Denitrification in Urban Coastal Environments: A Functional Gene Study

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LOYOLA UNIVERSITY CHICAGO

DENITRIFICATION IN URBAN COASTAL ENVIRONMENTS: A FUNCTIONAL GENE STUDY

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
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To Mom, Dad, & Danny
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................................ iii

LIST OF TABLES ......................................................................................... vi

LIST OF FIGURES .................................................................................. vii

ABSTRACT .............................................................................................. viii

CHAPTER ONE: INTRODUCTION .......................................................... 1

CHAPTER TWO: EFFECT OF EASTERN OYSTERS (*Crassostrea virginica*) ON *nirS*, *nirK*, AND *nrfA* GENE ABUNDANCE IN AN URBANIZED ESTUARY, JAMAICA BAY (NEW YORK CITY) ......................................................... 13

CHAPTER THREE: EFFECT OF WASTEWATER TREATMENT SHUTDOWN ON *nirS*, *nirK*, AND *nrfA* GENE ABUNDANCE IN THE WESTERN LONG ISLAND SOUTH SHORE ESTUARY (NEW YORK) ................................................... 44

CHAPTER FOUR: CONCLUDING COMPARATIVE ANALYSIS OF JAMAICA BAY AND THE WESTERN LONG ISLAND SOUTH SHORE ESTUARY .......... 80

APPENDIX A: SUPPLEMENTARY INFORMATION .................................. 90

REFERENCE LIST ................................................................................... 97

VITA ............................................................................................................. 108
LIST OF TABLES

Table 1. Mean physiochemical measurements for sampling sites in Jamaica Bay for fall and winter……………………………………………………………………………………………………28

Table 2. ANOVA p-values for sediment characteristics, N transformations, and gene abundance for oyster and control treatments………………………………………29

Table 3. Forward-stepping multiple regression results for gene abundance in Jamaica Bay…………………………………………………………………………………………..35

Table 4. Summary of relevant literature results …………………………………………………………………………38

Table 5. Average physiochemical measures for WLISS estuary sampling sites…………58

Table 6. Three-way ANOVA p-values for biogeochemistry and microbial gene abundance measurements at North Meadow and Cuba Island sites from marsh, intertidal, and subtidal habitats during summer, fall, and winter sampling seasons…….64

Table 7. Forward-stepping multiple regression results for denitrification and gene abundance data………………………………………………………………………………66

Table 8. Comparison of denitrification studies carried out in various environments……..86
LIST OF FIGURES

Figure 1. Diagram depicting various aspects of nitrogen cycling……………………………………12

Figure 2. Map of Jamaica Bay…………………………………………………………………………19

Figure 3. Gene copy numbers of Jamaica Bay samples from Spring Creek (SC), Mott’s Basin (MB), Wildlife Refuge (WR), and Floyd Bennett Field (FB)……………………………………31

Figure 4. PCA results for Jamaica Bay………………………………………………………………33

Figure 5. Map of the reference (Cuba Island; East Bay) and impacted (North Meadow; West Bay) study sites in the Western Long Island South Shore (WLISS) estuary ……51

Figure 6. qPCR results of absolute abundance and relative abundance (%) of nirS (as function of 16S abundance) in bacterial DNA from WLISS at the reference (Cuba) and impacted (North) sites ………………………………………………………………………59

Figure 7. qPCR results of absolute abundance and relative abundance (%) of nirK (as function of 16S abundance) in bacterial DNA from WLISS at the reference (Cuba) and impacted (North) sites ………………………………………………………………………60

Figure 8. qPCR results of absolute abundance and relative abundance, (%) of nrfA (as function of 16S abundance) in bacterial DNA from WLISS at the reference (Cuba) and impacted (North) sites ………………………………………………………………………61

Figure 9. qPCR results of absolute abundance of 16S in bacterial DNA from WLISS at the reference (Cuba) and impacted (North) sites ………………………………………………………………………62

Figure 10. PCA results for WLISS…………………………………………………………………………68

Figure 11. Mean (±SE) combined functional gene abundance for fall and winter sampling dates in Jamaica Bay (JBAY) and the Western Long Island South Shore Estuary (WLISS)………………………………………………………………………..81

Figure 12. Mean organic matter, chlorophyll a, and NO₃ for Jamaica Bay and WLISS………………………………………………………………………..82

Figure 13. Regressions for overall gene abundance for Jamaica Bay and WLISS………84

Figure 14. Relationship between nirS and nirK gene abundance and denitrification potential (DNP) for summer, fall, and winter sampling dates WLISS samples………..88
ABSTRACT

Eutrophication is a major anthropogenic stressor on aquatic ecosystems worldwide. Like many other estuaries, Jamaica Bay, which is located in the southeastern portion of New York City (NYC), NY, is impacted by anthropogenic N inputs from various sources. Despite eutrophic conditions, improvements in water quality over the last few decades have prompted government agencies to promote oyster restoration to help reduce anthropogenic N in NYC waters. The effect of eastern oysters on sediment bacterial communities responsible for denitrification in Jamaica Bay was previously unknown. Sediments exposed to oysters were predicted to have high denitrification gene abundances, and thus a high potential for denitrification; however no significant relationship was found between denitrification gene abundance and denitrification potential. Our results showed oysters’ effect on abundance of denitrifiers was variable by site and season with no significant overall impact. A significant factor for denitrifier abundance in Jamaica Bay was availability of organic matter.

This project also explored post-hurricane Sandy denitrification dynamics in a nearby estuary, the Western Long Island South Shore estuary (WLISS). Following the damage caused by this superstorm, 68 million gallons of raw sewage were released into WLISS. This massive release of sewage was the result of damage caused to the Bay Park Sewage Treatment Plant which is responsible for wastewater treatment serving 5 million people. The release of this sewage greatly increased the nutrient load in the WLISS estuary and the environmental impacts are of interest. Our analyses showed a mixed
effect of site and season on the bacterial denitrifying community. Unlike Jamaica Bay, there is a significant correlation between denitrifier abundance and denitrification rate. This study employed a $^{15}$N tracing method to assay denitrification rate. This method more directly assayed the samples in conditions similar to those found in the environment and therefore may be more indicative of natural denitrification rates.
CHAPTER ONE

INTRODUCTION

The Nitrogen Cycle

The availability of biologically reactive nitrogen (N) plays a critical role in the regulation of global primary production, especially in coastal and marine environments which are typically considered N-limited (Herbert 1999). The N cycle is a series of oxidation and reduction reactions, some of which are solely carried out by enzymes produced by microorganisms (Zehr and Ward 2002). N is a key component of amino acids and proteins, however, it is often a limiting nutrient because the most abundant form, dinitrogen gas (N₂), is relatively inert. Nitrogen fixation is required to make N bioavailable by converting N₂ into ammonia (NH₃; Figure 1). After this, NH₃ can be used to construct organic molecules used for growth and metabolism. Following excretion or death of an organism, organic N compounds are decomposed back into NH₃ and ammonium (NH₄⁺), which can be transformed into several other inorganic compounds (Figure 1). For example, nitrification, an aerobic, microbially-mediated process, is the oxidation of NH₄⁺ to nitrate (NO₃⁻). From there, NO₃⁻ can be transformed via denitrification, or the anaerobic reduction of NO₃⁻ to gaseous forms of N (N₂ or nitrous oxide; N₂O). Denitrification requires electron donors and thus is often coupled with oxidation of organic carbon (C). Because nitrification requires oxygen while denitrification does not, these two processes often occur together in environments where there is an interface of high and low O₂ availability (Brettar and Rheinheimer 1992,
Nielsen et al. 1990, Seitzinger 2006). Alternatively, NO$_3^-$ can be transformed to NH$_4^+$ via dissimilatory NO$_3^-$ reduction to NH$_4^+$ (DNRA), or directly assimilated by organisms. In addition, N$_2$ can be produced via a recently discovered process, annamox, (anaerobic ammonia oxidation), the conversion of fixed inorganic N (NO$_3^-$ or NH$_4^+$) to N$_2$ without any intermediate products (Einsle and Kroneck 2004) (Figure 1). Finally, microbes, fungi, algae, and plants can directly assimilate inorganic N (NH$_4^+$ and NO$_3^-$) and small organic N compounds (e.g., urea) into cells or tissues to use for growth.

**Nitrate Reduction**

In addition to direct assimilation, the two major pathways for NO$_3^-$ reduction are denitrification and DNRA (Tiedje 1988). Both of these processes are dissimilatory, that is, the reduced N is not used by the cell. Denitrification is typically defined as a facultative anaerobic reaction in which NO$_3^-$ is reduced to N$_2$ (Robertson and Tiedje 1987). Primary drivers of denitrification include sources of organic C (Tiedje 1988; Azam et al. 2002), N oxide availability, and reducing conditions (i.e., low oxygen concentrations) (Zumft 1997, Seitzinger 1988). DNRA is a reaction in which NO$_3^-$ is reduced to nitrite (NO$_2^-$) and ammonium (NH$_4^+$) (Kelso et al. 1997, Kraft et al. 2011). While DNRA has been recognized as a pathway for NO$_3^-$ removal for over 25 years, but it has only gained attention from a variety of scientists in the past 15 years (Burgin and Hamilton 2007). Like denitrification, DNRA usually occurs under anaerobic conditions. However, DNRA is less sensitive to O$_2$ fluctuations than denitrification (Fazzolari et al. 1998). DNRA also seems to occur when NO$_3^-$ is limiting in relation to organic C (Cole and Brown 1980).

Denitrification and DNRA are both carried out by a wide variety of organisms. For both processes, research has focused on characterizing bacterial taxa with the
enzymatic capacity for \( \text{NO}_3^- \) and \( \text{NO}_2^- \) reduction. Bacteria capable of denitrification have been identified in a number of phyla including *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Zumft 1997). There are also a variety of fungi capable of denitrification as well as *Archaea* (Shoun et al. 1992, Philippot 2002). Many studies have also identified diverse heterotrophs that can complete DNRA, with a focus on marine sediments. Bacteria capable of DNRA have been identified in a number of genera including *Aeromonas*, *Vibrio*, and *Clostridium* (Herbert 1999). Soil fungi including *Fusarium oxysporum* have also been shown to be capable of DNRA (Zhou et al. 2002).

**Denitrification Genes**

Denitrification from \( \text{NO}_3^- \) to \( \text{N}_2 \) requires four transformations, and each is mediated by a different enzyme (Knowles 1982). First, \( \text{NO}_3^- \) is reduced to \( \text{NO}_2^- \). The enzyme that catalyzes this reaction is nitrate reductase. Next, the reduction of \( \text{NO}_2^- \) to nitric oxide (NO) is catalyzed by nitrite reductase enzymes. The third steps involves the reduction of NO to nitrous oxide (\( \text{N}_2\text{O} \)), carried out by nitric oxide reductases. In the fourth step, \( \text{N}_2\text{O} \) is reduced \( \text{N}_2 \) by nitrous oxide reductase (Zumft 1997). Denitrification also requires many cofactors and proteins, such as cytochromes, which are produced by bacteria and serve as electron transporters (Payne 1981).

The four microbial enzymes that mediate the heterotrophic process of denitrification are nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) (Figure 1). The enzymes are encoded by four genes; *nar*, *nir*, *nor*, and *nos* (Zumft 1997) (Figure 1). These catalytic enzymes are
produced by a wide variety of microorganisms, but not all denitrifiers contain the entire suite of genes. Some strains of *Pseudomonas, Neisseria, and Bacillus* lack nitrate reductase (*nir*), and are considered “nitrite dependent” (Payne 1981). As many as one third of denitrifiers lack the nitrous oxide reductase gene (*nos*) responsible for the reduction of nitrous oxide to N₂ gas (Jones et al. 2008). In addition, some microorganisms have only *nosZ* and no other denitrification genes (Sanford et al. 2012). These findings suggest that denitrification may, under some circumstances, be a community process in which closely-associated microorganisms are required to complete the entire process (Wallenstein et al. 2006).

