2014

DGGE Analysis of Denitrifying Bacterial Diversity in Response to Nutrients and Oil Contamination in Salt Marshes

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DGGE ANALYSIS OF DENITRIFYING BACTERIAL DIVERSITY IN RESPONSE TO NUTRIENTS AND OIL CONTAMINATION IN SALT MARSHES

A Thesis

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

in

The Department of Environmental Sciences

by
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B.S., Louisiana State University, 2011
May 2014

ACKNOWLEDGMENTS

I would like to express my most sincere thanks to my major professor and advisor Dr. Crystal Johnson for her guidance, support, patience and encouragement throughout my graduate studies. To the member of my committee: Dr. Portier, thank you for your help and advice, for providing me with MC252 oil, and the materials required for the microcosm. And to Dr. Hou, thank you for all the help and advice throughout both projects and for serving in my committee.

I would also like to thank Dr. Rivera-Monroy for giving me the opportunity to collaborate in the denitrification rates project, and for all his help. To Edward Castañeda for the sediment core samples in the first project and for all the help and guidance. To Dr. Laws for helping me with MATLAB and the statistics part. I also want to thank all the faculty and staff at the Environmental Sciences Department at LSU for their support and encouragement.

To all my friends and co-workers at the Johnson lab: Vanessa Molina, Krystal Cole, Drusilla Cowan and Hannah Midyett, thank you all for making every day fun and interesting. Also, special thanks to Angelica Hernandez for assisting me in the field collecting sediment cores; I truly appreciate all the help and hard work.

Finally I'd like to thank my loving family: my father Santiago, my mother Marta, my siblings Juan and Alejandro and my dogs Olafo and Addie for all their love and encouragement. To Michael Bruckner for his love and support and to all my friends at LSU.

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ABSTRACT

Salt marsh ecosystems located at the Mississippi River delta are exposed to high levels of point and nonpoint sources of nutrients such as nitrate-based fertilizers and to oil spills in the Gulf of Mexico. However, there is little information on the effects of eutrophication and oil contamination on the bacterial diversity of denitrifying bacteria in salt marsh sediments of coastal Louisiana. The effects of excess nutrients on the bacterial diversity of denitrifiers were analyzed by injecting sediment cores with high concentrations of nitrate (100μM NO_3- and 20,000μM NO_3-) and carbon (0% and 20% C concentration from the original sample). After 7 days of incubation, the diversity of denitrifying bacteria at the top (aerobic) and bottom (anaerobic) samples was analyzed with polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE). Denitrifying bacterial diversity was significantly lower (P<0.05) at the top than at the bottom when treated with 100μM NO_3- and no carbon, but not on the other treatments. The number of species at the top was also significantly higher (P<0.01) in treatments with a high nitrate concentration (20,000 μM NO_3-) and where a carbon source was added along with nitrate. The effects of oil contamination on the bacterial diversity of denitrifiers was analyzed by treating sediment cores from black mangroves (*Avicennia germinans*) with 2% v/wt. contamination with MC252 oil from the *Deepwater Horizon* spill. DNA was extracted after 24 hours, 21 days and 110 days from both top and bottom samples and followed by PCR and DGGE analysis. No significant changes in the number of species between top and bottom samples were observed, nor when oil addition alone was analyzed. However, significant changes were observed when exposure time was factored in. While samples exposed for 24 hours and 21 days had no significant changes in the number of species of

denitrifiers between oiled and un-oiled samples, un-oiled samples exposed for 110 days had a significantly higher (P<0.001) number of species than oiled samples. The decrease in the number of species was probably the consequence of a decrease in the C:N ratio needed for denitrification and bacterial activity.

CHAPTER 1. INTRODUCTION

1.1 The Nitrogen Cycle

Nitrogen (N) is an essential and limiting nutrient that governs wetland productivity and is available both in inorganic forms such as ammonia (NH_3), ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), nitric oxide (NO) and nitrous oxide (N_2O) and in organic forms such as amino groups ($-NH_2$) which are essential for the formation of amino acids and other catabolic activities (Figure 1)(Reddy and DeLaune 2008).

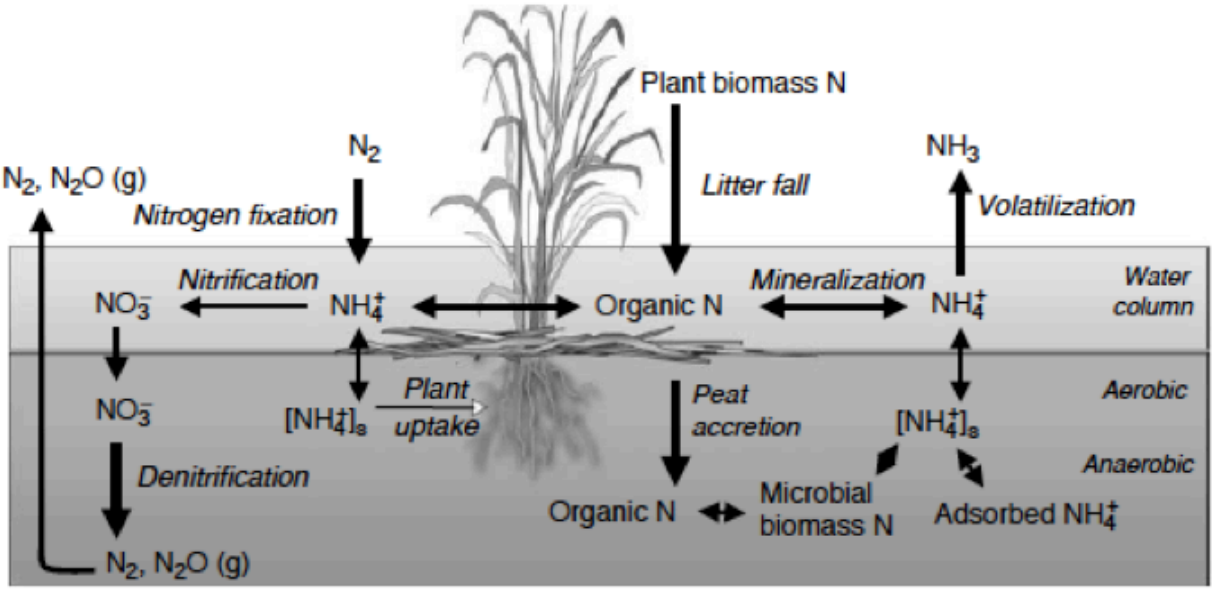

Figure 1. Schematic of the nitrogen cycle in wetlands (Reddy and DeLaune 2008).

Nitrogen gas (N_2) makes up ~78% of the atmosphere. Major nitrogen sinks include water bodies, soil and plants (Figure 2). The constant exchange of nitrogen between these nitrogen sinks and the atmosphere is also referred to as the nitrogen cycle (Reddy and DeLaune 2008) and it is crucial for maintaining healthy and highly productive ecosystems. Nitrogen is introduced into wetlands via nitrogen fixation by microorganisms as well as point and nonpoint sources of industrial forms of nitrogen such as fertilizers, which have

caused major shifts in the nitrogen cycle (Vitousek et al. 1997, Reddy and DeLaune 2008). In wetlands, the microbial conversion of ammonium to nitrate or nitrification provides denitrifiers with nitrate (NO_3-) as an electron acceptor (Reddy and DeLaune 2008). This process is key for completing the nitrogen cycle by connecting nitrogen fixation with denitrification, thus maintaining a balanced input and output of nitrogen into the environment.

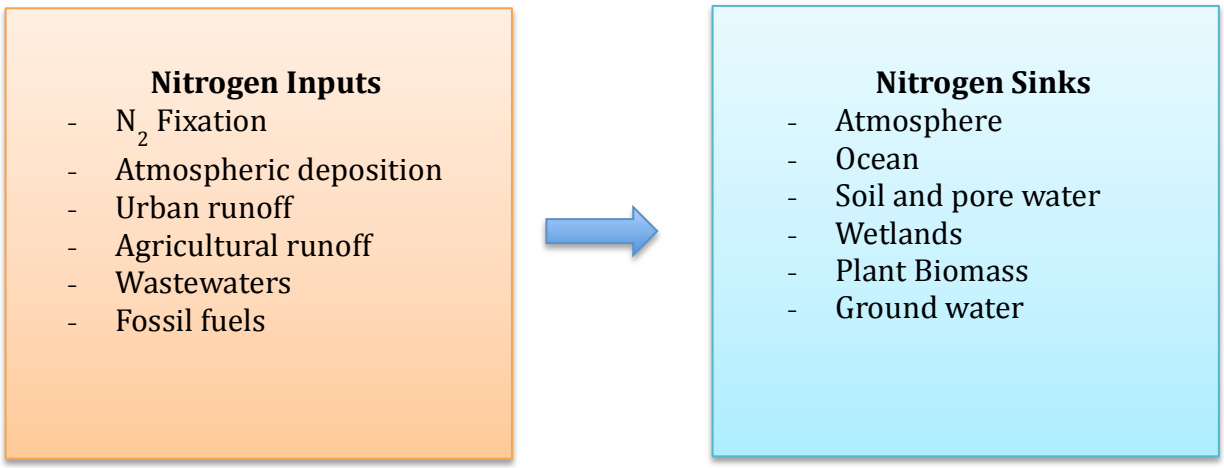

Figure 2. Main sources of nitrogen input and nitrogen sinks in the environment

1.2 Denitrification

Denitrification is the last step in the nitrogen cycle where fixed nitrogen returns to the atmosphere. Denitrification consists of the biological reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to nitric oxide (NO) and quickly converting to nitrous oxide (N_2O), which is then converted to nitrogen gas (N_2) (Figure 3). This stepwise reduction is mediated by facultative bacteria and distinguishes denitrifiers from nitrate respiring bacteria in the absence of oxygen (Hallin and Lindgren 1999, Heylen et al. 2006, Reddy and DeLaune 2008).

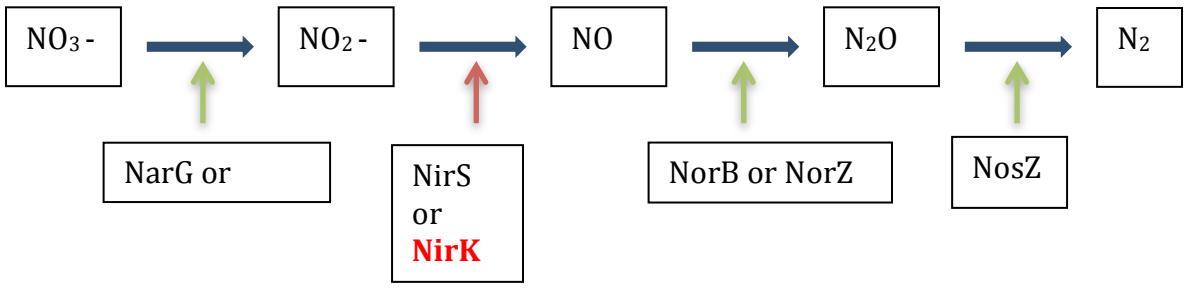

Figure 3. Schematic showing the denitrification pathway with the respective enzymes that catalyze each step.

1.3 Denitrifying Bacteria

Denitrification is a process performed by a wide variety of organisms including fungi and bacteria (Table 1). There are over 130 species from over 50 different genera that are capable of denitrification (Hallin and Lindgren 1999, Priemé et al. 2002). Most bacteria capable of denitrification belong to the *Proteobacteria* phylum (Peralta et al. 2010). Denitrifiers are known to be facultative aerobes, i.e. they have the ability to grow in both aerobic and anaerobic conditions because they can use either oxygen or an alternate electron acceptor (i.e., nitrate) (Reddy and DeLaune 2008). This allows them to survive in a wide range of oxygen concentrations. Denitrification occurs in the anaerobic layer of the soil where pore space is saturated and there is reduced diffusion of oxygen. Production of the proteins required for denitrification occurs in anoxic environments only and if exposed to oxygen their production is inhibited (Van Cleemput et al. 2006).

1.3.1. Nitrate Reductase Genes

Bacterial reduction of nitrite (NO_2^-) to nitric oxide (NO) is catalyzed by either one of the two nitrite reductase (Nir) enzymes found in denitrifying bacteria (Figure 2). The first nitrite reductase is a cytochrome (cd) enzyme containing heme c and d_1, encoded by *nirS,* and the second is a copper (Cu) containing enzyme encoded by *nirK*. Although structurally different, both enzymes catalyze the same reaction (Braker et al. 1998, Braker et al. 2000,

Priemé et al. 2002, Qiu et al. 2004). The *nirS* gene is the most predominant nitrite reductase in denitrifying bacteria. However, *nirK* varies in molecular weight, encompasses in 30% of the known denitrifiers, and is found in more frequently in taxonomically unrelated bacteria (Coyne et al. 1989, Hallin and Lindgren 1999, Qiu et al. 2004, Heylen et al. 2006).

Table 1. List of known species of denitrifying bacteria (Reddy and DeLaune 2008).

Actinobacillus	*Escherichia*	*Peptococcus*
Actinomyces	*Eubacterium*	*Photobacterium*
Aeromonas	*Ferrobacillus*	*Planobiospora*
Agrobacerium	*Flavobacterium*	*Planomonospora*
Alicagenes	*Fusobacterium*	*Plesiomonas*
Arachnia	*Gerodermatophilus*	*Propionibacterium*
Arthobacter	*Haemophilus*	*Proteus*
Bacillus	*Halobacterium*	*Pseudomonas*
Bacterionema	*Halococcus*	*Rhizobium*
Bacteroides	*Hyphomicrobium*	*Rothia*
Beneckea	*Hyphomonas*	*Salmonella*
Bordetella	*Klebsiella*	*Selenomonas*
Branhamella	*Lactobacillus*	*Serratia*
Brucella	*Leptothrix*	*Shigella*
Campylobacter	*Listeria*	*Simonsiella*
Cellulomonas	*Lucibacterium*	*Spirillum*
Chromobacterium	*Microbiospora*	*Sporosarcina*
Citrobacter	*Moraxella*	*Streptonmyces*
Corynebacterium	*Mycobacterium*	*Streptosporangium*
Cytophaga	*Neisseria*	*Thiobacillus*
Dactylosporangium	*Nocardia*	*Thiomicrospira*
Enterobacter	*Paracoccus*	*Veillionella*
Erwina	*Pasteurella*	*Vibrio*

Although both reductases are mutually exclusive at the strain level, any given species can encode either one of two types of *nir* genes but not both (Heylen et al. 2006). The mechanisms that cause a strain in one organism of one species to carry *nirS* and another strain in another organism of the same species to carry *nirK* are not yet understood. Environmental factors such as water saturation levels, salinity, pH, oxygen tolerance and carbon sources could have an effect on denitrification rates in soil (Heylen et al. 2006, Cao et al. 2008) as well as which enzyme is encoded (Heylen et al. 2006). For example, Archaean denitrifiers, which are known halophiles, are more likely to encode *nirK* as opposed to *nirS* (Priemé et al. 2002). This suggests the influence of environmental factors such as salinity in the process of selecting which nitrate reductase enzyme is encoded.

1.3.2. Denitrifiers in Microbial Ecology Studies

While some studies on the microbial ecology and denitrification processes have been conducted in relation to hypoxia in the Gulf of Mexico (Carl et al. 2002), little is known about the structure of denitrifying bacterial communities in salt marsh sediments in coastal Louisiana and how they respond to changes in nutrients and oil contamination. Choosing denitrifying bacterial communities as models for microbial ecology analysis is adequate for several reasons. First, there is a high quantity of taxonomic diversity among denitrifiers (Philippot and Hallin 2005). Second, there is an increasing level of understanding of the denitrification process and the genes involved (Philippot and Hallin 2005). Third, denitrifiers have an important role in nutrient cycling that can also affect global climate change. Finally, examination of the denitrifying bacterial community using molecular methods such as denaturing gradient gel electrophoresis (DGGE) are now more precise and

provide a better visualization of microbial communities compared to other molecular methods (Throbäck et al. 2004).

1.4 Role of Denitrification on a Global Scale

Denitrification is of global concern due to its implication in global climate change, agriculture, water quality and nutrient cycling. Both nitric oxide (NO) and nitrous oxide (N_2O) are important greenhouse gases responsible for the destruction of the ozone layer (Hallin and Lindgren 1999, Braker et al. 2000, Priemé et al. 2002, Henry et al. 2004). The release of NO and N_2O from both sediment and water into the atmosphere during denitrification plays a role in global climate. Environmental factors such as O_2 levels and water saturation can possibly have an effect on what denitrification genes are expressed and on whether denitrification is completed or not. Microorganisms possessing the NosZ enzyme, which catalyzes the reduction of N_2O to N_2, reduce nitrous oxide emissions into the atmosphere (Sanford et al. 2012). Usually, complete denitrification is more predominant in anoxic and saturated environments while incomplete denitrification is more prevalent in drier and aerobic environments (Peralta et al. 2010).

