also supported the findings of the qPCR. Two thirds of the eighty-five oil-degrading colonies identified were gram-positive, with 48% of the identified colonies belonging to phylum *Firmicutes* and 18% belonged to the phylum *Actinobacteria.* The genus *Bacillus* was by far the most prevalent member of the community and constituted 38% of the sequences isolated.

Relatively large populations of gram-positive oil-degraders in coastal sediment following hydrocarbon contamination have been previously reorted. Alonso-Gutierrez et al. (2009) studied

sediments a year after an oiling event and found that 28% of the oil degrading species were gram-positive *Actinobacteria*. Zhuang et al. (2003) reported that at least 69% of the population of observed naphthalene-degrading bacteria in oil-contaminated tropical coastal sediment was gram-positive. Kotska et al. (2011) observed a shift away from alkane degrading bacteria of the genus *Alcanivorax* and toward gram-positive oil degraders (including *Bacillus* and *Microbacterium*) in a study of oil-impacted beach sands of Florida following the BP spill. The authors of this study suggest that the shift is indicative of latter stages of the biologically-driven attenuation of hydrocarbon-contaminated soils. The shift occurs once easily degradable n-alkanes are depleted while PAH crude-oil components remain plentiful. The decreasing *alkB* abundance and increasing *PAH-RHDα* abundance in this study indicate that a similar succession is likely taking place in the marsh soils of Barataria Bay. The extremely high and virtually unchanged TPH concentrations in the soils also support the idea that there are copious amounts of persistent and recalcitrant PAHs in the soils.

Beazley et al. (2012) reported that gram-positives increased over the course of a study but that *Firmicutes*, unlike the *Actinobacteria*, continued to increase once TPH levels were below detection. The authors echo a hypothesis of gram-positives dominating later stages of oil-attenuation in coastal environments but specifically cite members of *Firmicutes* as key players. Salt-marsh community enrichment with *Firmicutes* has also been observed in a laboratory setting (Pearson et al., 2008) and members of the phylum of the genus *Bacillus* have been found capable of degrading high molecular weight PAHs (Maiti, Das, and Bhattacharyya, 2012). This study supports the theory of *Firmicutes* playing a key role in the attenuation of crude-oil in salt marshes as it revealed that bacterial populations thirty months after the initial event were dominated by members of *Firmicutes* and in particular *Bacillus*.

The dominance of gram-positive PAH-degraders in this study is likely due in large part to the volatile nature of the experimental sites. The peptidoglycan cellular envelope of the gram-positive cell makes it more fit for survival than its gram-negative counterpart in some variable and volatile environments including intertidal environments (Alonso-Gutierrez et al., 2009; Zhuang et al., 2003). Alonso-Gutierrez et al. (2009) also suggest that the gram-positive anatomy may also provide extra protection against toxic agents which could increase capacity to breakdown recalcitrant hydrocarbons.

4.2 Increased Oil-Degrading Genes in Reference Sites

Table 4.1 summarizes several studies which were concerned with bacterial populations in oil-contaminated marsh soils. The table illustrates the fact that this study involved analysis of populations in soil unusually saturated with oil. In our case, the use of the term "reference" site is misleading, as our reference sites contain enough oil to have been deemed "highly oiled" in many previous works. However, the reference sites were defined by NOAA and were categorically different than the highly oiled sites. Most notably the sites did contain lower TPH levels and contained more vegetative coverage than the highly oiled sites.

Table 4.1: Observed TPH levels in other oil-spill soil studies (**DWH* spill study).