The presence of the nitrite reductase gene (which reduces NO₂⁻ to NO) is considered an important indicator for the presence of denitrifying bacteria (Braker 1998). These genes differentiate denitrifying bacteria from NO₃⁻ respiring bacteria that do not possess *nir* genes or other genes required for denitrification. Measurements of *nir* genes are common in studies of denitrifying bacteria because *nir* genes mediate the first step in denitrification in which a gas is produced (Wallenstein et al. 2006).

There are two functionally homologous *nir* genes: *nirS* and *nirK*. These genes are mutually exclusive and are thought to exist primarily as single copy genes (Coyne 1989). However, the *nirS* and *nirK* genes both encode nitrite reductase and are therefore functionally identical. This was confirmed by expressing *nirK* from *Pseudomonas aureofaciens* in a *P. stutzeri* mutant in which *nirS* had been removed and observing that nitrite reductase was still properly produced (Glocker, Junst, and Zumft 1993). Although *nirS* and *nirK* share the same function, they have different structures. The *nirS* gene encodes a dimeric cytochrome cd₁ nitrite reductase composed of a c and d₁ heme, while *nirK* encodes a trimeric copper containing nitrite reductase (Zumft 1997). *nirS* is
frequently found in estuary or seawater samples and has been found in a greater percentage of environmental samples from a diversity of ecosystems (Braker et al. 1998). *nirK* is most frequently detected in terrestrial environments and, although not found in as many habitats as *nirS*, it is detected in more phylogenetically diverse bacteria (Braker et al. 1998). Because functional genes are frequently used in denitrification studies, *nirS* and *nirK* are widely studied. A number of PCR primers have been developed to target *nirS* and *nirK* gene fragments. Some of the more widely used primer sets include those by Ward (1995), Hallin and Lindgren (1999), Braker et al. (1998), and Throback et al. (2004).

**Factors Controlling *nirS* and *nirK***

Previous research on environmental factors controlling denitrifier populations has shown that organic C and O₂ availability are important factors which regulate relative abundance of *nirS* and *nirK* in a variety of environments. Organic C is positively correlated with *nir* gene abundance in estuarine, forest, and arctic habitat types (Dang et al. 2009, Levy-Booth and Windor 2010, Henry 2004, and Kandler 2006). Desnues et al. (2007) and Knapp et al. (2010) showed that *nirS* genes are more abundant in permanently anoxic samples while *nirK* is more frequently detected in samples with fluctuating O₂ and pH levels. The cytochrome cd₁ nitrite reductase encoded by *nirS* catalyzes a single electron reduction of NO₂⁻ to NO as well as a four electron reduction of O₂ to H₂O while the copper containing nitrite reductase encoded by *nirK* catalyzes only the reduction of NO₂⁻ to NO (Einsle and Kroneck 2004). These enzymatic differences contribute to the ability of *nirS* and *nirK* denitrifiers to reduce NO₂⁻ to NO in variable O₂ conditions.

In addition to C and redox conditions, NO₃⁻ availability and denitrifier abundance are expected to be positively correlated. However, studies on this topic have shown
equivocal results. Liu et al. (2003) found NO$_3^-$ and O$_2$ had the strongest influence on the
distribution of nirS and nirK genes in a marine environment. Kana (1998) and Seitzinger
(1993) also found direct relationships between denitrification rate and NO$_3^-$
concentrations in estuarine habitats. More recently, Mosier and Francis (2012) found nirS
and nirK gene abundance to be positively correlated to NO$_3^-$ in forest soils. However, nir
gene abundances and NO$_3^-$ levels have frequently been found to have no significant
relationship. Two forest soil studies found that NO$_3^-$ levels had no significant
relationship with nir gene abundance (Garzio-Hadzik 2012, Wallenstein 2004). Abell et
al. (2009) and Zhang et al. (2014) found no correlation between the abundance of nir
genes and NO$_3^-$, in highly eutrophic estuaries. Instead, nir gene abundance was related to
other environmental factors like temperature, salinity, and chlorophyll-a (Abell et al.
2009, Zhang et al. 2014). The mixture of results indicate that factors other than NO$_3^-$ are
of equal or greater importance in determining nir abundance across a variety of
ecosystem types including estuaries, forest soils, and streams.

Like the relationship between NO$_3^-$ and nir gene abundance, studies comparing nir
gene abundance with denitrification rates have produced equivocal results. A number of
studies have found correlations between nir gene abundances and denitrification rates
(Cuehl et al. 2010, Dong et al. 2009, Peterson et al. 2012). However, no correlation
between potential denitrification rate and nir gene abundance is also a common finding
potential denitrification rates do not correlate with nir gene abundances when rates are
very high or stable (i.e., not variable). Therefore, Peterson et al. (2012) proposed that
functional denitrification gene abundances may be better predictors for overall
denitrification potential in highly variable ecosystems or across large gradients, but have
limited application in studies conducted across small spatial and temporal scales. It is also possible that shifts in the dominant bacterial functional groups can obscure the relationship between denitrification rates and gene abundance (Brankatschk et al. 2011).

The method used to measure denitrification may also affect the possibility of detecting a relationship between nir gene abundance and denitrification rate. Some of the most frequently used methods include mass balance (i.e., difference between inputs and outputs of N), acetylene inhibition, N₂ flux measurements, pore water profiles, and ¹⁵N tracers (Groffman et al. 2006, Myrold 1990). The acetylene inhibition method stops the reduction of N₂O to N₂ and measures N₂O production as denitrification potential. It is a widely used method because of its sensitivity, relatively low cost, and ability to replicate samples (Herbert 1999). Some of the negative aspects of the acetylene inhibition method include the inhibition of nitrifying activity and conditions that do not mimic environments in situ. More direct measures of denitrification can be obtained using ¹⁵N tracer methods and direct N₂ measurements, which require fewer manipulations of the environmental conditions during the measurements. ¹⁵N tracer methods also allow denitrification, nitrification, ammonification, and N mineralization to be measured in a single experiment (Lohse et al. 1996). Those methods which maintain environmental conditions may be more likely to detect a relationship with nir gene abundance, although this has not previously been tested.

Aside from the primary drivers of carbon, redox, and NO₃⁻, other factors important to nir gene abundance include salinity, temperature, and seasonal variation. Chon et al. (2009) found nirS abundance was higher in summer than winter samples, and that in coastal environments, low salinity may be associated with higher nir gene diversity (Santoro et al. 2006). Jones and Hallin (2010) also speculate that nirS and nirK
respond differently to physiochemical gradients based on their environmental preferences. Overall, many factors have been found to influence nir gene abundance and distribution, which vary according to environmental conditions and habitat.

**Dissimilatory Nitrate Reduction to Ammonium (DNRA) Gene**

The nitrite reductase used in DNRA is encoded by the *nrfA* gene. This gene has been found in a wide variety of bacteria (Smith et al. 2007). Although it has generally been assumed that the majority of NO$_3^-$ in the environment is reduced via denitrification, DNRA can account for a large portion of NO$_3^-$ reduction in some cases. For example, Silver et al. (2001) found 75% of NO$_3^-$ reduction in a tropical forest was attributed to DNRA. In two studies in salt marshes, an average of 50% and 30% of NO$_3^-$ was consumed by DNRA (Koop-Jacobsen and Giblin 2010, Tobias et al. 2001). However, the overall contribution to NO$_3^-$ consumption or NH$_4^+$ production due to DNRA can be as low as 0-3% in soils (Silver et al. 2005, Inselsbacher et al. 2010).

Several studies have recently examined the relationship between *nrfA* abundance and DNRA rate. Dong et al (2009) found *nrfA* abundance and DNRA rate were positively related using $^{15}$N-labeling methods in the Colne Estuary, UK. The same pattern was also found for sediment the Arabian Sea (Jensen et al. 2011). In general, DNRA has received increased attention from researchers only recently (Burgin and Hamilton 2007). The development of *nrfA* primer sets by Mohan et al. (2004), Smith et al. (2007) and Takeuchi (2006) along with improving technologies will likely lead to more studies that explore the relationship between *nrfA* abundance and DNRA rate measures.

Understanding the relative rates of denitrification and DNRA is of interest due to its implications for eutrophication because DNRA returns NH$_4^+$ to the ecosystem while denitrification removes NO$_3^-$ via the production of N$_2$O or N$_2$. Therefore, DNRA allows
biologically active N to persist in the aquatic environment, which can further enhance eutrophication. Because of this, the competition between denitrifying bacteria and those capable of DNRA is a recent focus of study. Denitrification may be favored over DNRA in ecosystems that have high NO$_3^-$ concentrations (Burgin and Hamilton 2007, Morrissey et al. 2013, Stevens and Laughlin 1998). In salt marsh sediments, DNRA decreased from 50% to 4% of NO$_3^-$ reduced when NO$_3^-$ concentrations were increased (Smith et al. 1982). Conditions of low NO$_3^-$ and abundant labile C can favor DNRA over denitrification because DNRA transfers eight electrons per mole of NO$_3^-$ while denitrification transfers five electrons (Tiedje 1988). This would make DNRA more efficient than denitrification under low NO$_3^-$ and high C concentrations (Tiedje 1988, Burgin and Hamilton 2007). In general, more information is needed regarding competition between denitrification and DNRA communities under fluctuating environmental conditions (Burgin and Hamilton 2007, Gardner et al. 2009, Smith et al. 2009).

**Denitrification and Eutrophication**

Denitrification is an important step in the N cycle because it removes excess NO$_3^-$ from aquatic environments. Excess NO$_3^-$ is a common environmental concern in aquatic ecosystems exposed to anthropogenic N input from wastewater treatment plants, fertilizers, and urban runoff. Estimates suggest anthropogenic N enters the environment at twice the rate of N-fixation (Seitzinger 2008). Excess N in aquatic environments increases phytoplankton and macroalgae growth (Herbert 1999), and subsequent death and settling of primary producers stimulates microbial decomposition and O$_2$ consumption. At large scales, eutrophication can lead to widespread hypoxia in coastal environments, for example, expansive “dead zones” such as those that form annually in
the Gulf of Mexico and Chesapeake Bay (Seitzinger 2008). Understanding
denitrification and DNRA dynamics in eutrophic habitats is critical for developing
management and restoration strategies to protect ecosystem health.

Estuaries can accumulate excess N from watersheds, resulting in eutrophication
and associated environmental impacts (Seitzinger et al. 2006). However, estuarine
sediments are biogeochemical hotspots of denitrification (Piehler and Smyth 2011,
Seitzinger 1987, Valiela and Bowen 2002,), and may have the capacity to mitigate N-loading to coastal waters. Enhanced susceptibility to eutrophication and capacity for
denitrification make estuarine habitats important sites for denitrification research. With
an increase in urbanization in coastal environments worldwide, it is important to
understand denitrification in urban estuaries, and what factors are influencing NO₃⁻
removal and recycling at those sites.

**Thesis Project**

To quantify the influence of oysters on sediment N transformations and nir gene
abundance, we established an experiment at four sites across a gradient of N load in
Jamaica Bay. For the first research chapter of my Master’s thesis, I worked
collaboratively with Drs. Castignetti and Hoellein to quantify the influence of oysters on
nitrite reductase gene (nirS, nirK, and nrfA) abundance. I used qPCR to quantify the
number of nirS, nirK, nrfA, and 16S genes at four sites within Jamaica Bay in fall and
winter. Five research questions were explored:

(1) Do oysters affect nitrite reductase gene abundance?
(2) Does nitrite reductase gene abundance differ among study sites?
(3) Is there seasonal variation in denitrification gene abundance?
(4) Are nitrite reductase gene abundances correlated with denitrification potential or other N cycling rate measures?

(5) Are nitrite reductase gene abundances related to N pool measurements and N cycling rates?

For the second research chapter of my thesis, I analyzed denitrification and DNRA gene abundance in sediment samples from another estuary near Jamaica Bay, the Western Long Island South Shore Estuary (WLISS). This site experienced a large nutrient pulse when damage from Hurricane Sandy shutdown the Bay Park Wastewater Treatment Plant in fall-winter 2012. This event presented an opportunity to analyze the environmental and bacterial response to a large input of excess nutrients. Samples collected from WLISS allowed us to expand the questions asked in Jamaica Bay.