In agriculture, denitrification leads to significant losses of nitrogen, an important crop nutrient (Qiu et al. 2004) and this leads to extensive use of nitrogen fertilizer. Denitrification is also crucial in the removal of excess nutrients and pollutants in coastal zones such as marshes and mangrove ecosystems (Gilliam 1994, Rivera-Monroy et al. 1999). Intensifications of anthropogenic activities near coastal waters and river basins lead to the increase of nutrient and pollutants inputs. Denitrification aids in the removal of excess nitrate from fertilizer applied to agricultural fields and releases it back in the atmosphere as N_2, thus removing excess nutrients before they leach to neighboring waters

where they could lead to eutrophication-related problems. Eutrophication is not only detrimental to water quality but it also has important ramifications in the health of nurseries and fisheries. It could potentially change the biological and physical factors that could allow the environment to intrinsically recover.

1.5 Role of Denitrification on a Local Scale

In the United States, the ramifications of agricultural practices within the 31 states that contribute to the Mississippi River flows are observed in coastal Louisiana where the river reaches the Gulf of Mexico. Because the Mississippi River drains 41% of the area of the continental United States, it provides a crucial source of freshwater, nutrients and sediment transport (Rabalais et al. 2002, Rivera-Monroy et al. 2013). In coastal Louisiana, the Mississippi River delta region houses the largest wetland ecosystem in the United States (Shaffer et al. 1992, Visser et al. 2000, Rivera-Monroy et al. 2013). Part of the high productivity in this area is due to its role as a carbon, phosphorous and nitrogen sink (DeLaune and White 2012, Rivera-Monroy et al. 2013). This environment is rich in nutrients and it serves as nursery and breeding grounds to a large number of economically important species of fish, shellfish, crustaceans, and bivalves. However, too much nutrient input could lead to eutrophication, which could be detrimental to fisheries.

Historically, coastal Louisiana has been receiving large amounts of nitrate fertilizer from farms all across the country since the 1940s. Fertilizer use has increased 7-fold since the 1950s and the amount of nitrate measured in the lower Mississippi basin has increased from approximately 20 μM to 140 μM in the past four decades (Rabalais et al. 2002). Plant uptake of fertilizer is not 100% efficient, thus resulting in excess fertilizer leaching to nearby basins and water systems that connect with the Mississippi River until it reaches

wetlands in the Mississippi River delta. Most of the nitrate inputs in the Mississippi River come from nonpoint sources, mainly agriculture, and 61% of the nitrogen flux that enters the Mississippi River comes in the form of nitrate (Rabalais et al. 2002). An average concentration of 100μM NO_3- has been reported in coastal Louisiana (Rivera-Monroy et al. 2010). This high concentration of nitrate loading in the wetland ecosystem can potentially have major effects in the biogeochemical processes that help maintain a healthy ecosystem and its ability to recover.

Denitrification in wetlands is crucial for mitigating such large incursions of nitrate, thus achieving balance in the ecosystem's biogeochemistry and improving water quality. Studies in riparian wetlands have measured increases in denitrification rates when exposed to nitrate enrichments from underground sources (Hanson et al. 1994). In this case, 50% of the nitrate that entered the wetland was successfully removed by denitrification. This same study also found higher denitrification rates in poorly drained sediments (i.e., wetlands) than well-drained sediments from upland forests.

Studies on denitrification rates in Louisiana wetlands have found similar results. Salt marshes in coastal Louisiana are capable of high rates of denitrification when exposed to high concentrations of nitrate (Rivera-Monroy et al. 2010). A 2006 study comparing the denitrification potential of wetlands and riparian ecosystems reported up to 70% nitrate removal in the Mississippi delta region (Mitsch and Day Jr 2006). A study by Rivera-Monroy et al. (2013) predicts that denitrification in coastal Louisiana can potentially remove 19% to 25% of the total nitrate being loaded before it can reach the Gulf of Mexico. This buffering capacity of wetlands to absorb many excess nutrients and pollutants is

crucial in minimizing eutrophication problems such as the hypoxic dead zone found in the Gulf of Mexico (Rabalais et al. 2001, Rabalais et al. 2002, Rivera-Monroy et al. 2010).

In addition to nutrient loading, stressors such as sea level rise, pollution, subsidence, hurricanes and anthropogenic activities such as fisheries and oil and gas exploration are all stressors that can also potentially decrease the benefits from denitrification via wetland loss. Although many studies have been performed on denitrification potential and denitrification rates in Louisiana, few studies have examined bacterial denitrification changes in response to nutrient fluxes. Understanding both denitrification potential and bacterial communities is fundamental in ecosystem management practices.

1.6 Other Important Biogeochemical Mechanisms in Wetlands

Recently, alternate forms of denitrification have been detected with the help of new molecular and ^{15}N methods, thus revolutionizing the understanding of how nitrogen is cycled in wetland environments (Figure 4). These include chemodenitrification, which is characterized by the conversion of ammonium to nitrite and dissimilatory nitrate reduction to ammonia (DNRA), which is characterized by the reduction of nitrate to ammonia (Macko and Estep 1984, Reddy and DeLaune 2008, Giblin et al. 2013). Another important process possibly occurring in coastal wetlands includes anaerobic ammonium oxidation (ANAMMOX). ANAMMOX is the process of converting nitrate or ammonium to dinitrogen gas (Burgin and Hamilton 2007). However, it has been estimated that ANAMMOX is only responsible for about 5% of the total N_2 production (Rivera-Monroy et al. 2013). It is important to highlight these forms of nitrate removal since it is believed that they could play an important role in, or even govern, certain wetland ecosystems under particular environmental conditions.

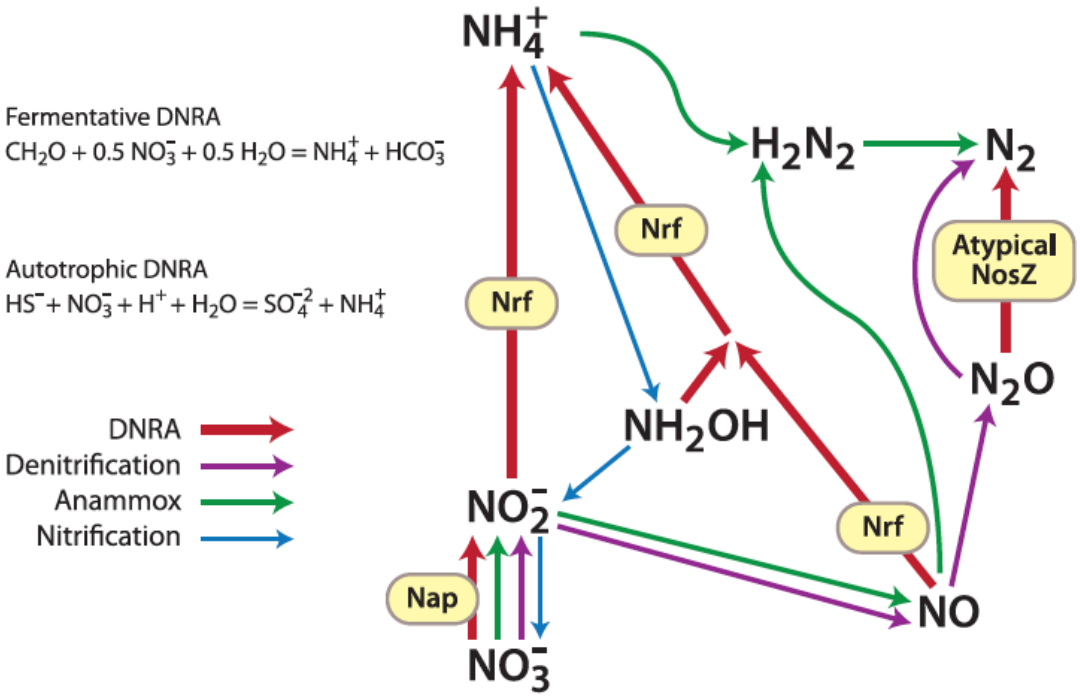

Figure 4. Diagram depicting the different pathways including denitrification, DNRA, ANAMMOX, and nitrification (Giblin et al. 2013).

In the case of DNRA, the reduction of nitrate to ammonia allows the environment to conserve N in the form of NH_4^+ that can later be recycled into nitrate by bacteria, and other microorganisms (Giblin et al. 2013). The first step in the DNRA pathway is the conversion of nitrate to nitrite and it is catalyzed by the NapAB enzyme, which is subsequently reduced to ammonium by a pentaheme cytochrome C nitrite reductase enzyme (NrfA) (Einsle et al. 2002) encoded by *nrfA* that is found in several species of bacteria (Mohan et al. 2004). Another enzyme that catalyzes nitrite to ammonium is octaheme tetrathionate reductase (Otr), which is commonly found in metal-reducing bacteria (Atkinson et al. 2007).

What causes DNRA or denitrification to dominate in certain environments is not yet well understood. This could possibly be related to abiotic factors such as temperature, nutrients,

oxygen levels or salinity. For example, recent studies have identified the prevalence of DNRA over denitrification where salinity increases (Giblin et al. 2010, Giblin et al. 2013). The same study also found evidence that higher temperature selects DNRA over denitrification. One of the most significant implications of DNRA and denitrification is the evidence of the coexistence of both *nirK* and *nrfA* in the same bacterial genome (Sanford et al. 2012).

CHAPTER 2. ANALYSIS OF THE DENITRYING BACTERIAL DIVERSITY IN RESPONSE TO NUTRIENT ENRICHMENTS

2.1 Hypotheses

In the past, several studies have identified nitrogen to be a limiting nutrient in salt marsh ecosystems (Kiehl et al. 1997). However, in soils where nitrogen is found in substantial quantitates, studies have found that other essential nutrients like phosphorous and carbon can become limiting (Van Wijnen and Bakker 1999). From previous studies, denitrification rates in wetland sediments have increased in response to increase concentrations of NO_3- (Rivera-Monroy and Twilley 1996). Nitrate additions in these experiments were based on previous measurements and studies on nitrate input from the Mississippi and Atchafalaya Rivers into coastal Louisiana (Rivera-Monroy et al. 2013). In addition high nitrate concentrations were chosen in order to promote bacterial denitrification activity.

Heterotrophic bacteria obtain their cellular energy from amino acids and amino sugars containing high energy compounds (Macko and Estep 1984). Higher carbon content in such compounds will result in more cellular energy; therefore, bacteria utilizing lower number carbon sources will be required to metabolize more amino acids and sugars to achieve the same amount of cellular energy as those compounds with high carbon numbers (Macko and Estep 1984). Since nitrate amounts in coastal Louisiana are so high, carbon is expected to a limiting nutrient. In order to encourage bacterial activities such as reproduction, metabolism and denitrification, it is important to incorporate a high carbon content source into the experiment.

Different groups of bacteria are expected to live at different depths depending on their adaptability to the different concentrations of organic and inorganic compounds and

oxygen levels. For this reason, it is important to take into account depth of the sampled sediments. It is also known that denitrification rates are much higher in anoxic environments (Reddy and DeLaune 2008).

H_0: Addition of nitrogen, glucose and/or core depth will not cause a significant change in the denitrifying bacterial community.

H_1: Addition of nitrate will cause a significant increase in the number of denitrifying bacterial species.

H_2: Addition of glucose will cause a significant increase in the number of denitrifying bacterial species.

H_3: Bottom samples will be populated by a larger number of denitrifying bacterial species.

2.2 Significance

The Mississippi River delta is home to a diverse and highly productive ecosystem that includes one of the biggest wetland systems in the U.S. This biome is crucial for the protection against hurricanes and houses some of the most productive fisheries in the country. However, anthropogenic activities have caused eutrophication, erosion and land loss, which have forced the entire ecosystem to adapt to changes in nutrient input and increasing sea levels and other variables. Thus, it is important to understand the role of the different contributing factors and participating mechanisms responsible for these environmental changes.

Several studies have looked into denitrifying bacteria communities in a wide range of ecosystems including salt water marshes (Flores-Mireles et al. 2007, Cao et al. 2008), marine sediments (Braker et al. 2000), freshwater (Braker et al. 1998), upland forests and

wetlands (Priemé et al. 2002), activated sludge and waste water (Hallin and Lindgren 1999), and agricultural fields (Henry et al. 2004). However, there are no studies to date that have examined the changes in denitrifying bacterial communities in response to nitrate and glucose additions in wetland sediments from coastal Louisiana. The aim of this study is to better understand how nutrient changes may alter regulation mechanisms in wetland ecosystem and the role denitrifying bacteria play within the nutrient cycles.

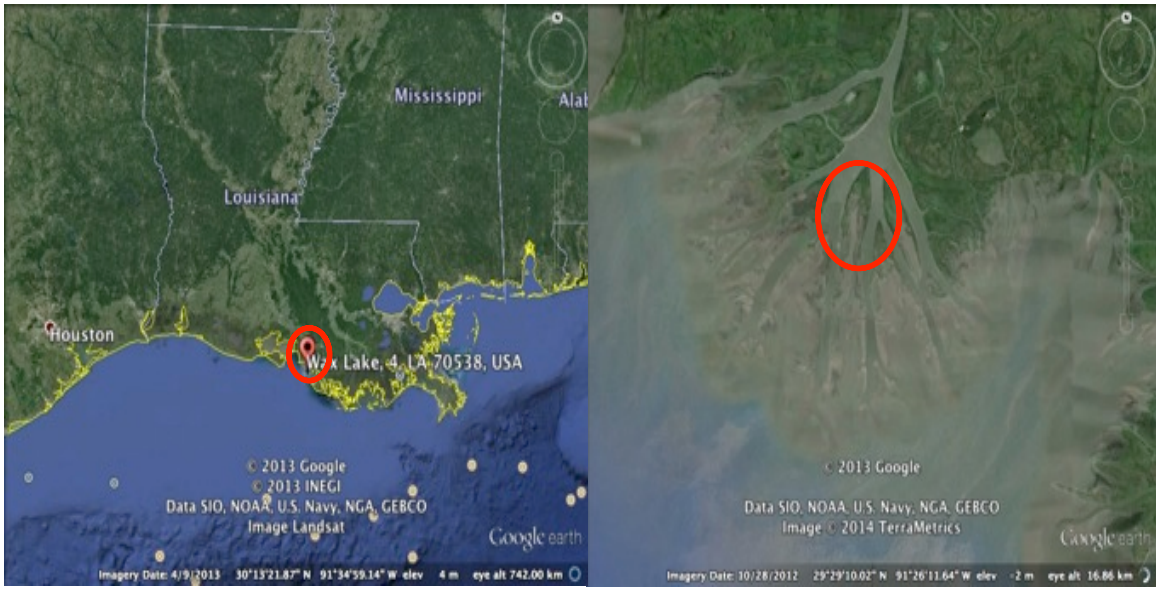

Figure 5. Satellite image of the Wax Lake Delta on the left and the Mike Island section of Wax Lake Delta on the right in Louisiana. Courtesy of Google Earth.

2.3. Materials and Methods

2.3.1 Site of Study

Sediment core samples were collected on March 22, 2012 at Mike Island, Wax Lake Delta, in South Louisiana (29º30'36. 76'' N, 91º26'41. 69'' W) (Figure 5). The vegetation in this area is mostly composed of willows, cattails, and arrowheads. Dr. Castañeda and Dr. Rivera-Monroy from the Department of Oceanography and Coastal Sciences at Louisiana State University provided these samples.

2.3.2 Sediment Core Collection

Dr. Rivera-Monroy and lab members collected twelve sediment cores using 30 cm long Plexiglas cylinders by carefully inserting approximately 25 cm deep into the sediment as explained by Rivera-Monroy and Twilley (1996). To minimize spatial variability, cores were sampled in close proximity to each other. Once the cores were collected, they were capped with a rubber lid and subsequently transported to the laboratory.

2.3.3 Nutrient Enrichments: Nitrate and Carbon

Once cores were transported to the lab, Dr. Rivera-Monroy and lab members performed the core enrichment process. Two of the cores were enriched with 100 μM NO_3- (treatments 1 and 3). The other two cores (treatments 2 and 4) were enriched with a very high concentration of nitrate (20,000 μM NO_3-) in order to supersaturate the sediments with nitrate and induce an increase in denitrification rate. Similarly, two of the cores (treatments 3 and 4) were enriched with a 20% carbon increase in the form of glucose expecting to promote denitrification. This carbon enrichment represents a 20% increase from the initial carbon concentration in the sediment core samples.

While cellulose and chitin are common sources of carbon naturally found in wetland environments, decomposition must take place before microorganisms are able to utilize these sugars. In nature this can be a lengthy process; therefore, a more readily available form of carbon, such as glucose, can be used experimentally in order to speed up this process.

The four different treatment combinations used in this experiment are illustrated in Figure 6. Enrichments were injected into the cores using a needle at multiple locations to a depth of 2 and 9 cm as performed by Rivera-Monroy and Twilley (1996). Ambient

floodwater was used to cover 1-2 cm of the sediment surface to simulate the wetland environment. The cores were then incubated at ambient temperature for 7 days in a sealed cooler for homogenization of the enrichments prior DNA extraction.