COMPARISON OF TPH LEVELS OF OIL SPILL STUDIES

Study	Min TPH (mg/kg)	Max TPH(mg/kg)	Soil Type
This Study	920	877,023	La. Salt Marsh
Anderson et al. (2001)	800	46,000	New York Salt Marsh
Andrade et al. (2004)	1,700	8,000	Spanish Estuary
*Beazley et al. (2012)	31	189	Alabama Salt Marsh
*Kotska et al. (2011)	3.1	4,500	Gulf of Mexico Beach Sand
Paisse et al. (2008)	42	286	Retention Sediment

On two occasions functional gene copy numbers were significantly greater in the reference sites than in the highly oiled sites- *alkB* at thirteen months and the gram-negative *RHDα* genes at twenty-two months. A likely contributing factor for this difference is the observed vegetative cover. Field observations and estimations of the highly oiled sites indicated that at thirteen months (when the *alkB* abundance was greater in the reference sites) only one of these sites had more than about 5% vegetative coverage. At twenty-two months (when *GN PAH-RHDα* abundances were greater in the reference sites) only two had more than about 1% coverage. By contrast only one reference site from the first two samplings was estimated to have less than 50% coverage. At twenty-nine months, the vegetation seemed to have recovered somewhat and nine of the thirteen highly oiled sites had more than 5% coverage. No significant differences were detected between genes in highly oiled and reference sites for this sampling.

Bacterial degradation of hydrocarbons is generally increased when a healthy plant community is present (Ho and Banks, 2006; Ho, Applegate, and Banks, 2007; Joner et al., 2001). Overall bacterial populations are supported via provision accessible organic material from the plants (Joner at al., 2001) while the healthier rhizosphere can promote oil-degrading genes' expressions and secrete degradation-facilitating oxidative enzymes (Ho, Applegate, and Banks, 2007; Joner et al., 2001). A healthier plant/microbe system in the reference sites also may have contributed to more robust oil-degrading communities via nutrient provision. Nutrients including nitrogen and phosphorous are proven limiting factors in hydrocarbon degradation rates (Atlas, 1981 and Toccalino, Johnson, and Boone, 1993). Inhibition of these nutrients' cycles as a result of decreased vegetative biomass could be a key contributing factor in the smaller oil-degrading populations in the sparsely vegetated and highly oiled sites.

4.3 Negative *RHDα* Correlations at Twenty-two Weeks

Though the vegetative coverage may help explain the disparity between the high oil and reference sites the correlation analysis reveals that TPH levels at twenty-two months had a significant effect on both *RHDα* gene abundances for this sampling. The negative correlation may be due to the toxic effects of the hydrocarbon contamination being more severe where the contamination is most dense. The increased toxicity may impede certain PAH-degrading members of the communities in the highly oiled sites as they evolve to cope with the spill.

The negative correlation between 16S rRNA and TPH during the first sampling suggests that the soil bacteria communities were diminished due to toxic effects from the oil contamination. The positive correlation between the same two factors in the next sampling and the lack of any significant correlation in the final sampling suggest that the communities evolved over time to cope with the oiling. A similar pattern is found with the *RHDα* genes, though not in the same samplings. Both highly oiled samples' *RHDα* genes relative abundance decreased as TPH levels increased at twenty-two months but no such correlation existed by the final sampling. Group comparisons also revealed that the *RHDα* abundance in the highly oiled sites increased significantly from twenty-two to twenty-nine months to levels comparable to the reference sites. These findings are illustrative of the theory that the response of the oil-degrading populations in the highly oiled sites was subdued due to the increased toxicity of the heavier oiling.

It is common for crude oil components to exhibit toxic effects on total and oil-degrading bacterial populations. Sikkema et al. (1995) attributed to this effect of hydrocarbons to the ability of lipophilic hydrocarbon components to penetrate and disrupt the cytoplasmic membrane and increase the cells susceptibility to hazards including protons and ions (Sikkema,de Bont, and

B Poolman, 1995). It is also possible that heavy metals including vanadium and nickel have been deposited by the *DWH* spill (Floyd, Lungu, and Gohlke, 2012). These metals are known to be toxic to many microbes (Baath, 1989 and Andrade et al., 2004) and could have resulted in the observed negative correlations.