Questions I explored in this phase of the project included:

(1) Will denitrification be influenced in a site impacted by the treatment plant shutdown versus a relatively unaffected site?

(2) Will abundances of nirS, nirK, and nrfA differ between the two sites?

(3) Are there differences in nirS, nirK, and nrfA gene abundance among estuary habitats (subtidal, intertidal, and marsh)?

(4) Are nirS and nirK gene abundances correlated with denitrification rates?

(5) What are the environmental factors controlling nirS, nirK, and nrfA gene abundance, and are they similar to those found in Jamaica Bay?
Figure 1. Diagram depicting various aspects of nitrogen cycling. Different N cycling processes are listed along with key microbial genes for steps of these processes listed in italics. Diagram adapted from Canfield et al. 2010 and Purvaja et al. 2008.
CHAPTER TWO

EFFECT OF EASTERN OYSTERS (*Crassostrea virginica*) ON *nir*<sub>S</sub>, *nir*<sub>K</sub>, and *nrf*A GENE ABUNDANCE IN AN URBANIZED ESTUARY, JAMAICA BAY (NEW YORK CITY)

**Introduction**

**Oyster Reefs**

Oysters are important ecosystem engineers in coastal environments worldwide (Beck et al. 2011). In addition, oysters provide multiple ecosystem services including water filtration, removal of suspended solids, increased water clarity, and shoreline stabilization (Newell et al. 2007, Grabowski and Peterson 2007). Recent studies have shown filter-feeders, such as oysters, can simulate removal of excess nutrients via enhanced denitrification in sediments exposed to oyster biodeposits (i.e., feces and pseudofeces; Newell et al. 2007, Kellogg et al. 2013).

Approximately 85% of oyster reefs have been lost globally (Beck et al. 2011). Causes of oyster reef loss include overfishing, cultural eutrophication, shoreline alteration, habitat destruction, and disease (Cerco and Noel 2007, Levinton et al. 2011). Oyster reef conservation and restoration is ongoing at many locations around the world. Strategies include preserving oyster reefs in protected areas, prohibition of oyster harvests in endangered areas, oyster reef mapping, and invasive species prevention (Beck et al. 2011). Other organizations construct new reefs by placing ‘spat on shell’ (i.e., juvenile oysters attached to shells) in areas where populations are low.
The natural range of the eastern oyster (*Crassostrea virginica*) extends from the Gulf of St. Lawrence to the Gulf of Mexico on the east coast of North America. Like other oysters species, populations of *C. virginica* greatly declined in the 19th century due to harvesting (Kennedy and Breisch 1981). Considered ‘functionally extinct’ in the Hudson-Raritan Estuary of New York and New Jersey, oysters have been the focus of many recent restoration projects in New York City (NYC) waters. Although subject to introduced diseases and contamination by heavy metals, oyster restoration has been promoted to help reduce anthropogenic nitrogen (N) concentrations in coastal environments.

**Cultural Eutrophication and Denitrification**

Denitrification, or the microbially-mediated anaerobic respiration of nitrate (NO$_3^-$) to dinitrogen (N$_2$) gas, is a focus of study in eutrophic ecosystems, because it represents a loss of biologically active N. Denitrification is primarily controlled by the availability of organic carbon (C), nitrate (NO$_3^-$), and favorable redox conditions (Barnard et al. 2005, Groffman 1991, Tiedje 1988). In many marine habitats, nitrification (i.e., the autotrophic oxidation of ammonium (NH$_4^+$) to NO$_3^-$) and denitrification are closely coupled, where the NO$_3^-$ produced by nitrification is used as a substrate for denitrification (Wallenstein et al. 2006). However, in eutrophic habitats, nitrification and denitrification may become decoupled (Seitzinger et al. 2006). Coupled nitrification-denitrification requires adjacent oxic-anoxic microsites, where conditions vary across small spatial scales. Decoupling can occur because denitrification takes place in suboxic conditions, while oxic conditions required for nitrification can be eliminated in eutrophic sediments and (Seitzinger et al. 2006).
Another N transformation of interest in eutrophic environments is the dissimilatory reduction of NO$_3^-$ to NH$_4^+$ (DNRA). This process competes with denitrification for NO$_3^-$, but rather than removing N from an ecosystem in an inert form (N$_2$), DNRA retains N in the form of biologically active NH$_4^+$ within the aquatic environment (Giblin et al. 2013). The implications of N removal versus retention of NH$_4^+$ within eutrophic habitats are of interest because when N is retained via DNRA, it can provide a positive feedback and further enhance eutrophication. Previous research studies have shown nirS functional genes outnumber nrfA genes in a variety of habitats including an estuary, wetland, and river (Dong et al. 2009, Huang et al. 2011, Morrissey et al. 2013). These results suggest that denitrification is predominant than DNRA in these aquatic habitats.

Oyster Feeding and N Cycling

Like other filter feeders, oysters couple benthic and pelagic processes by delivering biodeposits to sediments as pseudofeces and feces (Haven and Morales-Alamo 1966, Newell and Jordan 1983). During feeding, oysters filter particles from the water column and sort the material on their gills. Those particles deemed unpalatable are rejected as pseudofeces, which are aggregated with mucus and expelled from the oyster shell (Newell and Jordan 1983). Feces are formed when nutritious particles are ingested, digested, and defecated, usually within 24 hours (Langdon and Newell 1996). Biodeposits can sink as much as 40 times faster than unaggregated particles (Newell et al. 2005). Biodeposits contain 2-3 times more C and N than naturally settling particles and can help deliver chemical substrates important to denitrifying bacteria in the sediments (Newell et al. 2005).
Oyster biodeposition may affect sediment N cycling in several ways. One possibility is that accumulation of biodeposits promotes anoxia in the sediments by enhancing microbial oxygen demand at sediment surface. In this scenario, oysters would stimulate uncoupled denitrification, where microbes use C from oyster biodeposits and water column NO$_3^-$ as chemical substrates (Hoellein and Zarnoch 2014). However, oyster biodeposits also contain organic N and NH$_4^+$, which could be mineralized and nitrified. If the NO$_3^-$ from nitrification is used as a chemical substrate for denitrification, oysters would stimulate coupled nitrification-denitrification, or coupled denitrification. This process requires sediment containing adjacent oxic-anoxic microsites. In areas with elevated NO$_3^-$, oysters may have little impact on DNRA because of enhanced denitrification and the hypothesized preference for low NO$_3^-$ levels of DNRA bacteria (Burgin and Hamilton 2007, Kelso et al. 1997, Silver et al. 2001). However, in habitats with relatively low levels of NO$_3^-$, it is possible that oysters could increase DNRA relative to denitrification by delivering concentrated organic C and NO$_3^-$, two substrates necessary for DNRA. The effect of oysters on sediment N cycling has been well studied by measuring the chemical substrates and products of microbial N transformations (Higgins et al. 2011, Kellogg et al. 2013, Hoellein and Zarnoch 2014), however, the effect of oysters on the abundance of microbial genes responsible for denitrification and DNRA (i.e., nirS, nirK, and nrfA) have not previously been measured.

Microbial gene abundance can be measured as the amount of DNA while the amount of gene expression can be measured via RNA in the environment. Studies which use rRNA and mRNA abundance are thought to be most valuable for measuring short-term responses to variable environmental conditions because RNA is less stable than DNA and indicates gene expression (Morales et al. 2014, Peterson et al. 2012, Zhang et
al. 2013). Quantitative DNA measures have been used as indicators of how environmental factors shape microbial communities because DNA is used to measure the size of gene pools related to particular microbial processes (Morales et al. 2014). In this study, we chose to utilize quantitative polymerase chain reaction (qPCR) targeting microbial DNA to study the effect of oyster presence on denitrifying bacteria across a range of environmental conditions.

Our hypotheses were:

(1) Oyster presence will increase nirS, nirK, and nrfA abundance in sediment as oyster biodeposits could enhance conditions needed for sediment denitrifiers and those capable of DNRA.

(2) nirS, nirK, and nrfA abundance will be highest at the site with the highest nutrient concentrations, and the abundance of nrfA to be lower than nirS or nirK, but follow similar distribution patterns due to similar metabolic needs.

(3) nirS, nirK, and nrfA abundance will be greater in fall samples relative to winter, due to decreased availability of water column nutrients and lower temperatures in winter (Braker et al. 1998)

(4) nir gene abundance will correlate with potential denitrification rates and nrfA abundance will correlate negatively with potential denitrification rates because bacteria capable of DNRA are possible competitors of denitrifying bacteria.

(5) nirS will be more abundant than nirK, as previous studies have shown dominance of nirS in aquatic ecosystems. In addition, nrfA will be less abundant than nir genes because recent studies have detected fewer DNRA functional genes than denitrification functional genes in aquatic habitats (Dong et al 2009, Huang et al. 2011, Morrissey et al. 2013).
Methods

Study Site and Sample Collection

This study represents a subset of dates and experimental approaches used for a larger analysis of the effect of oysters on sediment N cycling in Jamaica Bay (Hoellein and Zarnoch 2014). Jamaica Bay is located on the south shore of western Long Island in NYC (Figure 2). Jamaica Bay is connected to the Atlantic Ocean by the Rockaway inlet near the western portion of the Bay. This 5,260 hectare bay is considered a highly urbanized water body by the United States Geological Survey and receives anthropogenic N in the form nitrate (NO\textsubscript{3}\textsuperscript{-}), nitrite (NO\textsubscript{2}\textsuperscript{-}), ammonia, and organic N (Benotti, Abbene, and Terracciano 2007). These inputs originate from wastewater treatment plants, combined sewer and storm water overflows, landfills, and the JFK airport which is located on the eastern shore (Benotti, Abbene, and Terracciano 2007). The four study sites that span a gradient of N load in Jamaica Bay (Figure 1). Floyd Bennett Field (FBF) had the lowest concentrations of nutrients due to its close proximity to the Atlantic Ocean inlet. The most eutrophic site, Spring Creek (SC), is near a wastewater treatment plant and has the highest nutrient concentrations. Mott’s Basin (MB) and Wildlife Refuge (WR) are removed from direct wastewater inputs and are classified as moderately eutrophic in the context of this study.
A full description of the experimental design, sampling procedure, and biogeochemical measurements can be found in Hoellein and Zarnoch (2014). Briefly, at each site, 660 cm$^2$ plastic sediment trays were filled with 11 kg of Pavestone playground sand (Paverstone Company, Atlanta, GA) and exposed to two treatments (oyster and control). For the oyster treatment, sediment trays were fitted with 27.5 x 30 x 10 cm mesh bags (16 mm openings) on top, filled with oysters at a density of 125 oysters m$^{-2}$. The oysters were five-years old and came from Frank M. Flower and Sons (Bayville, NY). Control trays were fitted with empty mesh bags for deployment, each tray was connected to a trotline that anchored parallel to the shore by cinder blocks. This arrangement kept the trays at a depth of 1-1.5 m (mean low water depth), and were accessible by wading.
only at low spring tide each month. The trays were deployed on May 26 or 27, 2010 and sampled every two to three months through November 2011. Control and oyster trays were replicated three times at each site (N= 6 site⁻¹) for this study.

We sampled sediment from control and oyster trays in winter (December 2010 for WR and FBF and January 2011 for SC and MB) and autumn (September 2011 for WR and FBF and October 2011 for SC and MB) (N=48 sediment samples). On each collection date, three replicate samples were taken from each of the two treatments at all four sites. Boxes were carried by hand to the shoreline for processing. We remove the mesh bags from the top of each tray. Then, we collected a composite sediment samples from the top 2-3 cm of sediment from 3 random locations within each tray using a 28 cm² corer and a trowel (total volume = approximately 150 mL of sediment per tray). After sampling, we added the same volume of playground sand evenly across the surface of the sediment tray. This ensured the total volume of sediment in the boxes remained constant. Within 2 h, mesh bags were reattached to sediment trays and trays were re-deployed in the water.