2.3.4 DNA Extraction

On day 7 of the incubation, cores were aseptically sectioned into 3 cm thick pieces, ranging from 0- 3cm (top) and 6-9 cm (bottom) in depth, using a sterilized knife. Each piece was placed in Whirl-Paks bags (Nasco, Modesto, CA) and manually homogenized for 60 seconds. DNA from cores was extracted using a previously described phenol-chloroform method (Holzmann et al. 2003). Due to presence of organic inhibitors in the DNA samples, further cleanup was necessary with a PowerClean DNA Clean-Up Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's protocol. Twelve cores were used, and from each, two sections were cut, representing the top (0-3cm) and the bottom (6-9cm). There were four treatments, and for each treatment there were triplicates for both top and bottom, for a total of 24 samples (Figure 6).

2.3.5 PCR Amplification of the *nirK* Gene

Polymerase chain reaction (PCR) was used to amplify the copper containing nitrate reductase gene encoded by *nirK*. The primers used were F1aCu and R3Cu (Throbäck et al. 2004). This set of primers was chosen in part because they yield amplicons of less than 500bp, which, according to Throbäck (2004) is the maximum length possible for appropriate DGGE analysis. A total volume of 50µL was used for PCR amplification using 5X GoTaq buffer, 1mM $MgCl_2$, 0.20mM dNTPs, 0.25µM of each primer, 0.80mg/mL of bovine serum albumin (BSA), 0.04units/µL of GoTaq DNA polymerase enzyme and 4µL of template DNA (Table 2).

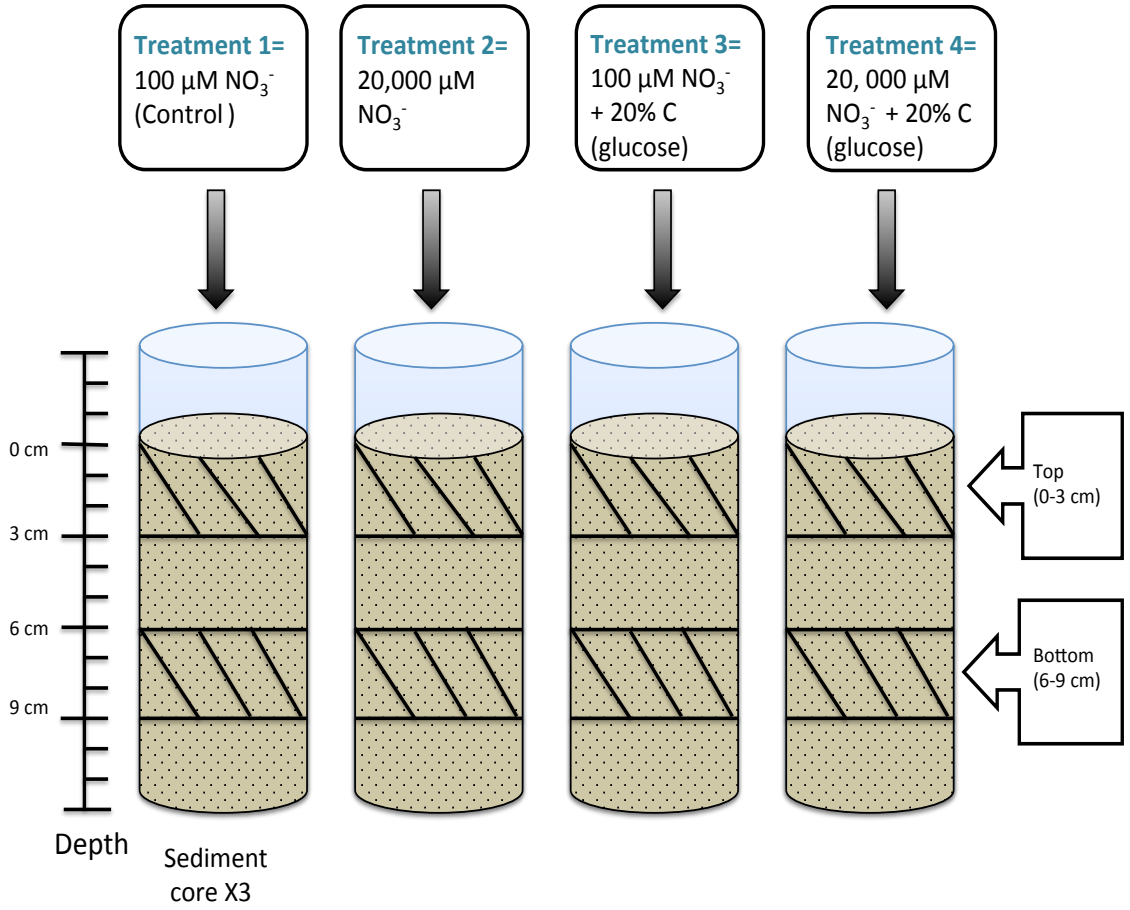

Figure 6. Core enrichments. Treatment 1= 100µM NO_3- (control), Treatment 2= 20,000µM NO_3-, Treatment 3= 100µM NO_3- +20% C (glucose), Treatment 4= 20,000µM NO_3- + 20% C (glucose).

Table 2. PCR preparation for a total volume of 50µL.

Component	Units	Final Concentration
5X GoTaq buffer	X	1
$MgCl_2$	mM	1
dNTPs	mM	0.2
F1aCu primer	µM	0.25
R3Cu-GC primer	µM	0.25
BSA	mg/mL	0.8
GoTaq	Units/µL	0.04
DNA template	µL	4

All samples were run at an initial denaturation temperature of 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing temperature of 57°C for 45 seconds, extension at 72°C for 45 seconds, and a final extension step at 72°C for 10 minutes (Table 3). PCR products were then run on a 2% agarose gel (6g of agarose, 300mL of 10,000X sodium borate buffer (SBB), and 30µL of 10,000 X SYBR Safe) and run at 300V for 1 hour. The agarose gel was analyzed under a BioDoc-It UV Transilluminator (UVP, Upland, CA). To confirm presence and correct size amplification of the denitrifying gene *nirK*, a strand of *Alcaligenes faecalis* (ATCC 8750) was used.

Table 3. PCR amplification conditions for *nirK.*

Step	Cycle (s)	Temperature (°C)	Duration (seconds)
Initiation	1	94	120
Denaturation	35	94	60
Annealing		57	45
Extension		72	45
Final Extension	1	72	600

2.3.6 DGGE Analysis of Denitrifying Bacteria

For DGGE, a 33-bp GC clamp was added to the 5'end of the R3Cu primer (5' GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC 3') was attached to prevent a complete denaturation of the PCR product. PCR conditions were the same as described earlier. The gel was prepared using a 58-70% denaturing gradient and run at 130V for 15 hours in a DGGEK-2001 electrophoresis system (C.B.S Scientific, San Diego, CA). Approximately 20µL of PCR product were added. Visualization of the DGGE bands was performed by staining with approximately 30µL of SYBR Gold in 400ml of 10X SBB and shaking at 75rpms for 45 minutes.

2.3.7 Statistical Analysis

DGGE bands were manually counted for each of the treatments. In this study, each band in the DGGE gel represents a species of denitrifying bacteria. A two-way analysis of variance (ANOVA) using MATLAB (MathWorks, Natick, MA) was used for the analysis of the interactions amongst the four different treatments and for the interactions of the treatments and depth of the sediment samples. A t-test was performed for each of the three treatments using MATLAB.

2.4 Results

2.4.1 Impact of Depth on Bacterial Diversity

The number of species in each core was used to determine the impact of depth in denitrifying bacterial diversity as well as the interactions of depth and the four different treatments using a two-way ANOVA. The two-way ANOVA showed no significant effect in the bacterial diversity of denitrifiers amongst the top and bottom samples. However, a statistically significant interaction (P=0.047) between treatments and depth was observed (Table 4). To further analyze the implication of such interactions, a *post hoc* t-test was used individually for each of the four treatments. This analysis revealed significant differences between the top samples of the four treatments (P=0.0027) but not when comparing the bottom samples of the four treatments. This suggests a change in the bacterial diversity of denitrifiers at the top with each nutrient enrichment experiment.

For treatment 1 (100μM NO_3-), the DGGE revealed differences in the number of species between the top samples (from 0-3 cm deep) and bottom samples (from 6-9 cm deep). Bottom samples contained a significantly higher (P=0.0464) number of bands than the top (Figure 7). This implies that bacterial diversity was significantly higher in the

bottom when both sample types were treated with nitrate concentrations representative of ambient, baseline conditions seen in salt marshes.

Table 4. Summary of results from the N-way ANOVA denitrifying bacterial diversity changes in response to nutrient enrichments. Star (*) indicates statistically significant differences at $P<0.05$.

Effect	Sum of squares	d.f.	Mean squares	*F* value	*P* value
Treatments (1,2,3,4)	123.576	3	41.192	1.52	0.2758
Depth (top or bottom	47.592	1	47.592	1.75	0.2183
Treatments X Depth	323.137	3	107.712	3.96	*0.047
Error	244.5	9	27.167		
Total	820.471	16			

DGGE analysis of samples containing a high concentration of NO_3- (20,000μM NO_3-) did not reveal significant changes in the number of species in either the top or the bottom. The average number of denitrifying species in treatment 2 was similar for both top and bottom and there were no major band pattern changes amongst the replicates (Figure 7). The t-test confirmed that bacterial diversity was not significantly different between top and bottom for this treatment.

The average number of species in treatment 3 (100μM NO_3- + 20% C) was similar for both top and bottom and the t-test confirmed that bacterial diversity was not significantly different between top and bottom sediments for this treatment. Similarly, the same results were found for treatment 4 (20,000μM NO_3- + 20% C) with no significant differences.

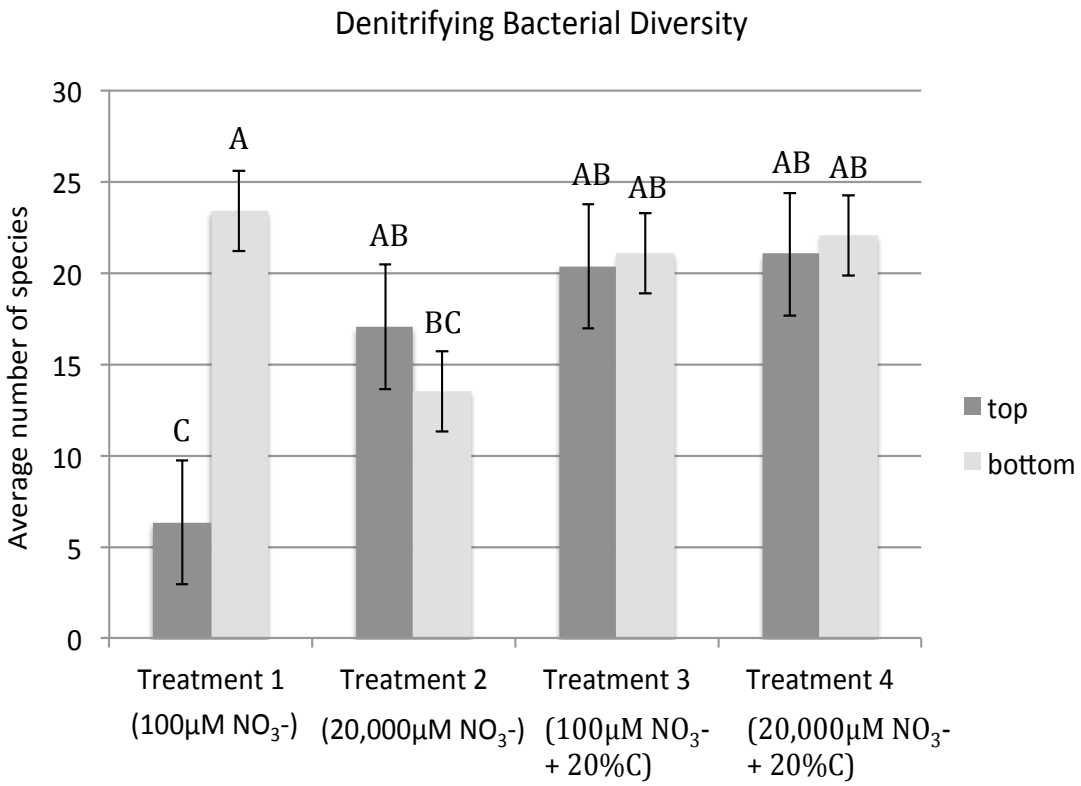

Figure 7. Bar graph shows the average number of denitrifying bacterial species per treatment with standard error bars. Different letters denote statistically significant difference (P<0.05).

2.4.2 Impact of Nutrient Enrichments on Bacterial Diversity

2.4.2.1 Impact of Carbon on Bacterial Diversity. In surface sediment samples, the average number of species in the presence of baseline-level nitrate (100µM NO_3-) plus a 20% C increase from the original carbon concentration in the samples (treatment 3) was significantly higher than the number of species in the absence of glucose (treatment 1) (Figure 7). This was not the case in bottom sediment cores. A t-test confirmed that top samples where both carbon and 100µM NO_3- were added had significantly higher bacterial diversity (P= 0.0046) than those with lower concentrations of nitrate and no carbon

additions (control). Thus, the addition of a carbon source significantly increased the bacterial diversity of denitrifiers in surface sediments but not in subsurface sediments.

The DGGE analysis also showed that the average number of species for the top sediment cores enriched with 20,000μM NO_3- and 20% C (treatment 4) appeared to be higher than those enriched with the same amount of nitrate but without carbon (treatment 2). However, the t-test showed that this difference in the number of denitrifying bacterial species was not statistically significant. Regardless of depth, the addition of carbon at high nitrate concentrations (i.e., 20,000μM NO_3-) did not cause any changes in bacterial diversity.

2.4.2.2 Impact of a Higher Nitrate Concentration and no Carbon Addition on Bacterial Diversity. DGGE banding patterns showed that the average number of species in the top samples enriched with 100μM NO_3- (treatment 1) were lower than those in treatment 2 which had a higher concentration of nitrate (20,000μM NO_3-). The t-test confirmed that this difference amongst top samples of treatments 1 and 2 was highly significant (P=0.0086). The diversity of denitrifying bacteria in the top samples was higher in cores treated with high concentrations of nitrate.

Though the average number of species in the bottom portion of the sediment core enriched with 100μM NO_3- (treatment 1) was higher than in the sediment cores enriched with a concentration of 20,000 μM NO_3- (treatment 2), the t-test showed that this decrease was not statistically significant (P=0.3020). Thus, increasing the concentration of nitrate did not increase the diversity of denitrifying bacteria in the bottom of the sediment cores.

2.4.2.3 Impact of Both Glucose Addition and High Nitrate Concentration on Bacterial Diversity. The average number of denitrifying species in the top core sediments enriched

with 100μM NO_3- + 20%C (treatment 3) was not significantly different from that in sediment cores enriched with 20,000μM NO_3- + 20% C (treatment 4). Similarly, the bottom core sediments did not show significant changes in the average number of species when the concentration of nitrate was increased and carbon concentrations remained the same. Thus, there were no changes in the diversity of denitrifiers regardless of the depth.

2.5 Discussion

2.5.1 Molecular Methods

Successful amplification of PCR products with wetland soil and marine sediment samples with both *nirS* and *nirK* has been achieved by other studies (Braker et al. 2000, Priemé et al. 2002), but amplification with the *nirS* gene fragment was not successful in this study. The absence of *nirS* genes in soil samples has also been reported in a number of diverse soil environments such as legume rhizospheres (Sharma et al. 2005), silt loam soil in Germany (Avrahami et al. 2002) and upland forest treated with fertilizer (Wolsing and Priemé 2004). In the current study, only the F1aCu-R3Cu primer pair of the *nirK* gene fragment achieved amplification of the correct size (472 bp) and was less than 500bp, the maximum length as recommended by Throbäck et al. (2004) for DGGE analysis. Environmental factors could play important roles in determining or selecting for either *nirS* or *nirK* (Priemé et al. 2002).

For example, a study by Braker et al. (1998) found a higher predominance of *nirS* in upland soils and *nirK* in marine sediments (Braker et al. 1998). However, more recent studies suggest that this is not always the case, and both *nirK* and *nirS* have been found in many different types of soils and environments (Throbäck et al. 2004). More research is

needed in order to understand the factors causing the expression of these nitrite reductase genes in a wide range of ecosystems.

Improvements in *nirS* and *nirK* primer development (Braker et al. 1998, Hallin and Lindgren 1999, Braker et al. 2000, Throbäck et al. 2004) have facilitated analysis of denitrifying bacterial communities for DGGE analysis. Throughout the PCR process, multiple bands were consistently present at 320bp in addition to the correct product size of 472 bp. Multiple bands were detected with positive control *A. faecalis*. Previous studies using different *nirS* and *nirK* primers have also experienced problems with multiple bands, in addition to the desired band size (Throbäck et al. 2004). The low *nirK* diversity in some of the treatments used in this study mirrors results by Priemé et al. (2002) possibly due to certain primers favoring conserved *nirK* genes that are not necessarily found in the environmental samples used in this study.