4.4 Incongruity of Culture-Based Data

Though it is a common practice to examine bacterial populations using traditional plating techniques it is widely recognized that they are not as powerful or informative as modern molecular genetic techniques including qPCR (Habi and Omori, 2003; Torvik et al., 1996; Ward et. al., 1990). The trends observed in the culture-based counts of total aerobic bacteria from the first two samplings support the trend presented by the qPCR data though the differences found in the culture-based data were not all significant. Likewise, the oil-degrading population relative abundance increased according to both the qPCR data and the culture-data though not all culture-based increases were significant. In addition the positive correlation between *16SrRNA* and TPH at thirteen months that was not supported by a significant correlation between total aerobic CFU and TPH levels. These results find a likely explanation in the aforementioned decreased sensitivity of culture-based techniques when compared to molecular-genetic analysis.

Not all of the culture-based data agreed with the qPCR data, however. At twenty-two months there were major differences between the correlations of total-bacteria abundances and TPH levels. The 16S rRNA copy number was positively correlated with TPH for this sampling but the aerobic CFU was negatively correlated with TPH. Anaerobic population fluctuations could partially account for this difference. Quantification of 16S rRNA includes genes from anaerobic cells while aerobic plating techniques do not account for anaerobic populations. More

likely, however, is that many of the dominant members of the new community structures in Barataria Bay's soils are not easily cultivated in a laboratory environment. It has been estimated that over 90% of effective oil-degraders are not culturable using current laboratory techniques (Mcnaughton et al., 1999). If, following the oiling event, many members of the resulting communities were nonculturable then the positive correlation between the total abundance and TPH levels found in the qPCR data may not be revealed using plating techniques. An increasing unculturable population as a function of TPH levels could cause the negative correlation between total aerobic CFU and TPH found in the twenty-two month samples of this study. It has also been found that bacteria may enter a viable but nonculturable (VBNC) state when starved for nutrients or introduced to other environmental stresses (Oliver, 2005). Such stress may have resulted from depleted vegetative coverage or the increased hydrocarbon concentrations at the site and may have resulted in the scope of the culture-based methods being limited.

CONCLUSION

This study determined that the oil-degrading populations made up an increasingly larger portion of the bacterial community in the salt marsh soils contaminated by the *DWH* oil spill as time went on. The relative abundance of alkane-degraders decreased as the relative abundance of PAH-degraders increased during the course of twenty-nine months after the spill and was likely due to the depletion of n-alkanes and persistence of recalcitrant PAHs in the soil. The oil-degrading populations were increasingly dominated by gram positive PAH-degraders over time. Based on these findings, the Louisiana salt marsh impacted by the *DWH* spill has come into its latter stages of naturally occurring microbial attenuation after twenty-nine months since the spill. Future research continuing to exploit changes in indigenous microbial populations in these marsh soils would determine the long-term impacts and recovery from the *DWH* spill on microbial communities and associated biogeochemical processes. The greater abundance of oil-degrading genes observed in the lightly oiled reference sites when compared to the highly oiled sites was likely attributed to a healthy oil degradation-facilitating rhizosphere and decreased toxicity associated with lower oil levels. The increased vegetation in the reference sites also likely supported more robust nutrient cycling which may have increased the abundance of limiting nutrients including nitrogen. The observed disparity between the vegetated and unvegetated sites warrants future research of the nutrients in the contaminated soils.

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VITA

Kristopher Nolan Ackoury was born in Lake Charles, Louisiana in 1987. As a young boy he always enjoyed the unique environment that south Louisiana provided. In what can be seen as an indicator of what was to come, his childhood was full of make-shift scientific investigations into everything from pond water to the stars to dinosaur fossils. After enrolling in McNeese State University in Lake Charles in 2005, Kris' appreciation for the living world played a key role in his decision to earn a degree in Biology Education. Kris thoroughly enjoyed sharing his love of his chosen subject matter with high school students, but after graduating from McNeese State with honors in 2010 he knew that his academic career would continue.

In 2011 Kris enrolled at L.S.U. in hopes of obtaining a Master's Degree in Environmental Science. His background in life sciences lead him to research in microbiology under Dr. Aixin Hou and which lead to a job researching biopolymer production for TMD Technologies starting in the Summer of 2012. He is currently working with TMD on groundwater remediation and wastewater treatment. After graduating, Kris hopes to use his professional and academic experiences to continue to grow his career in the private sector and provide for a family.