We collected water samples were also for use in measurements of denitrification potential, nitrification, water column nutrient concentrations, and chlorophyll a. Between 3-7 L of unfiltered site water was collected for denitrification potential and nitrification. Three 20 mL samples were collected and filtered (0.7 µM pore size, Whatman GF/F, Piscataway, NJ) for measurement of NH₄⁺, NO₃⁻, and soluble reactive phosphate (SRP). These samples were frozen until measurement. We also collected 2-3 L of site water for chlorophyll a measurements and stored the samples at -20°C. This study represents a subset of 4 sampling dates conducted for the larger study (N=15 collection dates), and results for all sediment C and N dynamics are presented in Hoellein and Zarnoch (2014).
Sediment Biogeochemistry

All sediment samples were thoroughly homogenized within their containers before completing measurements of sediment carbon (C) and N dynamics and microbial community composition. After sample collection, measurements for sediment ash-free dry mass (AFDM) exchangeable ammonium ($\text{NH}_4^+$), nitrification (via nitrapyrin inhibition), $\text{NH}_4^+$ mineralization, denitrification potential (acetylene block method), and C and N limitation of denitrification potential were started within 48 hours (for all methods see Hoellein and Zarnoch 2014). In addition, we collected sediment for bacterial DNA extraction by filling a 1.5mL microcentrifuge tubes approximately half full and storing tubes at -80°C.

Spectrophotometric assays were used to determine nitrite ($\text{NO}_2^-$), $\text{NO}_3^-$, SRP, and $\text{NH}_4^+$ with an autoanalyzer 3 (Seal Analytical, Mequon, WI). To measure $\text{NO}_2^-$ and $\text{NO}_3^-$, we determined $\text{NO}_x^-$ using cadmium reduction (APHA 1998). SRP was determined using antimony tartrate and $\text{NH}_4^+$ was measured using phenol hypochlorite (Murphy and Riley 1962, Solorzano 1969). Chlorophyll $a$ measurements were determined spectrophotometrically after an extraction at 4°C in 90% acetone overnight (Parsons et al., 1994).

DNA Extractions

We extracted DNA from sediment samples using the UltraClean Soil DNA isolation kit (MoBio, Carlsbad, CA). The 1.5mL microcentrifuge tubes containing the samples were weighed prior to extraction and the bead beating solution was added directly to the sample tube. DNA was extracted from 0.5-1 gram of sediment because the kit is optimized for 0.25-1 gram of sample. Protocols provided by the manufacturer were
followed. The sample tubes were weighed again after the DNA isolation to determine the weight of sediment used for DNA isolation.

Genomic DNA from reference organisms was used for nirS, nirK, and 16S rRNA gene standards. These controls were used to construct standard curves for absolute quantitation in quantitative polymerase chain reaction (qPCR). The standard used to generate nirS standard curves was *Pseudomonas stutzeri* (ATCC 17588). For nirK and 16S, the standard used was *Pseudomonas protegens* (ATCC BAA-477). The genomic DNA for both strains was isolated by growing the organisms first in liquid broth (nutrient broth for 17588 and tryptic soy broth for BAA-477). Overnight liquid cultures were then used for genomic DNA extraction using the Wizard Genomic DNA purification kit (Promega, Madison, WI) as directed by the manufacturer.

A cloned nrfA gene standards to construct standard curves for use in absolute quantification of nrfA genes in Jamaica Bay samples. Genomic DNA was extracted from *E.coli* strain MC4100 (Wang and Gunsalus 2000) and PCR was performed to amplify the nrfA gene following the methods used by Takeuchi (2006). The gene was then cloned into the pCR4-TOPO plasmid and transformed into competent *E. coli* cells using the TOPO TA Cloning Kit for Sequencing (Life Technologies, Grand Island, NY) according to the manufacturer’s instruction. The plasmid DNA was isolated using the Wizard Plus SV miniprep DNA purification kit (Promega, Madison, WI) as directed by the manufacturer.

**Preliminary PCR**

Prior to qPCR, traditional PCR was carried out to ensure positive amplification of standards and sample DNA for the nir genes, nrfA, and 16S genes. During this PCR, Amplitaq Gold DNA polymerase (Life Technologies, Grand Island, NY) was used. This
is the same polymerase used in the Power SYBR Green Master Mix (Life Technologies, Grand Island, NY) used for qPCR. Thermocycling conditions were set to mimic those used in the StepOne Plus machine (Life Technologies, Grand Island, NY). Reaction mixtures consisted of 25 µl Amplitaq Gold, 5 µl of 10µM forward primer, 5 µl of 10µM reverse primer, 8 µl water, and 2 µl undiluted DNA, for a total reaction mixture of 45 µl. Primers used are listed in Table A1. The cycling conditions used were one cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. These reactions were carried out in a Techne TC-412 thermocycler (Bibby Scientific, Burlington, NJ). PCR products were visualized and confirmed using gel electrophoresis. Approximately 10µl of PCR product was mixed with 2µl of 5X loading dye and electrophoresed in 1.5% agarose-TrisBorate gels. These gels ran at 100 volts and were stained with ethidium bromide.

Cloning and Sequencing of PCR Products

To confirm that DNA controls selected for qPCR standard curve generation were appropriate, PCR products were cloned for DNA sequencing. Also, the gene copy number per plasmid is equal to one, allowing for use in standard curve generation. The TOPO TA Cloning Kit for sequencing (Life Technologies, Grand Island, NY) was used and manufacturer protocols were followed. For all cloning reactions, PCR products generated within 48 hours of cloning were used. PCR amplicons for nirS, nirK, and nrfA were cloned into the pCR-4TOPO plasmid and transformed into E.coli TOP10 cells. These cells were plated on 50µg/ml kanamycin and ampicillin LB plates. Successful clones, indicated by colony growth, were selected from kanamycin selective LB plates and purified for DNA sequencing analysis using the Wizard Plus SV miniprep DNA purification kit (Promega, Madison, WI) according to the manufacturer protocol.
Plasmid DNA purified with the miniprep kit was sent to the University of Chicago for DNA sequencing. Samples were sequenced on an Applied Biosystems 3730XL 96-capillary automated DNA sequencer using M13 universal primers. Chromatograms were retrieved from the University of Chicago server and opened with Chromas Lite software. The DNA sequences from successful clones were analyzed using NCBI’s BLAST program to assess if the desired nir products were observed. Both Braker et al. (1998) and Throback et al. (2004) nirS and nirK primers were analyzed for use in qPCR along with Tackeuchi (2006) primers for nrfA. Throback primers for nirS and nirK were chosen based on the greater specificity for nitrite reductase genes as DNA sequence analysis indicated Braker primers may yield spurious results and non-nir amplicons (Table A2). After DNA sequence analysis of the amplicons generated by Braker primers, NCBI BLAST indicated the best match was a \textit{P. stutzeri} Tfp pilus assembly protein, not the expected nirS nitrite reductase. This ultimately led to our choice of Throback primers for qPCR analyses.

Quantitative PCR

Optimization of qPCR was performed prior to running the Jamaica Bay bacterial DNA samples to ensure efficient and successful amplification. All reactions were carried out a StepOnePlus Real Time PCR system (Life Technologies, Grand Island, NY). The qPCR reaction mixtures consisted of 20µl Power SYBR Green Master Mix, 4µl forward primer final concentration 0.3µM, 4µl reverse primer final concentration 0.3µM, 8µl water, and 4µl diluted 1:10 DNA for a total reaction mixture of 40 µl. From this mixture, reactions were pipetted in triplicate (10µl per well). MicroAmp optical 96-well reaction plates (0.1mL) and MicroAmp optical 8-cap strips were used (Life technologies, Grand Island, NY). Plates were centrifuged at 1500 rpm for 2 minutes prior to running the
reactions in a Hermle xl4000 centrifuge (Hermle, Mallabia, ES). The final cycling conditions used for the four primer sets are listed in Table A3 and were adapted from Hallin et al. (2005) and Kalvelage et al. (2013). An 80°C step for nirS, nirK, and 16S reactions and a 76°C step for nrfA reactions were added to each cycle flowing primer extension in order to help eliminate any non-specific amplification resulting from the environmental samples or possible primer dimers. A melt curve was performed from 65°C-95°C with a 0.2°C increase at the end of each reaction to check for specificity.

Initially, the Jamaica Bay samples had no qPCR amplification while genomic DNA standards did. We determined that environmental contaminants were likely inhibiting the SYBR Green chemistry and/or DNA polymerase and used the MoBio DNA Clean-Up Kit to remove PCR inhibitors for the extracted sediment bacterial DNA. After using the Clean-Up kit and diluting sample DNA 1:10, qPCR amplification was observed in the Jamaica Bay samples.

qPCR Standard Curves

Standard curves were generated for nirS, nirK, and 16S primer sets using dilutions of genomic DNA from two different species of bacteria with known copy numbers. Both strains were selected because they have fully sequenced genomes in NCBI and possess single copies of the nir gene and a known number of 16S rRNA copies. For nirS standard curves, genomic DNA from Pseudomonas stutzeri (ATCC 17588), which has a single nirS copy (NCBI Accession: NC015740.1), was isolated. The nirK standard curves as well as 16S standard curves were generated from genomic Pseudomonas protegens DNA which also has a fully sequenced genome with a single copy of nirK and five copies of the 16S rRNA gene (NCBI Accession: NC004129). The standard curves for nrfA were constructed using the pCR-4 generated plasmid with a single copy of nrfA per plasmid.
The *nrfA* gene was isolated from *E.coli* MC4100 (Accession: HG738867.1, Wang and Gunsalus 2000). Plasmid DNA was used here because we were unsure of the copy number per genome for *nrfA* in the *E.coli* MC4100 strain due to prior manipulation by Wang and Gunsalus (2000). However, the majority of genomes with *nrfA* analyzed have been found to possess a single copy of the gene (Welsh et al. 2014).

DNA concentration was determined with the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Hanover Park, IL). NanoDrop readings were taken in ng/µl and converted to genome copy number using the formula: number of copies = (ng DNA * 6.022x10^{23} molecules/mol) / (length genomic DNA bp * 1x10^9 ng/g * 650g/mol). This formula is from the University of Rhode Island Genomics & Sequencing Center (http://cels.uri.edu/gsc/cndna.html). DNA standard curves were constructed by calculating number of gene copies detected in 1µl of isolated DNA.

Dilution series of *P. stutzeri* DNA were used to generate *nirS* standard curves that covered a range of approximately 10^2-10^6 copies (Figure A1). A series of *P. protegens* genomic DNA was used to construct the *nirK* standard curves which covered a range of approximately 10^2-10^6 copies (Figure A2). The 16S rRNA gene standard curves were constructed by also using genomic DNA from *P. protegens* and covered a range of approximately 10^4-10^7 copies (Figure A3). The *nrfA* standard curves were generated using the constructed *nrfA* plasmid DNA and covered a range of approximately 10^1-10^5 copies (Figure A4).

The efficiency of standard curves used to determine *nirS, nirK, nrfA*, and 16S copy numbers in sample DNA was calculated using the formula qPCR efficiency = 10^{(-1/slope)} - 1. Efficiencies between 80% and 100% are generally accepted in the literature for studies related to these genes and were deemed acceptable for all qPCR experiments.
carried out (Banerjee & Siciliano 2012, Banner et al. 2011, Brankatschk et al. 2011, Morales et al. 2010, Morrissey et al. 2013). All curves used fell between 82% and 100% (Table A3). These curves were used to calculate copy numbers in Jamaica Bay DNA samples per microliter and were subsequently converted to gene copies per gram sediment (wet weight). Calculations of gene copies/µl template DNA were performed in the StepOne Software V2.2.2. Copy numbers of genes were calculated per gram of sediment by correcting the number of copies detected in each qPCR reaction for the DNA extraction volume from a known amount of sediment for each sample. We normalized gene copies to ng DNA (measured via NanoDrop) 

\[
\text{No. copies/g sediment} = \frac{\text{copies}}{\mu l \text{ template DNA}} \times \frac{\text{ng DNA}}{\mu l \text{ template DNA}} \times \frac{\text{ngDNA extraction}}{g \text{ sediment extraction}}
\]  


In general, 16S abundances were assessed to get a measurement of total bacteria present at the different site. We acknowledge that this is a general estimate of total bacteria because individual cells can contain anywhere from one to fifteen copies per genome (Kembel et al. 2012). The average number of 16S copies per bacterial genome (4.2) was used to calculate approximate percentages of nirS, nirK, and nrfA functional genes relative to overall bacterial abundance in our samples (Table A5, Vetrovsky and Baldrian 2013). We divided 16S copy numbers determined via qPCR by 4.2 prior to percentage functional gene calculations.