Another factor that can possibly explain the lack of *nirK* amplification in some of the replicates is the high presence of tannins in wetland sediments. Tannins can cause protein precipitation and inhibition of DNA extraction and amplification (Alongi 1987, Alongi et al. 1992, Rivera-Monroy and Twilley 1996, Reddy and DeLaune 2008). Although a cleanup kit was used after the DNA extraction process in order to eliminate tannins from the DNA, it is possible that the inhibitory effect had already affected the denitrifying bacterial DNA causing some of the replicates to produce low DNA quality. However, most samples produced a successful amplification of the desired length.

Due to the high number of unrelated taxonomic groups capable of denitrification (Braker et al. 2000) found in the environment, DGGE is capable of detecting subtle mutations in DNA, thus providing insight into subtle changes in the community's diversity.

However, it is important to remark that DGGE only provides information on the predominant populations in a community, and in some cases molecules have different melting configurations that cause them to have different migration patterns. For this reason, microbial studies such as the current one should use care when interpreting DGGE results (Throbäck et al. 2004). Manual quantification of DGGE samples can also lead to subjectivity, which should be taken into account in order to minimize bias. Nevertheless, DGGE was chosen over other methods such as terminal restriction fragment length polymorphism (T-RFLP) because it can analyze multiple samples and is ideal for examining population changes due to environmental modifications (Throbäck et al. 2004).

Another limitation of the current analytical PCR-based methods, such as DGGE and T-RFLP, is that they do not distinguish between functional genes and genetic material persistent in the environment even after the microorganisms are absent from the ecosystem (Recorbet et al. 1993, Philippot and Hallin 2005). However, such molecular methods have broadened our understanding of the denitrifiers' community and the genes related to each step of the pathway. In addition, these methods help us better understand how different species of denitrifiers are affected by ecological changes in nutrient cycling due to increasing eutrophication levels.

Although there have been important advances in the molecular methods used to study denitrifying bacterial communities, there are still many discoveries to be made. The development of new nitrite reductase primers can help identify even more taxonomically unrelated bacterial species that are not currently targeted by the existing primers. It is also important for future studies to take into account other pathways in the nitrogen cycle such as DNRA. These pathways seem to be more interconnected than what was previously

believed, and there is growing evidence that microorganisms may be capable of performing denitrification in more than one way. The capacity of these organisms to adapt to environmental conditions could one day provide the key to understanding and possibly predicting how wetland ecosystems will react to increasing anthropogenic activities and global climate changes.

2.5.1 Nitrate enrichments

For this study, two concentrations of nitrate were used to enrich the sediment cores. The first concentration, 100μM NO_3-, was chosen in order to simulate average nitrate concentration levels found in coastal Louisiana and the Mississippi River delta (Rivera-Monroy et al. 2010, Rivera-Monroy et al. 2013). This high concentration is due to runoff nitrate fertilizer used along the Mississippi River and tributaries as well as point and non-point sources of nutrients. The second concentration of nitrate used in this experiment was extremely high (20,000μM NO_3-) and not normally found in natural environments. Because such a high concentration of nitrate is not likely to occur in the environment, future studies concerning environmental impacts should use lower concentrations in order to simulate more realistic conditions. Nevertheless, for experimental purposes, the high nitrate concentration was used to supersaturate the system and promote high denitrification rates in a short period of time.

In treatment 1 (100μM NO_3-), the number of species was significantly higher in the bottom anaerobic samples than at the top aerobic samples (Table 5). Because 100μM NO_3- is the average nitrate concentration found in coastal Louisiana wetlands (Rivera-Monroy et al. 2010), these results suggest that at this concentration of nitrate, bottom sediments may have significantly higher denitrifying bacterial diversity compared to the top layer of

wetland sediments. This result is also consistent with the expectation that denitrification rates are higher in anaerobic conditions of wetland sediments than in aerobic conditions (Hanson et al. 1994, Van Cleemput et al. 2006, Reddy and DeLaune 2008). It is also possible that the low diversity of denitrifiers in the top layer was due to the inhibitory effect oxygen has on proteins in certain species grown anaerobically (Van Cleemput et al. 2006).

In treatment 2 (20,000μM NO_3-), which contained higher nitrate concentrations and no glucose, bacterial diversity increased significantly in the top samples but decreased in the bottom samples (Table 5). It is possible that in natural wetland ecosystems, as nitrate concentrations increase, the carbon ratio decreases to the point of becoming limiting. If increasing nitrate input with no additional carbon source could be continued for a longer period of time, the diversity of denitrifiers can be potentially decreased to a much more extreme degree than what was demonstrated in this experiment. A recent study by Suárez-Abelenda et al. (2014) found that high concentrations of nitrate resulted in an initial increase of microbial activity and subsequent decrease in organic carbon concentrations in wetland sediments. Other studies have also observed a positive association between denitrification rates and nitrate concentrations (Rivera-Monroy and Twilley 1996).

As nitrate concentration increases, bacteria require more carbon in order to metabolize the extra nitrate. The lower the C:N ratio is, the more likely nitrate assimilation by bacteria can be hampered if carbon becomes a limiting nutrient. However, when glucose was added in addition to 100μM NO_3– (treatment 3) and compared to the control (treatment1), it resulted in a significant increase in denitrifying bacterial diversity (Table 5). This study shows that by adding carbon to the system, thus increasing the C:N ratio, bacterial diversity increases for both top and bottom sediment samples. Similar results

were obtained by Rivera-Monroy and Twilley (1996) except that low denitrification rates were caused by high C:N ratios. Previous studies have also shown how organic matter composition leads to different rates of denitrification, therefore affecting the abundance and diversity of denitrifiers (Bowman and Focht 1974, Kandeler et al. 2006) and supporting the results of this experiment. Future experiments should incorporate measurements of C:N ratios in sediments to better understand how bacterial communities react to changes in nutrient concentrations and to better understand how liming nutrients affect bacterial diversity.

Table 5: Summary results of the effect of nitrate concentration changes and glucose addition in the diversity of denitrifying bacteria.

		$[NO_3^-]$	20% C	Diversity	P<0.01	
Treatment 1	Top	⟺	✖	⇩		
	Bottom	⟺	✖	⇧		YES
Treatment 2	Top	⇧	✖	⇧	YES	
	Bottom	⇧	✖	⇩		
Treatment 3	Top	⟺	✔	⇧	YES	
	Bottom	⟺	✔	⇩		
Treatment 4	Top	⇧	✔	⇧	YES	
	Bottom	⇧	✔	⇩		

2.5.2 Carbon Enrichments

Due to the high concentrations of nitrate used in this study, an increase in the levels of carbon source was used to increase C:N ratios and promote denitrification. A 20% carbon increase from the baseline carbon concentration in the original sediment samples was applied to treatments 3 and 4. Although natural sources of carbon found in wetland ecosystems include chitin and cellulose but not glucose, the latter was chosen because it provides a rich carbon source that is readily available for denitrifying bacteria to utilize.

Glucose has been repeatedly used as carbon source in many denitrification and nutrient enrichment experiments (Akunna et al. 1993, Riveramonroy et al. 1995, Rivera-Monroy and Twilley 1996, Cuervo-López et al. 1999); however, future studies should consider using chitin, which also has a high carbon content, in order to simulate a more natural scenario.

Following increases in nitrate (treatment 3) and in carbon (treatments 4), denitrifying bacterial diversity amongst top and bottom samples was similar (Table 5). This leveling in the denitrifying bacterial diversity is probably due to the fact that glucose is a carbon source that can easily break down to CO_2. These results indicated that regardless of the amount of nitrate being added, the presence of carbon was not associated with a significant difference between top and bottom samples. However, in the absence of carbon (treatment 2), the bacterial diversity in the top and bottom samples was different from treatment 1. The potential of nitrate reduction is low if there is a minimal source of carbon (Leahy and Colwell 1990, Hanson et al. 1994).

It is also important to analyze how changes in nutrient levels affect the nitrogen cycle and denitrification. For example, in a study by Rivera-Monroy & Twilley (1996) in temperate marshes with high nitrate input and low C:N ratios, a shift was detected from NO_3- ammonification to denitrification. Evidence suggests the coexistence of the dissimilatory nitrite reductase (*nirK*) gene and the pentaheme cythochrome C nitrite reductase (*nrfA*) gene on the same genome of certain bacteria (Giblin et al. 2013). It is possible that these bacteria have the ability to switch between denitrification and DNRA or even possibly perform both at the same time. However, it is important to highlight that the presence of a gene (i.e. *nirK* or *nirS*) does not necessarily entail its expression (Sharma et al.

2005). The mechanisms associated with the differential expression of one over the other are not yet well understood; thus, more research is necessary on these organisms.

Denitrifying bacteria are crucial for nutrient cycling, water quality improvement and resilience of salt marsh ecosystems. Understanding the response of the bacterial community to changes in the concentrations of nutrients such as nitrate and carbon is fundamental as food demand increases. Sediment core enrichments were carried out with 100μM NO_3-, the baseline nitrate concentration found in coastal Louisiana, or with 20,000μM NO_3-, a very high concentration of nitrate in order to promote denitrification. In addition, a 20% increase in the total carbon concentration in the original sample was also added in order to increase the C:N ratio. As, expected, the bacterial diversity in the bottom samples was significantly higher than top samples treated with 100μM NO_3-. In addition, bacterial diversity of denitrifiers was positively correlated with nitrate and glucose additions in top sediments but not in bottom sediments.

CHAPTER 3. ANALYSIS OF THE DENITRIFYING BACTERIAL DIVERSITY IN BLACK MANGROVES (*AVICENNIA GERMINANS*) IN RESPONSE TO OIL SPILL IN A MICROCOSM SETTING

3.1 Background Information

3.1.1. Mangrove Ecosystems

Mangroves are found in over 123 countries and cover about 152,000 km^2 (Ghizelini et al. 2012) in tropical and subtropical regions along estuarine zones (Marcial Gomes et al. 2008). Their aerial roots allow them to tolerate and adapt to a wide range of salinity, temperature, tide, pH, nutrient and oxygen conditions (Ghizelini et al. 2012). Mangrove sediments are often permanently inundated by water and therefore they have adapted to highly anaerobic sediment environments.

In North America, three species of mangroves have been described. Red mangroves (*Rhizophera mangle*) are characterized by curving roots that allow them to grow in deeper waters, round leaves and elongated seeds called progagules. White mangroves (*Laguncularia racemosa*) are characterized by the ability to grow farther inland. Black mangroves (*Avicennia germinans*) are found in Louisiana, and are characterized by dark green elongated leaves (Figure 8), dark stems and long straw-like roots called pneumatophores that allows them to perform gas exchange (Figure 9). In addition, black mangroves have the ability to grow farther inland than red mangroves (Reddy and DeLaune 2008).

Before the 1980s, black mangroves (*Avicennia germinans*) in Louisiana were limited to the islands off the coast due to lower temperatures in the mainland. Since the 1980s, however, there has been a significant expansion of mangroves towards inland regions, possibly as a result of global climate changes (Perry and Mendelssohn 2009). Increasing

global temperatures have favored the rapid expansion of black mangroves into marshes predominantly composed of *Spartina* sp.

Figure 8. An elongated leaf characteristic of black mangroves (*Avicennia germinans*) secreting salt.

Figure 9. Pneumatophores characteristic of black mangroves (*Avicennia germinans).*

3.1.2 Ecological Benefits of Mangroves and Marsh Ecosystems

Mangroves are known for their nutrient transport and retention capabilities. In sediments, mangrove roots produce 40-60% of the total nitrogen needed (Flores-Mireles et al. 2007) with a net primary production of 9.3 tons per hectare (Ghizelini et al. 2012). This highly productive and efficient ecosystem plays crucial roles in the estuarine food webs by providing protection and breeding and feeding grounds to a wide range of organisms including fish, amphibians, arthropods, and birds (Ewel et al. 1998, Flores-Mireles et al. 2007, Ghizelini et al. 2012). Salt marshes support a multi-billion dollar seafood industry in Louisiana (Jackson and Pardue 1999) and have important cultural implications for the state. Due to the capacity of mangrove forests to support a wide range of organisms, mariculture and shellfish farming have intensified, thus causing damage to the ecosystem. Shrimp farming, for example, is responsible for the clearing of 50% and 21% of mangroves in Thailand and Ecuador, respectively (Ewel et al. 1998). Shrimp farming is also responsible for eutrophication problems in coastal waters of Brazil (Suárez-Abelenda et al. 2014) and Colombia (Rivera-Monroy et al. 1999). As the human population continues to increase rapidly, urban areas are now expanding towards mangrove forests such, as it is the case in Brazil and Asia. This rapid growth increases the mangrove's susceptibility to stress from clearing, dredging, eutrophication and contamination of sewage, toxic compounds and oil spills.

In coastal Louisiana, marsh ecosystems provide protection against floods and hurricanes and mitigate erosion with their sediment trapping capabilities (Ewel et al. 1998). Because many mangrove forests are located near residential and industrial areas, they often come in contact with untreated sewage, fertilizers, pesticides, toxic metals and

hydrocarbons (Marcial Gomes et al. 2008). These pollutants can be trapped, processed and transformed by mangroves and microorganisms, thus improving water quality by removing excess nutrients (Holguin et al. 2001, Marcial Gomes et al. 2008). This could be especially important in areas where fish and shellfish are harvested for human consumption (Ewel et al. 1998, Holguin et al. 2001, Suárez-Abelenda et al. 2014) as the case in coastal Louisiana.

The symbiotic relationship between benthic microorganisms and mangroves is crucial for the whole ecosystem. Mangroves depend on bacteria for nutrient cycling (Holguin et al. 2001), and bacteria occupy an important niche on mangrove roots and soil. Of all the microorganisms found in mangrove sediment, 91% consist of bacteria and fungi and 7% and 2% consist of algae and protozoa respectively (Ghizelini et al. 2012), which are responsible for most of the biogeochemical transformation of nutrients in the carbon and nitrogen cycles (Alongi et al. 1992). Mangrove sediments are often permanently inundated with water, hence saturating pore space with water. This creates a thin aerobic layer at the top and a thick anaerobic layer at the bottom. Each layer is composed of bacteria that are specifically adapted to redox conditions, which makes bacterial communities in mangrove sediments highly diverse and highly adaptable to a wide range of environmental conditions.

3.1.3. History of Oil Spills and their Environmental Impacts

In addition to offshore drilling, transportation across the ocean is also responsible for a large portion of oil contamination events (Table 6). Some estimates suggest that at least one million tons of oil are lost in the ocean during the transportation process (Barrios San Martín 2011). Only a small portion of such contamination events is due to accidents. One of the most significant spills occurred in 1989 when the *Exxon Valdez* impacted a reef

off the coast of Alaska releasing approximately 50 thousand tons of oil into Prince William Sound making it one of the largest oil spills in history until 2010 (Barrios San Martín 2011).

Table 6. Major oil spill events listed in chronological order.

Spill/ Tanker	Year	Location	Amount (in tons)	References
Deepwater Horizon	2010	Gulf of Mexico, USA	500,000	(Kostka et al. 2011)
Prestige	2002	Spain	63,000	(Barrios San Martín 2011)
Erika	1999	Breton, France	37,000	(Barrios San Martín 2011)
Exxon Valdez	1989	Alaska, Prince William Sound	50,000	(Barrios San Martín 2011)
Ixtoc I	1979-1980	Gulf of Mexico, Mexico	475,000	(Jernelöv and Olof 1981)
Atlantic Empress and Aegean Captain	1979	Trinidad and Tobago	287,000	(Horn and Neal 1981)
Amoco Cadiz	1978	Brittany, France	227,000	(Barrios San Martín 2011)

3.1.4 The *Deepwater Horizon* Oil Spill

On April 20, 2010, the United States experienced one of the largest environmental disasters when the *Deepwater Horizon* platform located in the Gulf of Mexico about 41 miles (66 km) off the coast of Louisiana exploded. The explosion occurred about 5000 feet below sea level at the Macondo well located in Mississippi Canyon (Kostka et al. 2011). It has been estimated that about 4.9 million barrels of light crude oil were released into the waters of the Gulf of Mexico for a period of 83 days until the rupture of the well was

successfully capped in September 19, 2010 (Figure 10) (2011, Valentine et al. 2012). It has been reported that the oil slick covered an area of approximately 75,000km^2 (29,000 mi^2) (Cleveland et al. 2010), thus making the *Deepwater Horizon* oil spill one of the world's largest oil spills.

Because the well was not sealed until months after the blowout, a large portion of this oil reached the coasts of Louisiana, Mississippi, Alabama and Florida, affecting a wide range of coastal environments including both marshes and sandy beaches. The highest levels of hydrocarbons in marsh sediments were measured in June and July of 2010 but decreased in September (Beazley et al. 2012). A study using GeoChip microarray analysis confirmed increases in both richness and abundance of oil degrading bacterial populations in salt marsh grass sediments followed by subsequent decreased to below detection levels (Beazley et al. 2012). These findings suggest the involvement of bacterial communities in the PAH degradation process.