Statistical Analysis

We used two-way analysis of variance (ANOVA) analyses to compare gene abundance and biogeochemistry measurements by oyster treatment and site for each season. Significant ANOVA results were followed by Tukey’s multiple comparison test. We used multiple linear regression to assess relationships between gene abundance, water column and sediment characteristics, and N transformation rates. Multiple linear
regressions were completed using a forward stepping method. Finally, principal components analysis (PCA) was used to analyze spatial relationships between samples and overall patterns in our entire matrix of data. ANOVAs and regressions were carried out in R using R Commander, while PCA was completed using the package FactoMineR. Models were tested for normality using the Shapiro-Wilk normality test, examining the normal probability plots, and checking plots of residuals. If needed, data were log transformed to meet the assumptions of normality.

**Results**

**Water Column Nutrients and Sediment Biogeochemistry**

Physiochemical measurements at each study site and date are summarized in Table 1. As expected, the sites differed in a number of water column measurements including NO$_2^-$, NO$_3^-$, and NH$_4^+$ concentrations. The highest levels of NO$_3^-$ were measured at Mott’s Basin in fall and Spring Creek in the winter (Table 1). Samples from Spring Creek in both fall and winter had the highest measurements for NH$_4^+$ (Table 1). Temperature was lower in winter, and ranged from 0.1-24.1°C.

<table>
<thead>
<tr>
<th></th>
<th>Mott’s Basin</th>
<th>Spring Creek</th>
<th>Wildlife Refuge</th>
<th>Floyd Bennett</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Fall</td>
<td>Winter</td>
<td>Fall</td>
<td>Winter</td>
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<tr>
<td>NO$_3^-$</td>
<td>152</td>
<td>135</td>
<td>142</td>
<td>245</td>
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<td>NH$_4^+$</td>
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<td>SRP</td>
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<tr>
<td>DIN</td>
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<td>185</td>
<td>248</td>
<td>584</td>
</tr>
<tr>
<td>Chl$\alpha$</td>
<td>57</td>
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<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Temp</td>
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<td>0.1</td>
<td>22.6</td>
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</table>

Table 1. Mean physiochemical measurements for sampling sites in Jamaica Bay for fall and winter. Abbreviations: NO$_3^-$ = water column nitrate, NO$_2^-$ = water column nitrite, NH$_4^+$ = water column ammonium, SRP = soluble reactive phosphorus, DIN = dissolved inorganic nitrogen, Chl$\alpha$ = Chlorophyll $\alpha$, Temp = temperature. All measures reported in $\mu$g N or P L$^{-1}$ and chl $\alpha$ units are mg L$^{-1}$. 
Study site had the strongest and most consistent effect on sediment biogeochemistry, while there were few significant effects of oysters or interactions between site and oysters (Table 2). In both fall and winter, there was a significant effect of site on sediment organic matter, exchangeable NH$_4^+$, and NH$_4^+$ mineralization, but no effect of oysters. In contrast, there was no effect of site or oysters on sediment C:N or nitrification in either season. Finally, denitrification potential was significantly different among sites in both fall and winter, and in the fall, DNP was higher in the oyster treatments compared to the control treatments (Table 2).

Table 2. ANOVA p-values for sediment characteristics, N transformations, and gene abundance for oyster and control treatments. Values where p≤0.05 are in bold. Abbreviations: %OM = percent organic matter, C:N = carbon to nitrogen ratio, xNH$_4^+$ = exchangeable ammonium, NH$_4^+$ Min = ammonium mineralization, NIT = nitrification, DNP = denitrification potential.

<table>
<thead>
<tr>
<th></th>
<th>Fall</th>
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<tr>
<td></td>
<td>Site Treatment</td>
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<td>Sediment characteristics</td>
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<tr>
<td>%OM</td>
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<tr>
<td>C:N</td>
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<tr>
<td>xNH$_4^+$</td>
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<td>Sediment N transformations</td>
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<tr>
<td>NH$_4^+$ Min</td>
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<tr>
<td>NIT</td>
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<td>0.722</td>
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<tr>
<td>DNP</td>
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<td>0.001</td>
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<tr>
<td>Gene abundance</td>
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<td></td>
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<tr>
<td>nirS</td>
<td>&lt;0.001</td>
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<tr>
<td>nirK</td>
<td>&lt;0.001</td>
<td>0.946</td>
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<td>nrfA</td>
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<td>0.600</td>
</tr>
<tr>
<td>16S</td>
<td>0.008</td>
<td>0.776</td>
</tr>
</tbody>
</table>

Gene Abundance

As expected, abundance of 16S genes were higher than abundance of nirS, nirK, or nrfA. Gene abundance was $7.6 \times 10^6$-$4.7 \times 10^9$ copies g$^{-1}$ sediment for 16S, $4.9 \times 10^4$-$1.4 \times 10^7$ copies g$^{-1}$ sediment for nirS, $2.7 \times 10^3$-$4.1 \times 10^6$ copies g$^{-1}$ sediment for nirK, and $1.1 \times 10^3$-$1.3 \times 10^5$ copies g$^{-1}$ sediment for nrfA, and . On average, nirS gene
abundance was five times greater than those of \textit{nirK}, and 300 times greater than abundance of \textit{nrfA} (Figure 3).

Similar to the results of sediment biogeochemistry, two-way ANOVA showed gene abundance was significantly different among sites in fall and winter and for all genes, but oysters had no effect on abundance of any of the four genes measured (Figure 3; Table 2). In fall, \textit{16S}, \textit{nirS}, and \textit{nirK} abundance had similar patterns, where Mott’s Basin had the highest abundance for all three genes (Figure 3). However, the greatest abundance of \textit{nrfA} genes for fall samples occurred at Floyd Bennett Field (Figure 3). In winter, Wildlife Refuge and Spring Creek had the highest abundance for \textit{nirS}, \textit{nirK}, and 16S, while only Wildlife Refuge had the highest abundance for \textit{nrfA} (Figure 3).

We used the number of copies of each functional gene relative to copies of the 16S rRNA gene to estimate the relative composition of the microbial community. The mean (±SE) \textit{nirS} percentage was 1.5 (±1.1)% in fall and 1.78 (±2.23)% in winter, with the highest at Mott’s Basin in both seasons. Mean (±SE) \textit{nirK} abundance was 0.46 (±0.40)% in fall and 0.34 (±0.44)% in winter, where Mott’s Basin was highest in fall (0.68%) and Spring Creek was highest in winter (0.48%). Finally, \textit{nrfA} mean (±SE) abundance was lower than for \textit{nirS} and \textit{nirK}, with mean (±SE) abundance of 0.01% (±0.01%) in fall and 0.01% (±0.02%) in winter. Wildlife Refuge had the highest percentage of \textit{nrfA} in fall and winter.
Figure 3. Gene copy numbers of Jamaica Bay samples from Spring Creek (SC), Mott’s Basin (MB), Wildlife Refuge (WR), and Floyd Bennett Field (FB). (A) nirS copy number in fall samples. (B) nirS copy number in winter samples. (C) nirK copy number in fall samples. (D) nirK copy number in winter samples. (E) nrfA copy number in fall samples. (F) nrfA copy number in winter samples. (G) 16S copy number in fall samples. (H) 16S copy number in winter samples. Letters that differ indicate significant differences among sites detected by Tukey’s tests for ANOVAs based on site (n=24, error bars represent standard error).
Principal Components Analysis

Principal components analysis (PCA) was used to explore the underlying relationships and patterns between gene abundance, sediment N transformations, and water column nutrient concentrations. The PCA included data for gene abundance, sediment organic matter, temperature, chlorophyll $a$, $NO_3^-$, $NO_2^-$, SRP, nitrification, $NH_4^+$ mineralization, denitrification potential, and C:N. All variables were included in the initial analysis, but those with no significant eigenvalues were excluded from final analysis. Variables were considered significantly related to a principal component if the eigenvalue was $>0.30$ or $<-0.30$ (McGarigal et al. 2000).

The first component of the PCA (PC1) explained 33.9% of the variation in the data while the second component (PC2) explained 17.5% of the variation (total variation in PC1 and PC2 = 51.4%) (Figure 4A). PC1 had a significant positive relationship with nirS, nirK, and 16S gene abundance, and water chemistry measurements including SRP, $NO_2^-$, chlorophyll $a$, and $NH_4^+$ concentrations (Table A4). Organic matter, temperature, and water column $NO_3^-$ concentration were significantly correlated to PC2. Organic matter and water column $NO_3^-$ concentration were positively correlated while temperature showed a negative correlation to PC2. The abundance of nrfA was significantly related to the third principal component, which explained 12.1% of the variation in the data. Other factors positively related to PC3 were nitrification, and $NH_4^+$ mineralization, while denitrification potential was negatively related.
Figure 4. PCA results for Jamaica Bay. (A) Factor map for variables included in the PCA from the Jamaica Bay data set. Circles indicate data from fall, squares indicate data from winter. (B) PCA factor map for individual Jamaica Bay samples from Mott’s Basin (MB), Spring Creek (SC), Wildlife Refuge (WR), and Floyd Bennett Field (FB) from fall and winter (n=48).
Samples clustered in the PCA diagram based on season and site (Figure 4B). Those samples collected in fall scored positively on PC1 and negatively on PC2 (Figure 4B). For samples collected in the fall, Floyd Bennett Field and Spring Creek samples formed clusters in close proximity to one another while Mott’s Basin and Wildlife Refuge clustered close together. Mott’s Basin and Wildlife Refuge scored high on PC1 based on their high overall gene abundance and NO$_2^-$ concentrations. Floyd Bennett Field scored low on PC2 because of the low NO$_3^-$ concentrations while Spring Creek also scored low on component 2 due to lower chlorophyll $a$ concentrations. In winter, samples displayed negative scores for PC1 and positive scores on PC2 and formed two clusters; one consisting of samples from Mott’s Basin and Floyd Bennett Field and the other made up of samples from Spring Creek and Wildlife refuge (Figure 4B). For these two clusters, there was considerable overlap among samples from different sites. Spring Creek and Wildlife Refuge scored high on PC2 due to high NO$_3^-$ concentrations. In contrast, Floyd Bennett Field and Mott’s Basin scored low on PC1 due to low average abundance of all four genes surveyed.

**Multiple Linear Regressions**

Relationships between gene abundance and environmental characteristics were explored using multiple linear regression (MLR). A separate MLR was carried out for each of the four genes. The model generated by MLR accounted for 71% of the variance observed in nirS gene abundance. The significant factors in this model included organic matter, chlorophyll $a$, exchangeable NH$_4^+$, and SRP (Table 3). Organic matter, chlorophyll $a$, and SRP had a positive relationship with nirS abundance, but the relationship with exchangeable NH$_4^+$ was negative. Organic matter explained the most variance (50%).
The MLR model generated for nirK accounted for 79% of the variation in nirK abundance. The significant factors in this model were chlorophyll a, temperature, and exchangeable NH$_4$$^+$ (Table 3). Chlorophyll a and temperature both had positive relationships with abundance of nirK, and exchangeable NH$_4$$^+$ was negatively related. Here, chlorophyll a explained the most variance (61%) in nirK abundance.

For nrfA abundance, MLR generated a model that explained 61% of the variance. The significant factors in this model were C:N, NO$_2^-$, NH$_4$$^+$, chlorophyll a, and NO$_3^-$: The most variance in nrfA abundance was described by C:N (12%) (Table 3). Explanatory variables showing negative relationships with nrfA abundance were chlorophyll a and NH$_4$$^+$. MLR indicated that C:N, NO$_2^-$, and NO$_3^-$ were all positively related to nrfA.
The last MLR model accounted for 52% of the variance in 16S gene abundance. Significant factors in this model were organic matter, \( \text{NH}_4^+ \), \( \text{NO}_3^- \), and \( \text{NH}_4^+ \) mineralization (Table 3). Organic matter accounted for the most variance in this model at 28%. All relationships in this model were positive.

**Discussion**

One of the primary goals of this study was to test the hypothesis that oysters would increase genes for denitrification and DNRA in underlying sediments. No significant effect of oysters on denitrification or DNRA gene abundance was found. These results are consistent with results from the larger project measuring the effect of oysters on sediment C and N dynamics in Jamaica Bay (Hoellein and Zarnoch 2014), which covered a longer time period and more oyster treatments (low and medium density) than the samples in this study. Despite the lack of oyster effects on gene abundance, the significant variation in gene abundance among sites and between seasons allows for exploration of which environmental factors were controlling \( \text{nirS} \), \( \text{nirK} \), \( \text{nrfA} \), and 16S gene abundance in this urban coastal environment.

The reason why oysters did not affect denitrification gene abundance at our study sites could be related to hydrology and the spatial and temporal scales of our experimental approach. Filter feeding bivalves are known to couple benthic and pelagic processes through deposition of their biodeposits to sediment microbes (Newell et al. 2002, Piehler & Smyth 2011, Smyth et al. 2013). This relies upon hydrologic conditions which deliver wastes directly to underlying sediment. It is possible that oysters may be better able to enhance denitrification in areas where water flow is low, and allows direct delivery of organic matter to the sediments for use by denitrifying bacteria. The effect of oyster biodeposits may be dispersed by moving water and occur in small microsites,
which would make detection using our experimental design very difficult. The effects of oysters on sediment microbes may also have been short lived or higher in other seasons (i.e., summer). We may have detected short-lived and microscale effects using RNA metrics, taking samples more frequently, or collecting many discrete small samples from each replicate tray. Hoellein and Zarnoch (2014) found that oysters increased sediment organic matter using year-round sampling covering an 18 month period. We found no previous studies quantifying the effects of oysters on the abundance of denitrifying bacteria. Therefore, these results can be used as a baseline for future analyses on the impacts of oysters on sediment bacteria, and can guide the spatial and temporal intensity of sampling in follow-up experiments.

Although no oyster effect was observed on gene abundance in this study, we found significant and consistent effects of site and season on denitrification potential and abundance of all 4 genes measured. Both nirS and nirK showed strong, positive relationships with sediment organic matter, although the factors were different for each gene. For nirK, the most important factor in MLR was water column chlorophyll a, which can serve as a labile source of organic matter if it sinks to the sediment surface (Eyre and Furgeson 2005, Nixon 1995). In contrast, the most variability in nirS abundance was accounted for by sediment organic matter. Our results are consistent with other studies have found nirS and nirK gene abundance to be strongly related to organic matter in many environments including estuaries, agricultural soils, glaciers, and streams (Abell et al. 2013, Barett et al. 2013, Banergee and Siciliano 2012) (Table 4).
<table>
<thead>
<tr>
<th>Results</th>
<th>Genes Assessed</th>
<th>Habitat</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic matter most important factor in denitrifier abundance</td>
<td><em>narG, nirS, nirK, nosZ</em></td>
<td>Glacier</td>
<td>Kandler et al 2006</td>
</tr>
<tr>
<td><em>nirS</em> and <em>nirK</em> very abundant in sites with high photosynthetic genes and algae</td>
<td><em>nirS, nirK, nosZ</em></td>
<td>Glacier</td>
<td>Brankatschk et al 2011</td>
</tr>
<tr>
<td>TOC and NO3 important to abundance of bacterial denitrifiers. Low C:N inhibits <em>nrfA</em></td>
<td><em>narG, nirS, nirK, nosZ, nrfA</em></td>
<td>River</td>
<td>Huang et al 2011</td>
</tr>
<tr>
<td>Denitrifier abundance related to OM lability. Nitrate addition enhanced effects of OM</td>
<td><em>nirS</em> and <em>nrfA</em></td>
<td>Wetland</td>
<td>Morrissey et al 2013</td>
</tr>
<tr>
<td>Organic carbon important control on denitrification potential</td>
<td><em>nirS, nirK, nosZ</em></td>
<td>Arctic Soil</td>
<td>Banerjee &amp; Siciliano 2012</td>
</tr>
<tr>
<td><em>nirS</em> and <em>nirK</em> positively correlated with OM, total C and total N</td>
<td><em>nifH, nirS, nirK</em></td>
<td>Forest Soil</td>
<td>Levy-Booth and Winder 2010</td>
</tr>
<tr>
<td>Carbon levels correlated with clustering of soil samples</td>
<td><em>nirS, nirK, nosZ, nifH</em></td>
<td>Agricultural soil</td>
<td>Morales et al 2010</td>
</tr>
<tr>
<td>Denitification rates controlled mainly by sediment OM content. Bacterial numbers not related to denitrification rate.</td>
<td><em>nosZ</em> and 16s</td>
<td>Stream</td>
<td>Baxter et al 2012</td>
</tr>
<tr>
<td>OM content related to gene abundance</td>
<td><em>amoA, nirS, 16s</em></td>
<td>Estuary</td>
<td>Abell et al 2013</td>
</tr>
<tr>
<td>Availability of DOC correlated with increasing <em>nirS</em>/K and <em>nosZ</em></td>
<td><em>nirS, nirK, nosZ, 16s</em></td>
<td>Groundwater</td>
<td>Barret et al 2013</td>
</tr>
<tr>
<td>DOM affected abundance of denitrifiers</td>
<td><em>nirS, nirK, 16s</em></td>
<td>Forest Soil</td>
<td>Barta et al 2010</td>
</tr>
<tr>
<td>Organic carbon, nitrogen and several metals influenced denitrification rates and <em>nir</em> abundance</td>
<td><em>nirS, nirK</em></td>
<td>Estuary</td>
<td>Mosier &amp; Francis 2010</td>
</tr>
<tr>
<td>Organic matter and chlorophyll a were important factors for denitrifier abundance while C:N was important to abundance of bacteria capable of DNRA</td>
<td><em>nirS, nirK, nrfA, 16S</em></td>
<td>Estuary</td>
<td>This study</td>
</tr>
</tbody>
</table>
In addition to sediment organic matter, we hypothesized that water column NO$_3^-$ concentrations would be a good predictor for denitrification gene abundance. However, we found no relationship between NO$_3^-$ concentrations and gene abundance $nirS$, $nirK$, or $nrfA$. This has been documented in studies from other habitats as well. For example, Kandler et al. (2006) also found that soil NO$_3^-$ availability did not explain any variation in denitrification gene abundance in MLR, while organic matter was the most important factor. In addition, $nirS$ and $nirK$ abundance in forest soils were highly correlated with organic matter but not NO$_3^-$ levels (Levy-Booth and Winder 2010).

As expected, our results showed abundance of $nrfA$ was much lower than denitrification genes. Other aquatic studies in estuary and river habitats have found $nrfA$ abundance to be lower than denitrification functional genes, although the differences are not always statistically significant (Dong et al. 2009, Huang et al. 2011, Smith et al. 2007). Therefore, we assume lower potential for DNRA than denitrification in Jamaica Bay. This would indicate that less NO$_3^-$ is recycled within the ecosystem as NH$_4^+$ than is removed in the form of N gasses. From a eutrophication management perspective, this is a positive finding. Return of NH$_4^+$ to the ecosystem within Jamaica Bay would enhance the effects of eutrophication by stimulating phytoplankton and other primary producers, possibly exacerbating algal blooms and anoxic conditions. Other studies have found denitrification rates to be higher than those for DNRA in aquatic environments. For example, Smyth (et al. 2013) found DNRA rates to be consistently lower than denitrification rates in the Bougue Sound, an estuary. In another study, denitrification rates were found to be higher than DNRA in a fertilized marsh platform (Koop-Jakobsen and Giblin 2010). Denitrification was also found to be dominant over DNRA in Florida Bay during the month of January (Gardener and McCarthy 2009).
The major controlling factors of \(nrfA\) abundance were sediment C:N (positive) and water column chlorophyll \(a\) (negative; Table 3), which may be attributed to competitive relationships between microbes which carry out denitrification and those which complete DNRA. Tiedje (1988) hypothesized that availability of labile C would favor denitrifying organisms because they use electron acceptors more efficiently than microbes which carry out DNRA. Denitrification transfers five electrons per mole \(\text{NO}_3^-\) reduced while DNRA transfers 8 (Tiedje 1988). Therefore, if C:N is low and most of the C is from labile sources, numbers of DNRA bacteria may decrease. In addition, Morrissey et al. (2010) found \(nrfA\) abundance to decrease in the presence of labile sources of organic matter in comparison to recalcitrant sources of organic matter, and concluded that DNRA bacteria may be outcompeted for labile organic matter (i.e., chlorophyll \(a\)), by denitrifying bacteria. In addition, Huang et al. (2011) found that low C:N inhibits DNRA and leads to decreases in \(nrfA\). Our results are similar, as the negative relationship of chlorophyll \(a\) with \(nrfA\) is opposite to the one observed for denitrifying bacteria in Jamaica Bay. However, more research is needed to correlate DNRA gene abundance with DNRA rates, and to measure competitive forces between the bacteria which carry out these metabolic pathways in eutrophic habitats.

As expected, 16S gene abundance was much higher than \(nirS\), \(nirK\), and \(nrfA\) gene abundance, and multiple linear regression revealed that sediment organic matter accounted for the most variance in 16S abundance. The results suggest that heterotrophic microbes dominate the bacterial community at these sites. Other studies which quantify gene abundance and related environmental factors have found that elevated C lead to increased 16S gene abundance both in terrestrial and aquatic environments (Peterson et al. 2012, Wu et al. 2012). In addition to organic matter, DIN (\(\text{NH}_4^+\) and \(\text{NO}_3^-\)) and 16S
gene abundance showed significant positive relationships. In contrast, there was no effect of DIN concentrations on functional gene abundance, which suggests that DIN may have been used primarily for assimilation and growth of the broad microbial community. This finding is supported by other studies showing bacterial growth is increased by the addition of N (Alden et al. 2001, Church et al. 2000, Zweifel et al. 1993).

Overall, the numbers of nirS and nirK gene copies g sediment$^{-1}$ in our samples were comparable to those found in other studies investigating denitrification functional genes in estuarine habitats. For nirK, 9.7 x 10$^3$ to 4.4 x 10$^6$ copies g$^{-1}$ sediment were found in the San Francisco Bay Estuary (Mosier and Francis 2010). Previous measurements for nirS include: 5.4 x 10$^5$ to 5.4 x 10$^7$ copies g$^{-1}$ sediment in the San Francisco Bay Estuary, 2 x 10$^5$ to 7 x 10$^7$ copies g$^{-1}$ sediment in Chesapeake Bay, and 10$^4$ to 10$^7$ copies g$^{-1}$ sediment in the Colne Estuary (Mosier and Francis 2011, Bulow et al. 2008, Smith et al. 2007).

PCA allowed for a multivariate exploration of the data to reveal underlying patterns and relationships among multiple variables from our data set and confirmed our seasonal hypothesis. There was a clear separation of samples taken in fall and winter. Clustering of samples based on season of collection was impacted by water column nutrients changing between seasons and shifts in the availability of organic matter. The data showed that there was a seasonal shift in these samples that resulted from changing environmental conditions. These patterns could be used to design future studies and support the use of multiple sampling seasons in microbial ecology projects.