In addition to the ecological impacts of the *Deepwater Horizon* oil, concerns were initially raised about residence time of PAH in the environment that could have potentially impacted pathogenic factors in some marine bacteria (Smith et al. 2012). Furthermore, concerns were raised regarding commerce and consumption of seafood specially filter feeding organisms such as shrimp and oysters. Concerns included the potential effects on human health through consumption of seafood contaminated with PAHs, toxic metals from dispersants, or pathogenic bacteria (Gohlke et al. 2011). In addition, pollutants can potentially inhibit important metabolic functions in a wide range of organisms and passed along the food chain to higher trophic level organisms.

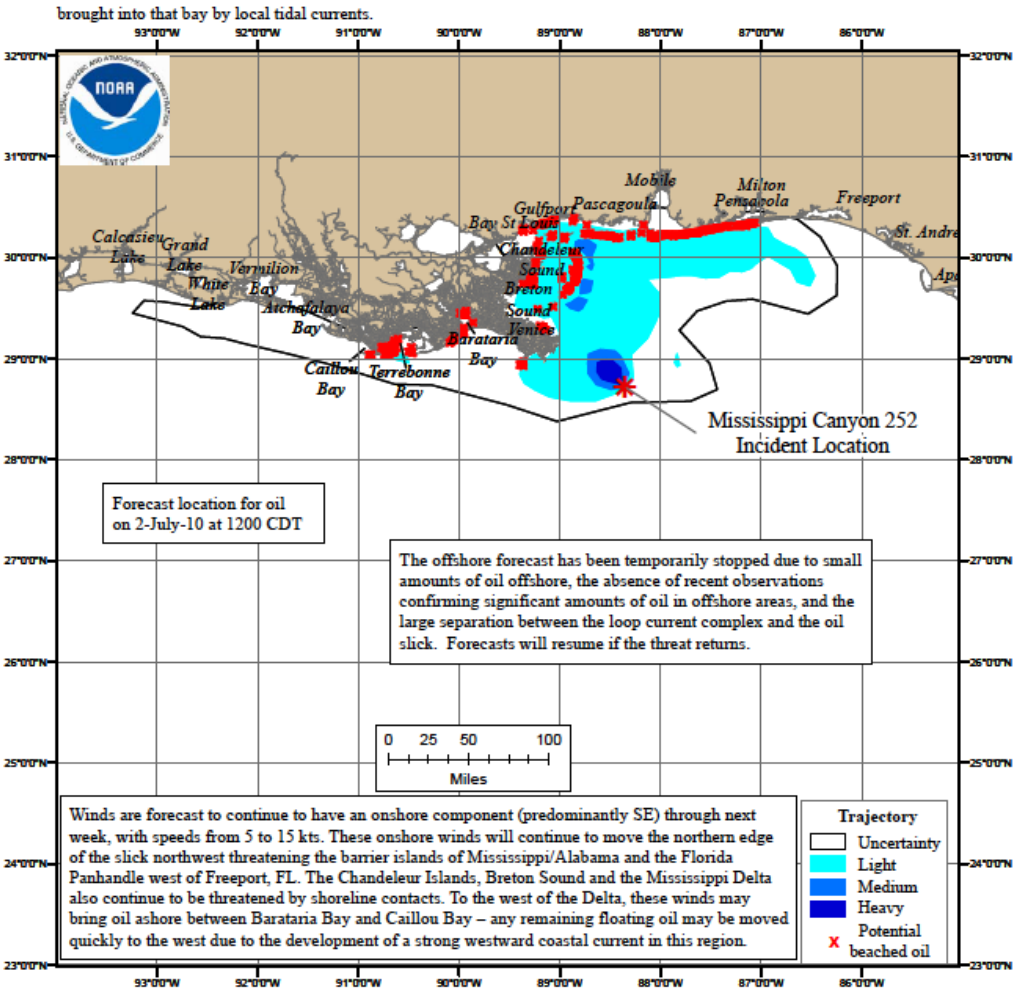

Figure 10. Trajectory map of the *Deepwater Horizon* oil spill in July 2010 (NOAA 2010).

3.1.4 South Louisiana Crude Oil Properties and Characteristics

There are several different types of oils that vary in chemical composition and toxicity. Knowing about the physical and chemical characteristics of the oil released by the Macondo well is important in order determine the potential effects to the fauna and flora impacted by the spill. Oil from the *Deeptwater Horizon* spill, also called MC252 oil, originated from the Mississippi Canyon block 252 of the Gulf of Mexico. MC252 oil belongs to a category of light crude oil also called south Louisiana sweet crude (SLSC). SLSC oil is considered “sweet” due to its low concentration of sulfur (NOAA 2010, Oil Spill Academic

Task Force) which gives it a syrup-like texture and color when fresh. SLSC oil is high in alkanes, or chains of single carbons bonds. MC252 oil has a lower concentration of polycyclic aromatic hydrocarbons, thus making it less toxic and more easily degraded by microorganisms compared to other types of heavier crude oils (NOAA 2010, Oil Spill Academic Task Force). Table 7 shows the chemical composition and properties of south Louisiana crude oil compared to a Kuwait crude oil that is much heavier and toxic. Although PAHs in south Louisiana crude oil are more easily degraded in the water column due to the volatile organic compounds like toluene, benzene and xylene, they can potentially endure for long periods of time once they reach the shore. Once there, they can subsequently penetrate into the sediments and persist longer (NOAA 2010).

3.1.4 Intrinsic Bioremediation of Hydrocarbons by Bacteria

A number of studies have demonstrated the ability of microorganisms to naturally degrade south Louisiana crude oil (Zobell and Prokop 1966, Miget et al. 1969, Kator et al. 1971, Atlas and Bartha 1973, Walker et al. 1975, Walker et al. 1976, Jackson and Pardue 1999). Intrinsic bioremediation is the process of breaking down contaminants to less hazardous compounds by achieving partial or total degradation through natural means (Barrios San Martín 2011). Chemically, some of the factors affecting the degradation potential of oil include contents of alkenes, asphaltene, nitrogen, sulfur and oxygen (Westlake et al. 1974, Walker et al. 1976). Natural removal of hydrocarbons involves dispersion, volatilization, chemical oxidation, diffusion and microbial biodegradation. The rate of natural bioremediation is usually slow. It can take months to decades depending on the chemical structure of the oil and the environmental conditions such as temperature, salinity, oxygen content, pH, wave action, and microbial communities.

Table 7: Chemical composition and characteristics of weathered south Louisiana crude oil compared to a Kuwait crude oil (Walker et al. 1976). Note the low percentage of sulfur in the SLSC oil versus Kuwait crude oil and the high concentration of saturates in the SLSC oil versus Kuwait crude oil.

Component	South Louisiana crude oil	Kuwait oil
Sulfur (wt. %)	0.25	2.44
Nitrogen (wt. %)	0.07	0.14
Nickel (ppm)	2.2	7.7
Vandalium (ppm)	1.9	28.0
Saturates (mg)	23.1	13.4
Aromatics (mg)	18.3	26.9
Resins (mg)	7.6	11.2
Asphaltenes (mg)	0.5	3.5
Total residue (mg)	49.5	55.0

Most PAH degrading microorganisms are bacteria; however, fungi and algae are also known to degrade PAHs (Table 8). Bacterial degradation of PAHs has been confirmed in mostly aerobic conditions; however, it has also been reported under anaerobic conditions (Haritash and Kaushik 2009). In sensitive habitats such as salt marsh ecosystems, intrinsic bioremediation is capable of removing toxic compounds instead of transferring them to other areas or organisms (Jackson and Pardue 1999) where toxic compounds continue biomagnifying in food webs. Recent studies have revealed the presence of oil degrading bacteria in sandy beaches after the *Deepwater Horizon* oil spill (Barrios San Martín 2011, Kostka et al. 2011). Many of these bacteria and microorganisms are used as bioindicators for oil presence and contamination, which can be useful in restoration and management efforts in affected areas.

Table 8. List of known oil degrading microorganisms (Barrios San Martín 2011)

Group	Genus	
Bacteria	*Acinetobacter sp*	*Lucila sp.*
	Alcaligenes sp.	*Marinobacter sp.*
	Alcanivorax sp.	*Methylophaga sp.*
	Aquaspirillum sp.	*Micrococcus sp.*
	Arthrobacter sp.	*Microscilla sp.*
	Azospirillum sp.	*Mycobacterium sp.*
	Bacillus sp.	*Neisseria sp.*
	Beggiatoa sp.	*Nocardia sp.*
	Cycloclausticus sp.	*Novospingobium sp.*
	Cytophaga sp.	*Oleiphilus sp.*
	Corynebacterium sp.	*Planococcus sp.*
	Desulfotalea sp.	*Porpyrobactes*
	Desulfovibrio sp.	*Pseudomonas sp.*
	Erythrobacter sp.	*Rhodoplanes sp.*
	Flavobacterium sp.	*Rubrivivax sp.*
	Geobacillus sp.	*Staphylococcus spp.*
	Geobacter sp.	*Sulfitobacter sp.*
	Halochromatium sp.	*Thioalcalovirbio sp.*
	Halomonas sp.	*Xanthomonas sp.*
Fungi	*Alternaria sp.*	*Penicillum sp.*
	Aspergillus sp.	*Phanaerocheate sp.*
	Chaetomium sp.	*Pleurotus sp.*
	Cladosporium sp.	*Rhizopus sp.*
	Fusarium sp.	
Yeast	*Candida sp.*	*Saccharomyces sp.*
Algae	*Chlamydomonas sp.*	*Dunaliella sp.*
	Chlorella sp.	

The success rate of intrinsic bioremediation not only depends on the chemical characteristics of the oil spilled but also the type of ecosystem where it is found. Dispersion and diffusion of oil depend on the oil type and the physical and abiotic characteristics of the environment. Sandy beaches for example, have relatively large pore water space to wetland sediments. This pore space allows oxygen to diffuse deeper into those sediments at beaches

than in wetlands. As a result, beaches have a thick aerobic layer. In wetlands, this pore space is saturated with water, thus limiting oxygen diffusion and creating a thin aerobic layer at the top and a thick anaerobic layer at the bottom.

Unlike sandy beaches, wetland ecosystems present unique challenges and advantages to clean-up efforts and natural biodegradation of crude oil. While diffusion and volatilization can be limited in wetland sediments, nutrient content in wetland sediments is higher, therefore facilitating microbial biodegradation of hydrocarbons. Due to the physical sensitivity of marsh ecosystems, mechanical clean-up methods often used in sandy beaches (e.g. trucks that collect tar balls and pressure washers) are not feasible (Jackson and Pardue 1999). In fact, such clean-up methods can lead to more harm than benefits by removing the intrinsic microorganisms capable of oil bioremediation. Because of the sensitivity of salt marsh ecosystems, natural biodegradation of hydrocarbons is preferred over mechanical clean-up mechanisms (Beazley et al. 2012). After the *Exxon Valdez* oil spill, extensive research was performed on nutrient and nitrogen-based fertilizer additions to enhance microbial biodegradation on sandy beaches (Atlas 1995, Jackson and Pardue 1999, Boopathy 2000) and on salt marsh ecosystems (Jackson and Pardue 1999).

Denitrifying bacteria are often capable of degrading a wide range of xenobiotic substances including hydrocarbons, fertilizers, toxic metals and organic compounds. In wetland soils, the biodegradation of many xenobiotics occurs in the absence of O_2 depending on the ability of the organism to use alternate electron acceptors such as nitrate (Reddy and DeLaune 2008). This process is important because it improves water quality by removing different types of toxic compounds and excess nutrients that cause eutrophication. Some denitrifying bacteria are also capable of degrading highly toxic

organic pollutants and hydrocarbons under anaerobic conditions such as naphthalene and phthalic acids derived from petroleum, resins, asphaltenes and PAHs (Figure 11) which can be further mineralized into carbon dioxide (Aftring et al. 1981, Throbäck et al. 2004, Barrios San Martín 2011). Some denitrifiers are also used for bioremediation of environmental contaminants (Braker et al. 1998).

Oil spills resulting from oil exploration and oil transportation are not uncommon in coastal Louisiana and the Gulf of Mexico. The Gulf of Mexico is considered a hot spot for oil spills and ranks among the top places with highest numbers of oil spills from vessels in the U.S. (Etkin 1997). However, oil spill events equivalent to the magnitude and duration of the 2010 *Deepwater Horizon* spill are rare, and can potentially have unseen effects in coastal ecosystems.

Because of the significant amount of oil-related activities and the natural seepage of oil from the ocean bed (Farwell et al. 2009) the likelihood of microorganisms evolving and adapting to such stressors could explain the degree of resilience of the whole ecosystem. Identifying changes in the microbial composition and diversity after a contamination incident is crucial for better understanding the environment's resilience and its natural ability to adapt to changes. In previous studies, microbial community assays have been successful at identifying potential bio-indicators of contaminants such as polycyclic aromatic hydrocarbons (Santos et al. 2010, dos Santos et al. 2011, Sodré et al. 2013). Other studies have shown that certain species of bacteria are effective in naturally degrading oil contamination (Kostka et al. 2011). However, there seems to be a lack of information to date about the impact of a major oil spill of the same magnitude of the *Deepwater Horizon* in the bacteria consortium in mangrove ecosystems.

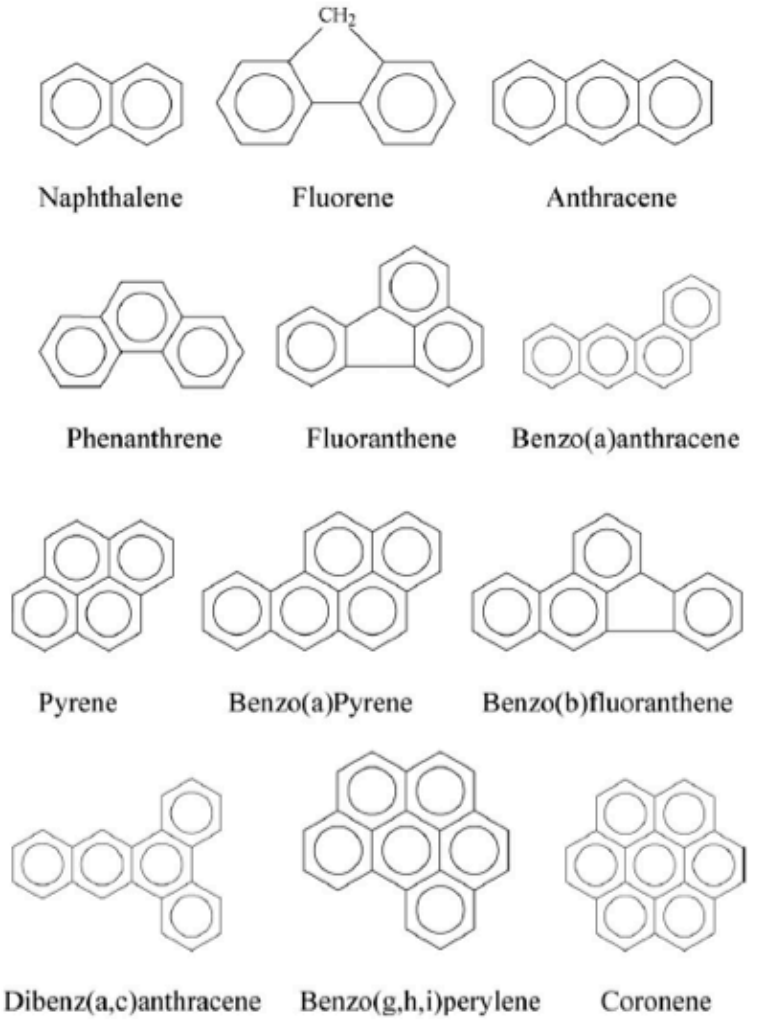

Figure 11. Chemical structure of some commonly studied PAHs (Haritash and Kaushik 2009).

3.2 Hypotheses

Several studies have looked at denitrifying bacterial communities in a wide range of ecosystems including mangroves (Flores-Mireles et al. 2007), but no studies to date have looked at the impact of oil spills on denitrifying bacterial communities in salt marsh and mangrove ecosystems. In Louisiana, most oil spill studies have focused on the effects on *Spartina alterniflora* and biogeochemical cycles, but few have focused on black mangroves and microorganisms living in mangrove sediments (DeLaune et al. 1979, Lin and Mendelssohn 1996, DeLaune and Wright 2011).

DGGE analysis using specific denitrifying genes (*nirK*) was chosen because of its ability to fingerprint the dominant denitrifying taxa in the environment and how the population changes in response to oil contamination. It also provides a comparison of the diversity composition amongst sediment samples treated with oil and pristine ones. However, because of the frequency of oil contamination events in the area, it is highly possible that even soil samples not treated are already populated with oil degrading bacteria.

H_0: Addition of oil will not cause a significant change in the denitrifying bacterial community.

H_1: Addition of oil will cause a significant change in the denitrifying bacterial community.