Contrary to our hypothesis, we found no significant relationship between nir gene abundance and denitrification potentials. This lack of relationship between potential rate measurements and nir abundance has been observed in a number of other studies (Dandie
et al. 2008, Baudoin et al. 2009, Attard et al. 2011). It is possible that the relationship between gene abundance and DNP would be stronger if conditions during DNP measurements more closely matched in situ conditions as can be obtained with other measurements such as $^{15}$N isotope tracers. For example, both nirS and nirK gene abundance were correlated with NO$_3^-$ uptake rates measured using $^{15}$N isotope assays in an urban stream (Knapp et al. 2009). Another study employed N$_2$O flux to measure denitrification and found a positive correlation to nirS gene abundance (Morales et al. 2010).

As expected in our final hypothesis, the abundance of nirS outnumbered nirK in all samples. This pattern may be caused by a variety of factors, but previous work has focused on how oxygen affects the nirS to nirK ratio. nirK prevails over nirS in environments that are conditionally exposed to oxygen, like agricultural soils, while nirS is more abundant in environments which are continuously anoxic (Desnues et al. 2007, Knapp et al. 2009). This is because the cytochrome cd$_1$ nitrite reductase encoded by nirS can catalyze two reactions, the single electron reduction of NO$_2^-$ to NO and the four-electron reduction of O$_2$ to H$_2$O (Einsle and Kronneck 2004). Unlike the cytochrome cd$_1$ nitrite reductase, the copper containing nitrite reductase encoded by nirK carries out only the reduction of NO$_2^-$ to NO (Einsle and Kronneck 2004). These enzymatic differences contribute to the ability of nirK-containing organisms to denitrify in the presence of elevated O$_2$ levels compared to nirS-containing organisms. The higher O$_2$ thresholds for bacteria containing nirK suggest they are better adapted to compete with aerobic heterotrophs in environments like agricultural soils (Prieme et al. 2002). In contrast, nirS seems to be better suited for aquatic habitats and is often much more abundant in many marine environments (Desnues et al. 2007, Huang et al. 2011, Mosier and Francis 2009,
Zhang et al. 2013). Although *nirS* outnumbered *nirK*, they showed similar relative abundance among study sites. This suggests that despite the overarching effects of $O_2$ threshold on their relative abundance, the two forms of nitrite reductase involved in denitrification have some similar environmental drivers.

**Conclusion**

Contrary to our predictions, oysters did not affect denitrification or DNRA gene abundance of in underlying sediments of this urban coastal environment. It is possible that the effect of oysters was missed due to the overriding influence of hydrology, the spatial and temporal limitations of our experimental approach, and high water column nutrients across all sites. However, results from this study were consistent with much recent research that shows organic matter, rather than NO$_3^-$ concentrations, are a critical controlling factor for denitrification gene abundance. Although we did not find a significant relationship between DNP rate measurements and denitrification functional gene abundance, we suggest that methods measuring *in situ* denitrification rates may be more beneficial for developing predictive relationships between gene abundance and rate measurements. Data on microbial communities is frequently excluded from models that address ecosystem effects of eutrophication, including the relative balance of genes for denitrification and DNRA. We showed that even at multiple sites within a eutrophic embayment, quantitative DNA measurements can help reveal underlying relationships and environmental factors related to critical sediment N transformations.
CHAPTER THREE
EFFECT OF WASTEWATER TREATMENT SHUTDOWN ON nirS, nirK, AND nrfA GENE ABUNDANCE IN THE WESTERN LONG ISLAND SOUTH SHORE ESTUARY (NEW YORK)

Introduction

Coastal Weather Events

Approximately 1.2 billion people, or 23% of the world’s population, live within 100 km of a coastal environment, and 50% of the population is projected to live near coastal environments by 2030 (Bender et al. 2010). Coastal populations are exposed to hazardous weather events including hurricanes and flooding (Adger et al. 2005). Statistical models have linked increased hurricane activity to the rise in sea surface temperatures (Mann and Emanuel 2006, Elsner et al. 2008, Emanuel 2005) influenced by the anthropogenic emission of greenhouse gasses (Solomon et al. 2007, Santer et al. 2006, Gillet et al. 2008). Climate change is likely to become an increasingly important factor to environmental and public health of coastal ecosystems (Lotze et al. 2006). For example, intense hurricanes (i.e., categories 4 and 5) related to global climate change are predicted to increase during the 21st century, with the largest increase in the western Atlantic Ocean (Bender et al. 2011).

Eutrophication and Nitrogen Cycling

In autumn 2012, Hurricane Sandy caused extensive damage in a number of coastal areas on the east coast of North America, generating approximately $50 billion in
damage in the United States alone (Blake et al. 2013). Heavily impacted states in the U.S were New York and New Jersey, including the Western Long Island South Shore (WLISS) estuary. The WL ISS estuary was hit by Hurricane Sandy on October 29, 2012. Damage to the Bay Park Sewage Treatment Plant, which treats waste from 5 million people, released 68 million gallons of raw sewage over the course of two days (Branca 2013). After this, the plant was in partial operation for 7 weeks until fully repaired (Branca 2013). The environmental impacts of this untreated sewage in the WL ISS estuary are unknown.

Enrichment of aquatic environments with excess nutrients (i.e., eutrophication) is typical in coastal environments with high human population density (Howarth et al. 1996, Nixon et al. 1996). Nitrogen (N) is often the primary limiting nutrient in marine ecosystems, which has affected estuary and coastal ecosystems globally (Nixon 1995). Under eutrophic conditions, algal blooms and hypoxia frequently occur (Herbert 1999). These blooms are responsible for large anoxic or “dead zones” observed in the Gulf of Mexico, Chesapeake Bay, and elsewhere (Alexander et al. 2000). Understanding how excess nutrients are processed by biota in eutrophic estuaries is important managing N loads and predicting fate of nutrients in coastal ecosystems.

A primary focus of research in eutrophic ecosystems is denitrification, or the oxidation of organic matter where nitrate (NO\textsubscript{3}^{-}) serves as a terminal electron acceptor and is reduced to the gaseous nitrogen (N) products dinitrogen gas (N\textsubscript{2}) and nitrous oxide (N\textsubscript{2}O) (Zumft 1997). Denitrification allows the NO\textsubscript{3}^{-} that contributes to eutrophication in enriched environments to be removed from the aquatic ecosystem. This heterotrophic process involves an anaerobic transfer of electrons mediated by microbial enzymes present in taxonomically diverse groups of bacteria (Seitzinger 2008). The four
microbial enzymes that mediate the heterotrophic process of denitrification are nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos). The enzymes are encoded by four separate genes; nar, nir, nor, and nos (Zumft 1997) and are produced by a wide variety of microorganisms. However, not all denitrifiers contain the entire suite of genes necessary for complete denitrification (Payne 1981). The presence of the nitrite reductase gene, responsible for the reduction of NO$_2^-$ to NO, is often viewed as an important indicator for the presence of denitrifying bacteria (Braker 1998). These genes differentiate denitrifying bacteria from nitrate respiring bacteria that do not possess nir genes or other genes required for denitrification.

Another microbially-mediated process may compete with denitrification for NO$_3^-$, but receives much less study in eutrophic ecosystems: dissimilatory reduction of NO$_3^-$ to ammonium (NH$_4^+$) (DNRA). DNRA conserves N within the aquatic environment in the form of biologically active NH$_4^+$. The NH$_4^+$ created by DNRA can then be used by plants, microbes, and algae (Giblin et al. 2013). The nitrite reductase enzyme used in DNRA is encoded by the nrfA gene. This gene has been found in a wide variety of bacteria (Smith et al. 2007). Previous research studies have shown nirS functional genes to outnumber nrfA genes in a variety of habitats including an estuary, wetland, and river (Dong et al. 2009, Huang et al. 2011, Morissey et al. 2013). Which environmental factors control the balance between denitrification and DNRA are not well understood.

Nutrient Loading and Estuary Bacterial Communities

Recent research has documented the response of microbial communities following environmental disturbances including the introduction of toxic chemicals, change in temperature, and addition of nutrients. Much research has focused on microbial community response to nutrient loading because of increased nutrient levels in many
natural habitats worldwide. In a meta-analysis of 38 studies examining the effects of N or phosphorous (P) fertilization, 84% showed the microbial community composition (i.e., richness, relative abundance, or phylogenetic structure of community composition), changed in some way (Allison and Martiny 2008). Overall, it is thought that microbial communities may be initially sensitive to nutrient loading (i.e., low resistance), but have the ability to quickly re-establish normal functions within the ecosystem (i.e., high resilience; Allison and Martiny 2008).

Individual studies of genes responsible for denitrification (nirS and nirK) have shown gene abundance changes following nutrient addition. For example, the addition of C and N to river biofilms increased the relative abundances of nirS and nirK (Chenier et al. 2006). The abundance of nirS was elevated in wastewater compared to clean groundwater (Zhou et al 2011), and denitrifier abundance increased in soils irrigated with wastewater (Guo et al. 2013). In addition, potential denitrification was positively related to denitrifier abundance under different wastewater irrigation practices (Guo et al. 2013). In contrast, Bowen et al (2011) found that N loading in two salt marsh plots did not affect the nirS community composition or abundance, and Bunnemann (et al. 2004) found that denitrifiers were not affected by fertilization with phosphorus (P) alone.

**Denitrification Varies by Habitat Type in Estuaries**

Estuaries are dynamic aquatic environments in which denitrification and DNRA can take place at high rates (Dong et al. 2009, Hopkinson and Giblin 2008). Seasonal changes in water stratification, organic matter quantity and quality, and variable hydrological cycles impact N cycling in estuaries (Santoro 2010). In addition, denitrification is highly variable among habitat types within estuaries such as salt marsh, seagrass bed, oyster reef, subtidal flat, and intertidal flat habitats (Piehler and Smyth...
Salt marshes are sites of interest for N cycling research because marshes act as buffers between terrestrial and aquatic environments where anthropogenic N can be intercepted and removed by plant growth and microbial pathways (Koop-Jakobsen and Giblin 2010). Denitrification rates in marshes have been found to be higher than in other types of marine sediments (Hopkinson and Giblin 2008, Piehler and Smyth 2011).

Factors which influence high NO$_3^-$ removal or retention in salt marsh habitats include organic matter availability, NO$_3^-$ loading, oxygen levels, macrofauna, and submerged plants (Corwell et al. 1999). Rhizospheres from aquatic plant growth in marsh sediments can enhance denitrification because roots and rhizomes exude labile organic carbon that can be used in denitrification (Koop-Jakobsen and Giblin 2010). DNRA may decrease in marsh rhizospheres due to elevated oxygen while coupled nitrification-denitrification may increase (Matheson et al. 2002). A synthesis of multiple Chesapeake Bay studies concluded that a decrease in marsh area and aquatic vegetation led to decreased denitrification (Cornwell et al. 1999). Intertidal and subtidal flats likely have lower rates of denitrification than adjacent salt marshes because they lack the plant life associated with salt marshes, receive fewer direct inputs of organic matter (i.e., root exudates and leaf litter), and water movement may prevent the settling of organic matter.

While rates of N cycling among estuary habitats have received increased research attention (Hopkinson and Giblin 2008, Piehler and Smyth 2011, Smyth et al. 2013), few studies have examined the abundance of denitrification genes among different estuary habitats. Understanding where genes are most abundant is critical for predicting the effects of eutrophication on N cycling in urban estuaries.

The aim of this study was to determine how the sewage released near the Bay Park Sewage Treatment plant post-Hurricane Sandy impacted the bacterial communities
responsible for denitrification and DNRA in the WL ISS estuary. From July 2013 to February 2014, we measured abundance of genes for denitrification and DNRA in the WL ISS estuary at two sites: one near the impacted Bay Park effluent outfall (North Meadow) and one un-impacted site (Cuba Island). At each site, samples were collected from 3 habitat types in each site: subtidal, intertidal, and salt marsh. We also measured physiochemical conditions and rates of denitrification and DRNA for each sample. Our hypotheses were:

(1) More denitrifying bacteria (determined by enumerating nirS and nirK gene copies) and DNRA-capable bacteria (determined by enumerating nrfA gene copies) will be detected in the impacted site (North) than the reference site (Cuba).