3.3 Significance

Previous studies have proven the ability of denitrifying bacteria to degrade oil in fresh water sediments (Rabus and Widdel 1995, Rabus et al. 1999) and marine sediments (Harayama et al. 2004). Given the importance of denitrifying bacterial communities in wetland sediments for the removal of excess nutrients and xenobiotics, no studies to date have looked at the potential impact in the diversity of such group of microorganisms after a major oil spill such as the *Deepwater Horizon* spill. Important advances in the molecular biology field have been made in regards to oil degrading bacteria such as the completion of the genome sequence of *Alcarnivorax borkumensis,* a known oil degrader that uses PAH as its the main carbon source (Golyshin et al. 2003). This shows how molecular diversity studies can provide important information about the functioning and regulation of entire ecosystems. Changes in bacterial diversity after a major contamination event can result in changes in both nutrient cycling and global climate regulation.

While studies on the effect of oil spills on general microbial communities in Brazilian mangrove ecosystems have used T-RFLP (Marcial Gomes et al. 2008)(Marcial Gomes et al. 2008)(Marcial Gomes et al. 2008)(Marcial Gomes et al. 2008), there are no studies to date on the effects of the 2010 *Deepwater Horizon* oil spill in the denitrifying bacterial diversity of mangrove sediment located off the coast of Louisiana. Microbial composition analysis studies on mangrove sediments are important for determining which species are key in the process of oil degradation and the biological restoration of the environment after a major oil spill event (Marcial Gomes et al. 2008). In addition, this study can provide some insight on how oil can impact the bacteria responsible for important nutrient cycling processes such as denitrification and how this can affect an already fragile ecosystem with eutrophication problems and land loss.

3.4 Materials and Methods

3.4.1 Site of Study and Core Extractions

Three sediment core samples were collected near Port Fourchon, South Louisiana (Figures 12 and 13)(coordinates: +29° 6' 15.95", -90° 11' 19.94") using a clear 50 cm long cylindrical PVC tube. Approximately 20cm of the core was manually inserted at the base of black mangroves (*Avicennia germinans*). Each core was inserted in close proximity to each other to reduce spatial variability. Top (0-3cm) and bottom (6-9 cm) samples were dissected for each of the three cores. Each section was carefully stored in a Ziploc bag and stored in a cooler with ice for transportation.

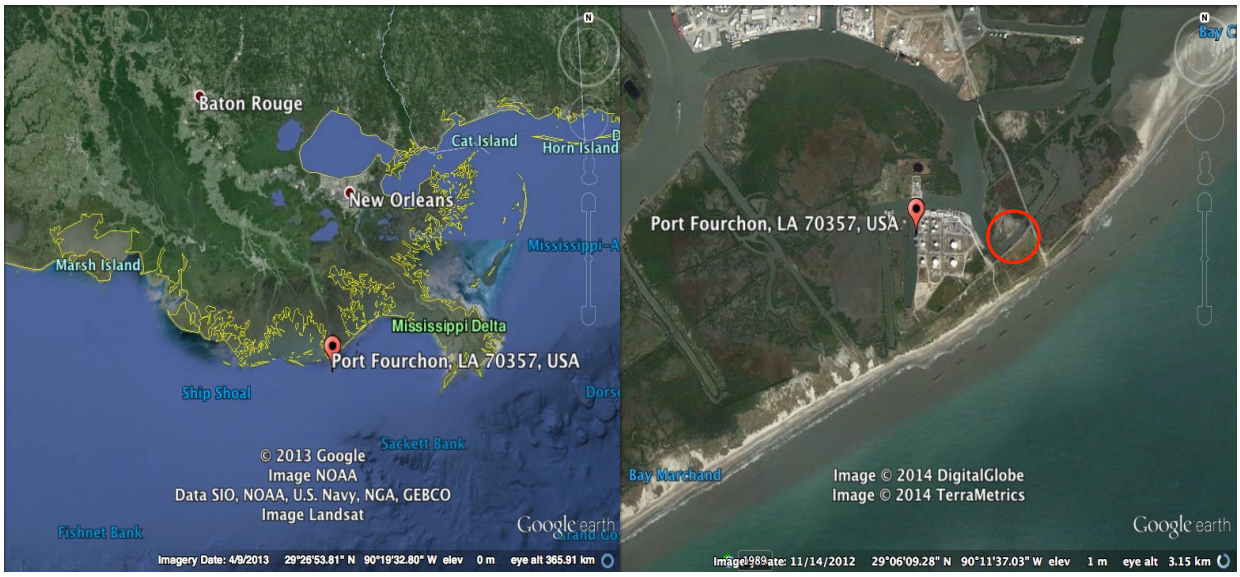

Figure 12. Satellite images of the mangrove sediment collection site. The image on the left shows the location of Port Fourchon, South Louisiana. Image on the right shows the exact location of the sampling site circled in red (courtesy of Google Earth).

Figure 13. Satellite image showing the site of mangrove core sediment collection in South Louisiana (courtesy of Google Earth).

3.4.1 Sample Processing and Microcosm Treatments

Once in the lab, each slice was manually homogenized for 30 to 60 seconds with approximately half of the sediments placed in small clear borosilicate glass vile with PTFE lined caps for CO_2 injection. From a total of twelve microcosms, six were treated with 2%

volume/weight (v/wt.) with MC252 oil (Santos et al. 2010, dos Santos et al. 2011), and six were not treated (Figure 14). All microcosms containing bottom sample sediment were flooded with CO_2 gas to maintain anaerobic conditions. Microcosms were incubated at room temperature and DNA was extracted after 24 hours, 21 days and 110 days from all twelve microcosms.

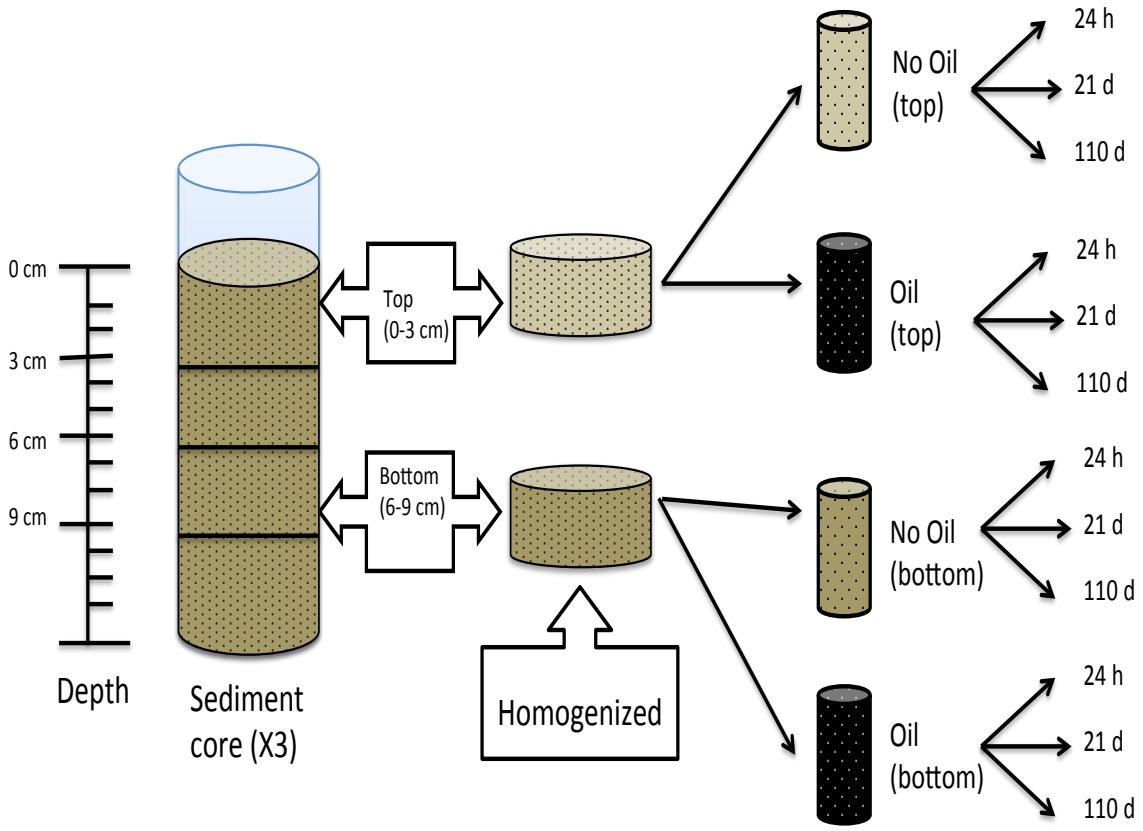

Figure 14. Microcosm setting. Three cores were extracted. From each core the top section (0-3 cm) and bottom section (6-9cm) were dissected and each slice was then split into two and manually homogenized. One half was treated with 2% v/wt. contamination with MC252 oil and the other half was not. Soil samples were taken after 24hrs, 21 days and 110 days for DNA extraction and subsequent PCR and DGGE analysis.

3.4.1 DNA Extractions

DNA samples were extracted from all samples after day 1, day 21 and day 110 respectively using an Ultra Clean Soil DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to protocol instructions.

3.4.2 PCR Amplification of the *nirK* Gene

Polymerase chain reaction (PCR) was used to amplify the copper-containing nitrate reductase gene encoded by *nirK*. The primers used were F1aCu and R3Cu (Throbäck et al. 2004). This set of primers was chosen in part because they yield amplicons of less than 500 bp, the ideal length possible for appropriate DGGE analysis according to Throbäck et al (2004). A total volume of 50 µL was used for PCR amplification using 5X GoTaq buffer, 1 mM $MgCl_2$, 0.20 mM dNTPs, 0.25 µM of each primer, 0.80 mg/mL of BSA, 0.04 units/µL of GoTaq and 4 µL of template DNA (Table 9). All samples were run at an initial denaturation temperature of 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing temperature of 57°C for 45 seconds, extension at 72°C for 45 seconds, and a final extension step at 72°C for 10 minutes (Table 10).

Table 9. PCR formulation in the microcosms experiment for a total volume of 50µL.

Component	Units	Final Concentration
5X GoTaq buffer	X	1
$MgCl_2$	mM	1
dNTPs	mM	0.2
F1aCu primer	µM	0.25
R3Cu-GC primer	µM	0.25
BSA	mg/mL	0.8
GoTaq	Units/µL	0.04
DNA template	µL	4

PCR products were then analyzed on a 2% agarose gel (6 g of agarose, 300mL of 1X SBB buffer, and 30µL of 10,000 X SYBR Safe) and run at 300V for 1 hour. The agarose gel was then analyzed using a Bio Doc It UV Transilluminator (UVP Laboratories, Upland, CA). To confirm presence and correct size amplification of the denitrifying gene *nirK*, a strain of *Alcaligenes faecalis* (ATCC 8750) was used.

Table 10. PCR amplification conditions for *nirK* in mangrove sediments.

Step	Cycle (s)	Temperature (°C)	Duration (seconds)
Initiation	1	94	120
Denaturation	35	94	60
Annealing		57	45
Extension		72	45
Final Extension	1	72	600

3.4.3 DGGE Analysis of Denitrifying Bacteria

For DGGE analysis, a 33-bp GC-clamp was added to the 5'end of the R3Cu primer (5' GGC GGC GCG CCG CCC GCC CCG CCC CCG TCG CCC 3') to prevent a complete denaturation of the PCR product. PCR conditions were the same as those mentioned above in section 2.3.5. The gel was prepared using a 58-70% denaturing gradient and run at 130V for 15 hours in a DGGEK-2001 Electrophoresis System (C.B.S. Scientific, San Diego, CA). Approximately 20µL of PCR product were added. Visualization of the bands was performed by staining with approximately 30µL of SYBR Gold in 400ml of 10X SBB and shaking at 75 rpms for 45 minutes. The DGGE gel was analyzed using a Bio Doc It UV Transilluminator. In this study, each DGGE band represented a separate species of denitrifying bacteria based on its GC content. All DGGE bands were manually counted.

3.4.4 Statistical Analysis

Data were analyzed using N-way analysis of variance (ANOVA) on MATLAB followed by *post hoc* t-tests.

3.5 Results

3.5.1 DNA Extraction and PCR

The 12 different treatments consisted of a combination of: (i) no oil versus oil, (ii) top (aerobic) samples versus bottom (anaerobic) samples of the core, and (iii) exposure time (24 hours, 21 days or 110 days). DNA was extracted from all microcosms using the Ultra Clean Soil DNA Isolation Kit (Mo-Bio, Carlsbad, CA). PCR products of the correct size (472 bp) that targeted the *nirK* gene were obtained from all samples except the three samples from the top core exposed to oil for 110 days. Because DNA amplification was successful in samples exposed to oil for both acute (24 hours) and chronic periods of time (21 and 110 days), the oil did not affect the quality of the DNA extracted and did not inhibit further PCR and DGGE processes.

3.5.2 DGGE Analysis of Microcosms

The DGGE banding patterns of the 12 different treatments were used to compare the bacterial diversity structure of denitrifiers encoding *nirK* in mangrove sediments exposed to oil for different periods of time (24 hours, 21 days, 110 days) compared to those not treated with oil (Figure 15). In addition, diversity structure amongst denitrifiers occupying the aerobic samples (0-3 cm deep) was compared to those in the anaerobic samples (6-9cm deep). DGGE fingerprinting for existing populations of denitrifiers was successful for all samples except for the three samples from the top core exposed to oil for 110 days in which PCR amplification was not successful. Changes amongst triplicate samples in the DGGE

profiles indicate heterogeneous populations of denitrifying bacteria within core samples, thus providing evidence of high taxonomic diversity within a small area.

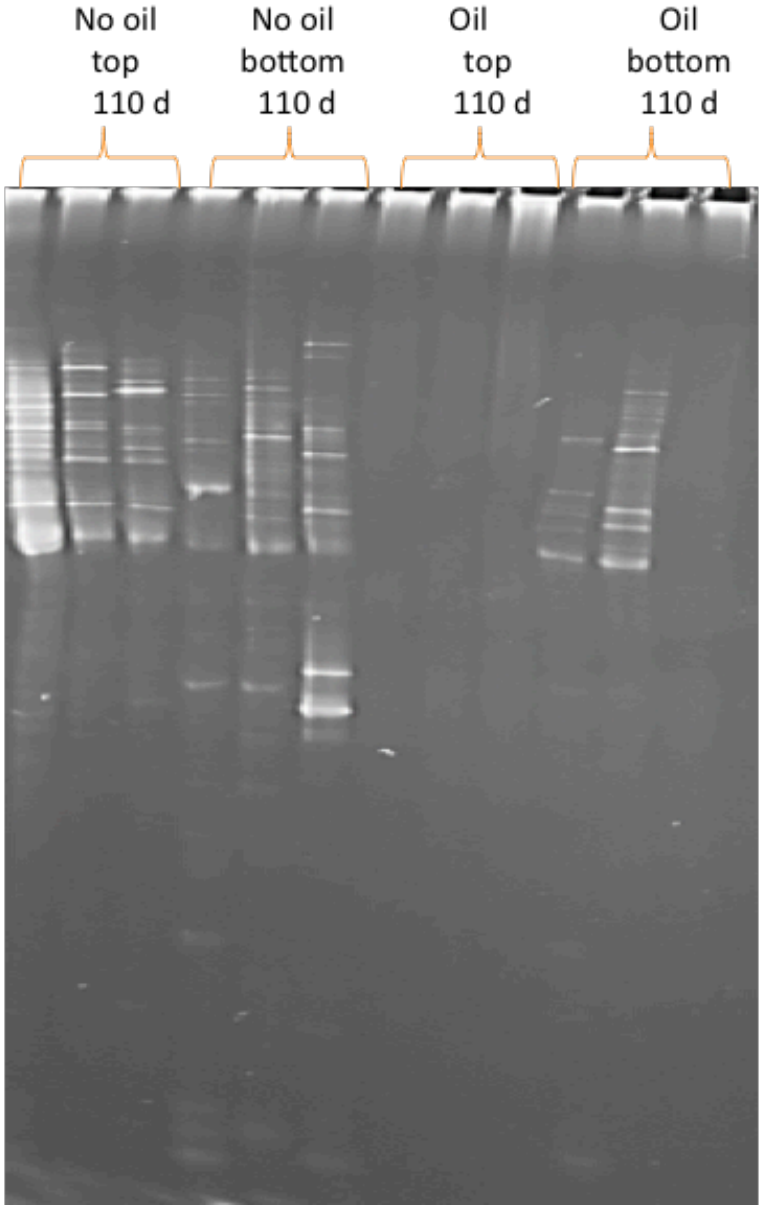

Figure 15. DGGE profile of denitrifying bacterial diversity in microcosms. No oil, top, 110d; oil, top, 110d; no oil, bottom, 110d; oil, bottom, 110d.

DGGE profiles of all microcosms exposed for 24 hours (acute exposure) to either oil or no oil (controls) had similar banding patterns regardless of whether they were from the

aerobic top (0-3cm) layer, or whether they were from the anaerobic bottom (6-9 cm) layer**.** Except for bottom sediments exposed to oil for 24 hours, all sediment samples exposed to either oil or no oil for 24 hours had low numbers of species.DGGE profiles of microcosms treated with either oil or no oil for 21 days had a higher species numbers than those samples exposed for 24 hours or those exposed for 110 days. Species numbers were also higher in the anaerobic (bottom) samples than in the aerobic (top) samples. However, there were no significant changes in band patterns amongst those microcosms treated with oil and the control groups.

After 110 days, banding patterns in microcosms from the top samples exposed to oil had differences in banding patterns when compared to the corresponding controls (no oil). Some bands that appeared in the no oil, top, 110 days microcosms seemed to disappear in those microcosms treated with oil. No bands were obtained for the microcosm from the bottom of the core that was treated with no oil for 110. Successful band fingerprinting was possible for microcosms within the bottom sediments that were treated with oil for 110 days. While banding patterns in all microcosms exposed for 24 hours and 21 days were consistent, banding patterns in all microcosms exposed for 110 days differ significantly form each other.

3.5.3 Treatment: Oil versus No Oil

A t-test comparing all non-oiled samples with all oiled samples did not show any significant difference between the number of bands in oiled samples and non-oiled ones (Figure 16). Although the average number of species was higher in microcosms not exposed to oil versus oiled ones, the results from the t-test indicate that oil contamination did not cause a significant change in the diversity of denitrifiers.

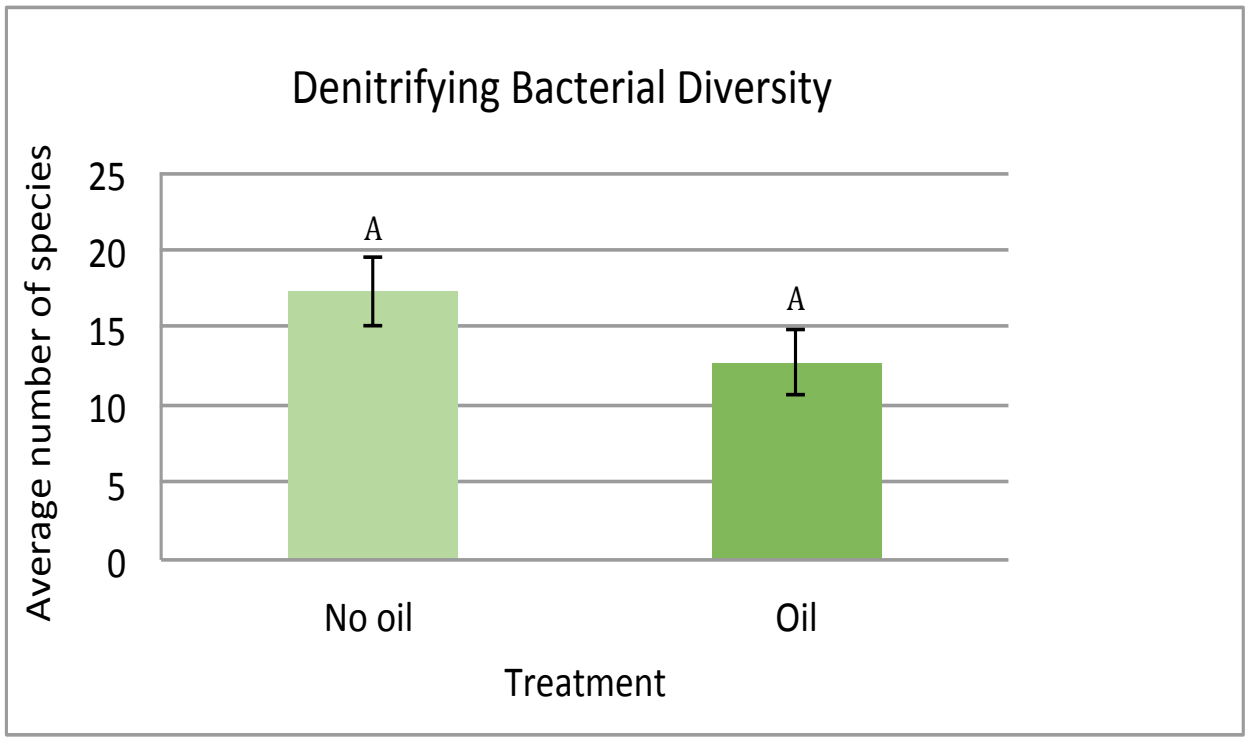

Figure 16. Bar graph comparing the average number of denitrifying bacterial species in microcosms treated with oil (2% v/wt. contamination) and no oil (control). There were no significant differences between oiled and non-oiled samples.

3.5.4 Depth: Top versus Bottom

The initial N-way ANOVA (Table 11) indicated no significant difference between top and bottom sediments (Figure 17). This result indicates that the diversity of denitrifying bacteria occupying the top aerobic samples is not significantly different than that at the bottom anaerobic samples. Oil does not seem to cause a significant difference in bacterial diversity when treatment alone is measured.

Table 11. N-way ANOVA results from the microcosm experiment.

Source	Sum Sq.	d.f.	Mean Sq.	F	*P* value
Treatment (oil, no oil)	196	1	196	10.2	*0.0039
Depth (top, bottom)	81	1	81	4.21	*0.0512
Exposure Time (24hrs, 21d, 110d)	848.167	2	424.083	22.06	* *P*<0.05
Treatment * Depth	2.778	1	2.778	0.14	0.7072
Treatment * Exposure Time	976.167	2	488.083	25.39	* *P*<0.05
Depth * Exposure	183.167	2	91.583	4.76	*0.0181
Treatment * Depth * Exposure Time	319.389	2	159.694	8.31	*0.0018
Error	461.333	24	19.222		
Total	3068	35			

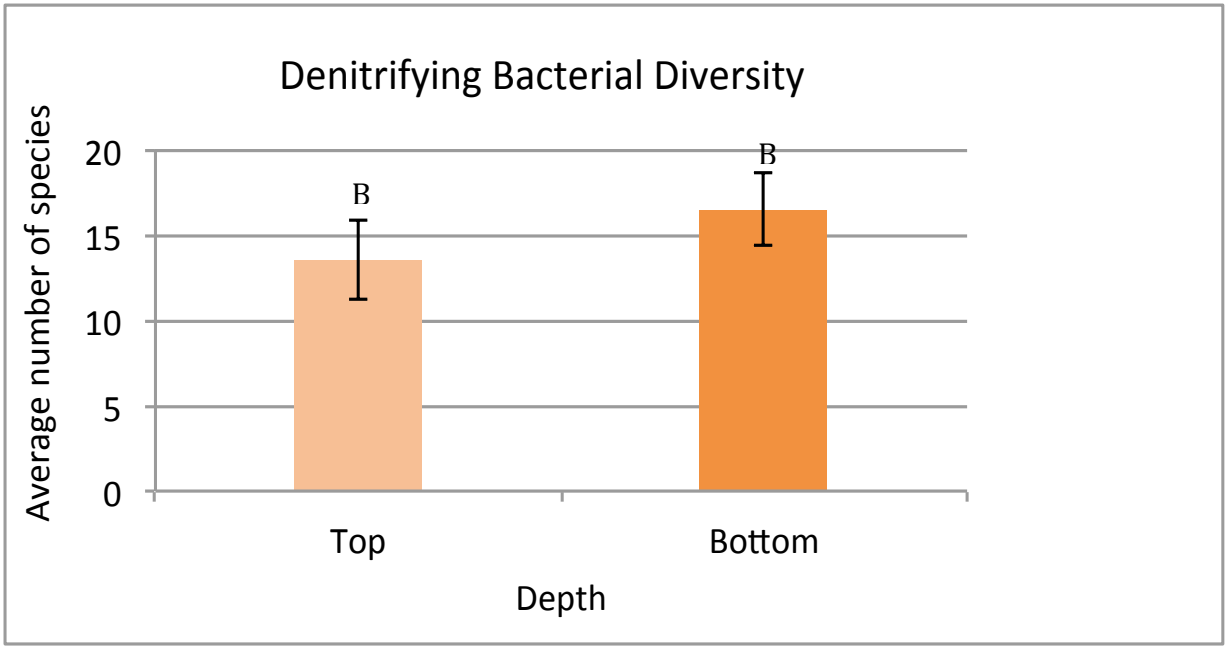

Figure 17. Bar graph comparing the average number of denitrifying bacterial species in the aerobic top layer of the core (0-3cm deep) and the anaerobic bottom layer of the core (6-9 cm deep). No significant difference was detected.

3.5.5 Exposure Time

The N-way ANOVA (Table 11) regarding the three different exposure times in the microcosm (24 hrs, 21 days and 110 days) showed highly significant results ($P<0.01$). To better understand the level of significance, a *post hoc* t-test was performed. The results from the t-test showed that the bacterial diversity of denitrifiers was significantly higher ($P<0.01$) amongst the denitrifiers sampled after 24 hours and 21 days but not after 110 days (Figure 18). Consequently, exposure time of denitrifiers in microcosms caused significant changes in the diversity after 21 days, but not after long exposures (i.e., 110 days).

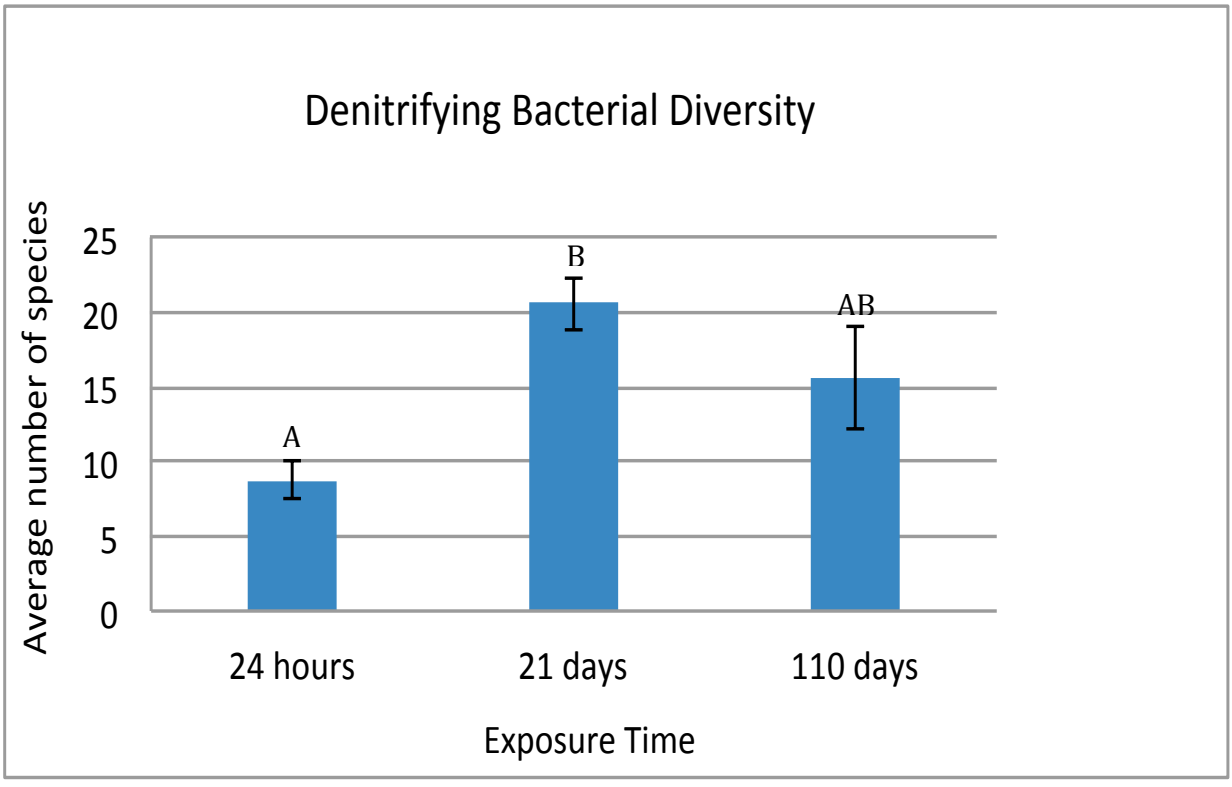

Figure 18. Bar graph comparing the average number of denitrifying bacterial species in microcosms exposed to treatments for 24 hours, 21 days and 110 days. Different letters indicate significant differences ($P<0.05$).

3.5.6 Interaction of Treatment and Depth

When analyzing the interaction between treatment (i.e., oil versus no oil) and depth (top versus bottom), the N-way ANOVA (Table 11) did not show any significant changes in the diversity of denitrifying bacteria. The *post hoc* t-test further supported this result. None of the interactions were significant as shown in Figure 19. Although average number of species was higher in bottom sediments versus the top, and microcosms not exposed to oil also had a higher average number of species, these results suggested that neither oil exposure nor depth caused significant changes in diversity.

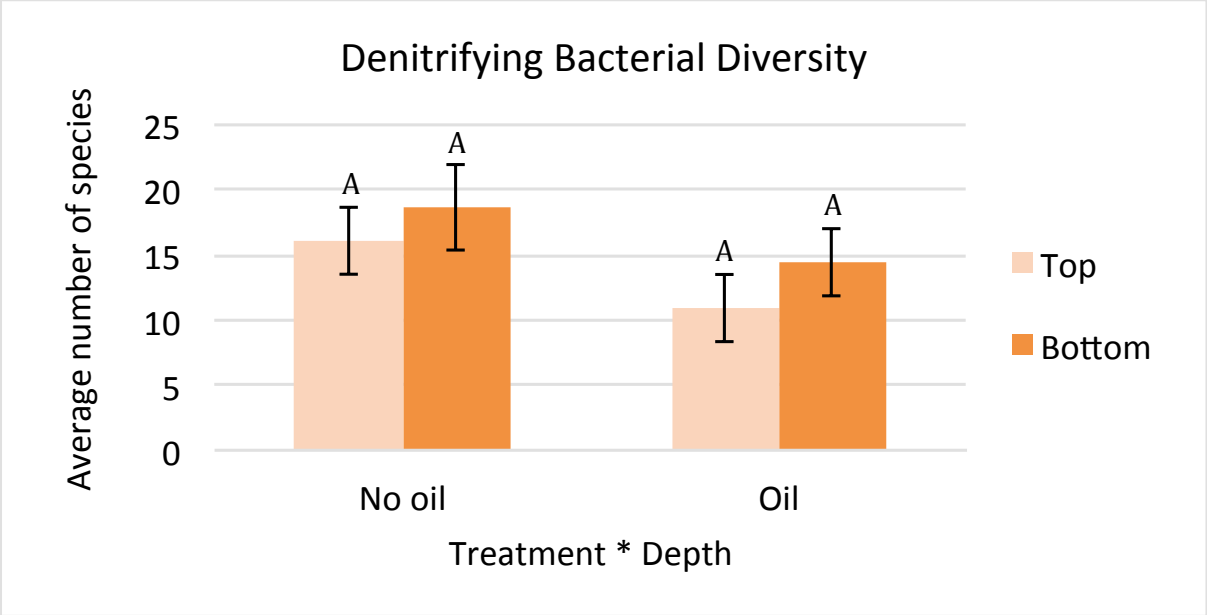

Figure 19. Bar graph with the interaction of the average number denitrifying bacterial species in microcosms exposed to treatments (oil or no oil) and depth (top or bottom).

3.5.7 Interaction of Treatment and Exposure Time

When analyzing the interaction between treatments (oil or no oil) and exposure time (24 hrs, 21 days and 110 days) the N-Way ANOVA (Table 11) suggested a highly significant effect ($P<0.01$) in the bacterial diversity of denitrifiers. The *post hoc* t-test also confirmed a highly significant difference ($P<0.01$) in the diversity of denitrifiers between

microcosms treated with oil for 24 hours and those treated with oil for 21 days (Figure 20). Highly significant differences in denitrifier diversity were observed amongst microcosms exposed to oil for 21 days and those exposed to oil for 110 days (Figure 20). In addition, there were no significant changes in the number of species between microcosms exposed to oil for 24 hours 110 days (Figure 20).

The t-test also indicated a highly significant effect between the microcosms exposed to no oil for 110 days and those exposed to oil for the same amount of time (Figure 20). These results suggest that the main factor driving denitrifying bacterial diversity is exposure time rather than presence or absence of oil except for those exposed for extended periods of time (i.e., 110 days).

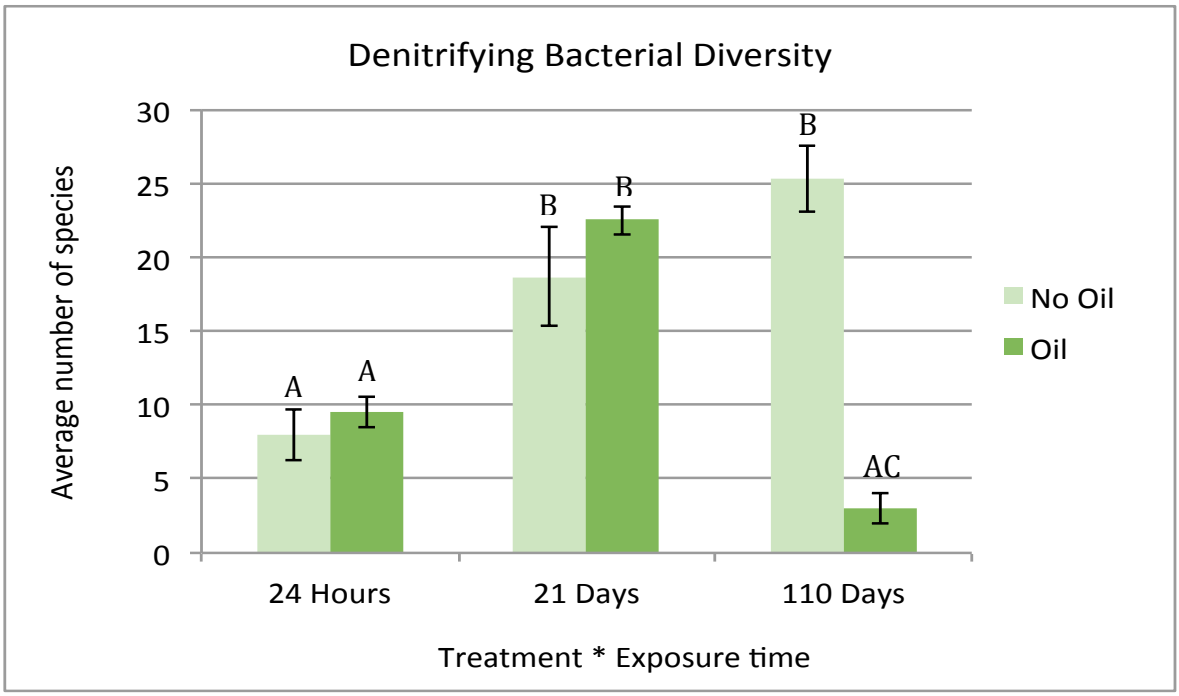

Figure 20. Bar graph showing the average number of denitrifying bacterial species in microcosms where the interaction of treatments (oil or no oil) and exposure time (24 hours, 21 days, 110 days) were accounted for. Different letters indicate significant differences (P<0.05).

3.5.8 Interaction of Depth and Exposure Time

The interaction of depth and exposure time shown in the N-way ANOVA (Table 11) suggested that there were significant differences in the number of species of denitrifiers. The *post hoc* t-test indicated highly significant differences (P<0.01) between bottom sediment microcosms exposed for 24 hours and bottom sediment microcosms exposed for 21 days (Figure 21). Similarly, significant differences (P=0.016) were observed between bottom sediment microcosms exposed for 24 hours and bottom sediment microcosms exposed for 110 days (Figure 21). In addition, significant differences (P=0.029) were observed amongst top sediment and bottom sediment microcosms exposed for 21 days (Figure 21). These results again suggest the predominance of exposure time as a leading factor for denitrifying bacteria diversity change.

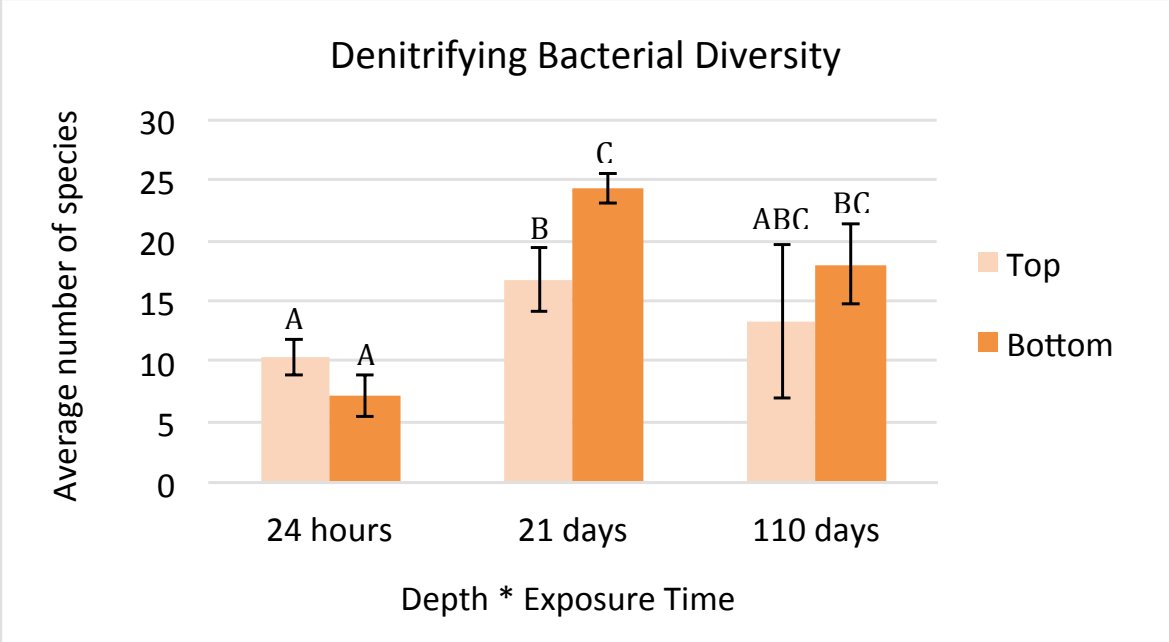

Figure 21. Bar graph showing the average number of denitrifying bacterial species in microcosms where the interaction of exposure time (24 hours, 21 days, 110 days) and depth (T= top and B=bottom) were accounted for. Different letters indicate significant differences (P<0.05).

3.6 Discussion

3.6.1 Oil Treatment Impact

When not considering depth nor exposure time, oil did not seem to have a significant effect on the diversity of denitrifying bacteria. These results suggest that when exposed for a short period of time, the south Louisiana crude oil used in this study did not have an acute toxic effect on the denitrifying bacteria. In a study by Walker et al. (1975), the ability to biodegrade was compared amongst four types of crude oils, south Louisiana crude oil, Kuwait crude oil, and two refined fuel oils No.2 and No.6. In that study, the highest biodegradability rate (82%) was observed in the south Louisiana crude oil. SLSC oil also supported the largest and fastest growing population of oil degrading bacteria compared to the other oils. The low sulfur and high saturates levels found in SLSC oil actually serves as nutrients for a wide range of bacteria (Walker et al. 1976). The present study supports the findings by Walker et al. (1975) in that acute exposure to SLSC oil did not harm the microbial community.

However, the drastic decrease in the number of species exposed to oil after 110 days could be the result of chronic toxicity or due to other factors such as imbalances in the C:N availability in the sediments. More studies are necessary to understand what factors caused the bacterial diversity to decrease in oiled microcosms after 110 days. In addition, future studies involving different types of crude oil, both light and heavy could help to better understand toxic tolerance levels in bacteria and the biodegradation capability of denitrifying bacteria in salt marsh ecosystems.

3.6.2. Depth Impact

Denitrifying bacteria are facultative aerobes; therefore, changes in denitrifying bacterial diversity in both aerobic and anaerobic samples were tested. Since anaerobic conditions are required for denitrification, this study resulted in a higher average number of denitrifying bacterial species at the bottom samples. However, the difference between the number of species at the top and bottom was not significantly different in this study.

The lack of changes in the diversity of denitrifiers across different depths could be explained by the high level of adaptation these microorganisms have in response to varying oxygen levels and their ability to switch between oxygen and nitrate as electron acceptors. This study supports previous research demonstrating the ability of facultative aerobes to switch between oxygen and nitrate as the terminal electron acceptor due to changes in redox potential measurements (Tam 1998, Rabus et al. 1999, Rockne et al. 2000, Ambrosoli et al. 2005). Studying the structure of denitrifying bacterial communities across salt marsh sediment depths is crucial for understanding how denitrifiers regulate nutrient balance. Thus, nitrate reducers in salt marshes can possibly play a more important role in the productivity and resilience of the whole ecosystem than as previously thought.

3.6.3 Exposure Time

Some of the most significant changes in the denitrifying bacterial communities in this study were observed in regards to exposure time (24 hours, 21 days and 110 days). Normally, the longer bacteria are present in a microcosm containing nutrients the more time they have to grow and reproduce. Such was the case in microcosms incubated for 21 days compared to those incubated for 24 hours. Microcosms incubated for 21 days had a significantly higher number of species of denitrifiers than microcosms incubated for 24

hours. It is highly possible that microorganisms experienced an initial shock when transferred from the field to the microcosm in the lab, thus causing the initial decrease in diversity. However, there were no significant differences in the bacterial diversity of denitrifiers between microcosms incubated for 24 hours and 110 days, nor between microcosms incubated for 21 days and 110 days. This suggests that once bacterial communities adapt to the environmental conditions the diversity increases. However, after prolonged exposure times (i.e., 110 days) the average number of species slightly decreased again. Future studies involving shorter sampling times could reveal the peak time of bacterial diversity.

3.6.4 Interactions Between Treatment and Depth

Degradation of PAHs by microorganisms in aerobic environments is well documented (Cerniglia 1993, Geiselbrecht et al. 1996, Van Hamme et al. 2003). More recent studies have also demonstrated degradation of naphthalene by bacteria under anaerobic conditions where mineralization of naphthalene was nitrate dependent (Rockne et al. 2000), thus providing evidence of oil biodegradation by denitrifying bacteria. The current study presents evidence of how denitrifying bacterial communities in both aerobic and anaerobic conditions respond to contamination with south Louisiana crude oil in wetland sediments.

Denitrifying bacteria are facultative anaerobes; therefore, changes in denitrifying bacterial diversity in both aerobic and anaerobic samples and the presence or absence of oil were tested. In general, there was a higher average number of denitrifying bacterial species at the bottom samples, as expected since anaerobic conditions are required for

denitrification. Similarly, there was a slightly higher average number of denitrifying species in non-oiled sediments than oiled ones. However, none of these differences were significant.

Nonetheless, the ability of denitrifiers to utilize crude oil as a carbon source and achieve degradation in both aerobic and anaerobic conditions is very interesting. In this study, oil treatment was associated with a small decrease in the number of denitrifying bacterial species for both aerobic and anaerobic samples. Regardless of oil presence, the bottom samples had a higher, though not significant, number of denitrifying bacterial species. Thus, while most oil degrading bacteria prefer aerobic conditions, this study shows that denitrifying bacteria capable of oil degradation were equally able to do so in aerobic and anaerobic sediments.

3.6.5 Interaction Between Treatments and Exposure Time

Due to the fact that anthropogenic activities have expanded into mangrove ecosystems, pollution and oil contamination studies in mangrove sediments have increased. Guo et al. (2005) found that degradation potential and ability to metabolize toxic organic pollutants such as PAHs depends on the type of bacteria present in the community and whether the community has had prior exposure to oil contamination rather than the amount of oil or the ring structure of the PAHs. Degradation rates are also enhanced when communities of microbes are capable of acclimatization after oil contamination exposure (Wilson and Jones 1993, Guo et al. 2005).

The results from the current study, concur with previous studies demonstrating no significant changes in the community of denitrifier bacteria treated with oil for 24 hours and 21 days However, significant changes did occur between oiled and non-oiled microcosm exposed for 110 days and between oiled microcosms exposed for 21 days and

oiled microcosms exposed for 110 days. Although chronic exposure to oil could potentially have a toxic effect on the bacterial community, other factors may also contribute to such a drastic decrease in the bacterial diversity. Such factors include exhaustion of essential nutrients and C:N ratio imbalance due to chronic oil exposure. However, further studies are needed to better determine the exact cause of bacterial diversity reduction. Nevertheless, this study was successful at demonstrating how exposure time to oil can play a significant role on bacterial diversity and how a large number of species of denitrifiers are capable of withstanding light crude oil contamination for at least 21 days of exposure.

Although it is generally believed that microbial diversity and population density of PAH degrading microbes is correlated to exposure time and the concentration of oil, Zou et al. (2009) found the opposite effect. In his study, both exposure time and PAH concentration (both low and high) reduced microbial diversity. While in some cases microorganisms are able to utilize PAH as carbon source, which in turn increases microbial activity and the diversity of oil degraders, petroleum hydrocarbons can be toxic to many microbes, thus reducing the microbial diversity (Zhou et al. 2009). While this is most certainly true for many types of heavy crude oils, south Louisiana crude oil is low in toxicity and therefore easily degraded by microorganisms. In the present study, there was a significant increase in the number of species of denitrifiers exposed to oil for 21 days compared to those exposed for 24 hours. This further supports the idea that bacteria diversity increases as exposure time increases.

Because of the rapid and easy biodegradation potential of Louisiana crude oil by denitrifying bacteria, future research applications should be conducted after 60 days of exposure but no more than 90 days. This could shed light on how denitrifying bacterial

communities react to light petroleum hydrocarbons before other essential nutrients such as nitrogen or phosphorus become limited.

3.6.6 Interaction Between Depth and Exposure Time

While there were no significant differences in the bacterial diversity of denitrifiers in terms of depth alone, when exposure time was included interesting results were observed. After incubation for 21 days, bottom sediments had significantly higher numbers of species than top sediments. This is probably due to the fact that even though denitrifiers are capable of living in aerobic areas, some proteins crucial for denitrification can be potentially inhibited in the presence of oxygen (Van Cleemput et al. 2006). This can also be explained by the fact that bottom sediments have the capacity of storing more nutrients as opposed to top sediments from simple accumulation, thus providing bottom sediment communities of denitrifiers with more essential nutrients to perform metabolic activities.

In addition, there was a significantly higher number of species in bottom sediments exposed for 24 hours than bottom sediments exposed for 21 days. Similarly, bottom sediments exposed for 110 days had a significantly higher number of species than bottom sediments exposed to 24 hours. These results suggest that after 21 days, denitrifying bacteria in bottom sediments reached a high number of species, most likely due to bacterial acclimation. However, as bacteria continue to grow, some of the essential nutrients can potentially become limited, thus decreasing the species diversity. After the initial shock when sediments are transferred from the natural environment, bacterial communities adapt and thrive, thus increasing their diversity.

CONCLUSIONS

Denitrifying bacteria are facultative aerobes and represent an important group responsible for a major biogeochemical pathway capable of naturally biodegrading a wide range of toxic substances, reducing excess nutrients from fertilizers and reducing greenhouse gas emissions to the atmosphere. However, low microbial diversity can reduce the rate of denitrification and bioremediation in wetland ecosystems. For this reason, studying the microbial diversity of denitrifiers in salt marshes under stressful circumstances is crucial for the management and development of wetland restoration programs.

Core sediments were enriched with 100 μM NO_3-, the average concentration of nitrate found in south Louisiana wetland ecosystems, or supersaturated with 20,000 μM NO_3- in order to promote denitrification. A 20% increase in the total carbon concentration in the original sample was also added in order to increase the C:N ratio and promote denitrification. At 100 μM NO_3-, bottom sediments had significantly higher numbers of denitrifying bacterial. In addition, bacterial diversity was compared between top and bottom samples. This study revealed that nitrate and glucose additions were associated with a significant increase in the diversity of denitrifiers at the top but not at the bottom.

Because of the important role denitrifying bacteria play in nutrient cycling in mangrove and salt marsh ecosystems, it is important to understand how oil spill contamination events can potentially affect the community of denitrifiers. For south Louisiana crude oil, the lower sulfur content and high concentration of saturates make it less toxic and more susceptible to microbial degradation. To date, this study is the first one

to analyze the diversity of denitrifying bacteria in mangrove sediments affected by the *Deepwater Horizon* oil spill using DGGE.

The diversity of denitrifying bacteria was higher in bottom anaerobic sediments and after 21 days. However, the oiled microcosm experienced a slightly lower diversity of denitrifying bacteria than those unoiled. In this study, denitrifying bacterial diversity was highest after 21 days, thus suggesting the ability of denitrifiers to adapt to oiled condition and their ability to utilize oil as a source of carbon, which they can metabolize and degrade within the first few weeks of exposure. Nevertheless, after prolonged exposure to oil (i.e., 110 days) the diversity of denitrifiers decreased significantly which could be explained by either exhaustion of essential nutrients and an imbalance in the carbon to nitrogen ratio or toxicity due to chronic exposure. However, more studies are needed to understand the exact factors that caused such a change in bacterial diversity over time.

As the Earth's human population continues to grow, the need for more nitrate based fertilizer and oil extraction and exploration will continue to expand, thus increasing the threat of eutrophication and oil spill contamination in coastal wetland ecosystems. The resilience of salt marsh ecosystems to such anthropogenic stressors can be partially attributed to the ability of denitrifying bacteria to regulate nutrients and metabolize a wide range of xenobiotic substances such as PAHs. Given the contributions of nutrient balance by denitrifying bacteria in salt marsh ecosystems, future management and restoration efforts should focus on the microbial community structure and their ability to mitigate contamination events.

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