(2) The abundance of nir genes will be positively correlated to NO$_3^-$ uptake and denitrification rates. Gene abundance will also be positively related to organic matter content and water column NO$_3^-$, which increases denitrification (Abell et al. 2013, Kandler et al. 2006, Mosier and Francis 2010). Abundance of DNRA bacteria will be negatively related to NO$_3^-$ levels because DNRA bacteria may outcompete denitrifiers when NO$_3^-$ concentrations are low (Morrissey et al. 2013).

(3) Rates of denitrification and denitrifier abundance will be highest for samples collected from marsh habitats because plants can stimulate denitrification (Koop-Jakobsen and Giblin 2010). Abundance of DNRA bacteria will be greatest in the subtidal environments where conditions that promote DNRA such as permanent anoxia and high sulfides are present (McGlathery et al. 2007). Elevated dissolved oxygen (DO) in marsh sediments from plant roots may also stimulate coupled nitrification-denitrification and suppress DNRA (Matheson et al. 2002).
Abundances of nirS, nirK, and nrfA will be greatest in summer and fall due to higher water column nutrients, temperature, plant growth and organic matter abundance.

**Methods**

**Study Site and Sample Collection**

Sediment samples were collected during three sampling dates from two sites. The site most impacted by the wastewater treatment plant shutdown was North Meadow which is adjacent to the Bay Park effluent outfall, and the reference site, Cuba Island, is east of Jones Inlet (Figure 5). At each site, we sampled subtidal, intertidal, and marsh habitats, collected on July 10, 2013, November 2, 2013, and February 19, 2014. We collected three sediment cores from each habitat type at the two sampling sites, resulting in 18 sediment cores per sampling date (total N=54). Cores were collected using a corer with a one-way valve designed to minimize disturbance of the sediment-water interface (Gardner et al. 2006). The cores were sealed with rubber caps, placed in a dark cooler with ice, and transported to the laboratory under dark conditions. We also collected 60L of unfiltered site water to be used in continuous-flow measurements and analysis of water chemistry.
Continuous-flow measurements were set up within 2-3 hours to measure nutrient and gas fluxes in each sediment core (Gardner and McCarthy 2009). All cores were incubated in the dark to avoid photosynthesis and approximately 230 mL of water was kept above the sediment water interface (SWI) in each core. Site water was passed over the intact cores for 24 hours, and then water was collected from each in-flow carboy and from each of the core outflows and filtered (0.2 µm nylon syringe filters, Thermo Scientific, Rockwood, TN, USA) into three, 20 mL scintillation vials and frozen for measurement of NH$_4^+$, NO$_3^-$, and soluble reactive phosphorus (SRP). We also collected water from the inflows and outflows into triplicate vacutainers for measuring dissolved gasses. After sampling under ‘control’ conditions, $^{15}$NO$_3^-$ tracer was added to inflow water (+10 µM $^{15}$NO$_3^-$-N) and then passed over the three replicate cores (from two sites and three habitat types each) for a second 24 hour period. Inflow and outflow samples were taken as described above.

Figure 5. Map of the reference (Cuba Island; East Bay) and impacted (North Meadow; West Bay) study sites in the Western Long Island South Shore (WLISS) estuary.

$^{15}$N Isotope Tracing
An Autoanalyzer III (Seal Analytical, Inc., Mequon, WI) was used to measure dissolved inorganic nutrients. We used the phenol hypochlorite technique for NH$_4^+$ (Solorzano 1969), the antimonyl tartrate method for SRP (Murphy and Riley 1962), and the cadmium reduction method for NO$_3^-$ (APHA 1998). Dissolved $^{28}$N$_2$, $^{29}$N$_2$, $^{30}$N$_2$, O$_2$, and Ar were measured with membrane inlet mass spectrometry (MIMS; Kana et al. 1994). Nutrient and gas fluxes were equal to the concentration in the outflow minus concentration in the inflow, and corrected for surface area of the core and pump flow rate. Net retention is indicated by a negative value and a positive value represents net production out of the sediment. A value of zero indicates a balance in the sediment core where no net retention or production of a particular gas is occurring. Control cores show net N$_2$ flux and sediment oxygen demand (i.e., respiration). N-fixation was calculated following An et al. (2001) in the $^{+15}$NO$_3^-$ cores. We calculated denitrification (DNF) from the control cores as the sum of net N$_2$ flux and N-fixation, while denitrification potential (DNP) was the sum of $^{28}$N$_2$, $^{29}$N$_2$, $^{30}$N$_2$ in the treatment with $^{15}$NO$_3^-$ added (McCarthy et al. 2007).

After flow-through measurements were completed, we removed the plungers and tubing to collect sediment for organic matter measurements and bacterial community analysis. We removed 15 ml of sediment from the top 3 cm of sediment from 3 locations within each core. The sample was homogenized, and organic matter and C:N ratios were measured from sediment. To measure ash-free dry mass (AFDM), sediment samples were placed into pre-weighed and ashed tins, sediment was dried at 60°C for a minimum of 4 days, samples were re-weighed for dry mass, and then the samples were combusted at 550°C for 3 hours for AFDM. The C:N ratio was determined by using a CHN analyzer after acidification and drying of the samples. Finally, we collected approximately 1 ml of
homogenized sediment to measure gene abundance using a sterilized stainless steel spatula. Sediment was placed into a sterile 2 ml microcentrifuge tube and frozen at -80°C until analysis. Chlorophyll a measurements were determined spectrophotometrically after an extraction at 4°C in 90% acetone overnight (Parsons et al. 1994).

DNA Extraction

Bacterial DNA was extracted from sediment samples using the PowerSoil DNA isolation kit (MoBio, Carlsbad, CA) according to the manufacturer’s protocol. We weighed the 2 mL microcentrifuge tubes containing the samples prior to extraction. The bead beating solution was then added directly to the sample tube. We extracted DNA from 0.3-0.7 g of sediment because the kit is optimized for 0.25-1 g of sediment sample. The sample tubes were weighed again after the DNA isolation to determine the weight of sediment used for DNA isolation. We used genomic DNA from reference organisms for nirS, nirK, and 16S rRNA to construct standard curves for absolute quantitation in quantitative polymerase chain reaction (qPCR). The reference organism used to generate nirS standard curves was Pseudomonas stutzeri (ATCC 17588). For nirK and 16S, the reference organism used was Pseudomonas protegens (ATCC BAA-477). Genomic DNA for both bacterial species was isolated by growing the organisms first in liquid broth (nutrient broth for 17588 and tryptic soy broth for BAA-477). We used overnight liquid cultures for genomic DNA extraction with the Wizard Genomic DNA purification kit (Promega, Madison, WI) as directed by the manufacturer. These bacterial species were selected because P. stutzeri has a single copy of nirS and P. protegens has a single copy of nirK.

A cloned nrfA gene was used to construct standard curves for use in absolute quantification of nrfA genes in WLISS samples. We extracted genomic DNA from E.coli
strain MC4100 (Wang and Gunsalus 2000) and PCR was performed to amplify the \textit{nrfA} gene according to methods by Takeuchi (2006). We cloned the gene into the pCR4-TOPO plasmid and transformed the plasmids into competent \textit{E.coli} cells using the TOPO TA Cloning Kit for Sequencing (Life Technologies, Grand Island, NY) according to the manufacturer’s instruction. The plasmid DNA was isolated using the Wizard Plus SV miniprep DNA purification kit (Promega, Madison, WI) as directed by the manufacturer. We used DNA sequencing to confirm the presence of a single copy of \textit{nrfA} in the plasmids.

**Quantitative PCR**

Optimization of qPCR was performed prior to running the WL ISS bacterial DNA samples to ensure efficient and successful amplification. All reactions were carried out in a StepOnePlus Real Time PCR system (Life Technologies, Grand Island, NY). The qPCR reaction mixtures consisted of 20µl Power SYBR Green Master Mix, 4µl forward primer (final concentration 0.3µM), 4µl reverse primer (final concentration 0.3µM), 8µl water, and 4µl diluted 1:10 DNA for a total reaction mixture of 40 µl. From this mixture, we pipetted reactions in triplicate (10µl per well). MicroAmp optical 96-well reaction plates (0.1mL) and MicroAmp optical 8-cap strips were used (Life technologies, Grand Island, NY). We centrifuged the plates at 1500 rpm for 2 minutes in a Hermle xl4000 centrifuge (Hermle, Mallabia, ES) prior to running the reactions. The final cycling conditions used for the four primer sets are listed in Table A3 and were adapted from Hallin et al. (2009) and Kalvelage et al. (2013). An 80°C step for \textit{nirS}, \textit{nirK}, and 16S reactions and a 76°C step for \textit{nrfA} reactions were added after primer extension to each cycle in order to help eliminate any non-specific amplification resulting from the environmental samples or possible primer dimers. A melt curve was performed from 65°C-
95°C with a 0.2°C increase at the end of each qPCR reaction to check for specificity. We also used agarose gels periodically to check that the expected amplicons were present.

qPCR Standard Curves

We generated standard curves for nirS, nirK, and 16S primer sets using dilutions of genomic DNA from two different species of bacteria with known copy numbers. Both strains were selected because they have fully sequenced genomes in NCBI and possess single copies of the nir gene and a known number of 16S rRNA copies. For nirS standard curves, genomic DNA from *Pseudomonas stutzeri* (ATCC 17588), which has a single nirS copy (NCBI Accession: NC015740.1), was isolated. The nirK standard curves as well as 16S standard curves were generated from genomic *Pseudomonas protegens* DNA which also has a fully sequenced genome with a single copy of nirK and five 16S rRNA copies (NCBI Accession: NC004129). We constructed the standard curves for nrfA using the pCR-4 generated plasmid with a single copy of nrfA per plasmid. The nrfA gene was isolated from *E.coli* MC4100 (Accession: HG738867.1, Wang and Gunsalus 2000). Plasmid DNA was used here because we were unsure of the copy number per genome for nrfA in the *E.coli* MC4100 strain due to prior manipulation by Wang and Gunsalus (2000). However, the majority of genomes with nrfA analyzed have been found to possess a single copy of the gene (Welsh et al. 2014).

DNA concentration was determined with the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Hanover Park, IL). NanoDrop readings were taken in ng/µl and converted to genome copy number using the formula: number of copies = (ng DNA * 6.022x1023 molecules/mol) / (length genomic DNA bp * 1x109 ng/g * 650g/mol). This formula is from the University of Rhode Island Genomics & Sequencing Center and allowed us to determine genomic copy number in our isolated
reference DNA (http://cels.uri.edu/gsc/cndna.html). Standard curves were constructed by calculating number of gene copies detected in 1µl of DNA.

Dilution series of *P. stutzeri* DNA were used to generate nirS standard curves that covered a range of $10^2$-$10^6$ copies (Figure A1). The *P. protegens* genomic DNA was used to construct the nirK standard curves which covered a range of approximately $10^2$-$10^6$ copies (Figure A2). The 16S rRNA gene standard curves were constructed by also using genomic DNA from *P. protegens* and covered a range of approximately $10^3$-$10^7$ copies (Figure A3). The nrfA standard curves were generated using the constructed nrfA plasmid DNA and covered a range of approximately $10^1$-$10^5$ copies (Figure A4).

The efficiency of standard curves used to determine nirS, nirK, nrfA, and 16S copy numbers were calculated using the formula $\text{PCR efficiency} = 10^{(1/\text{slope})} - 1$. Standard curves falling between 83% and 100% were used to calculate copy number in WLISS DNA samples. These copy numbers were normalized to DNA extraction yield and were subsequently converted to gene copies per gram sediment (wet weight) (Mosier 2011, Peng et al. 2013).

In general, 16S abundances were analyzed to assess total bacteria present at the different sites. We acknowledge that this is a very general estimate of total bacteria because bacteria can have 1-15 copies per genome (Kembel et al. 2012). The total number of 16S copies per environmental sample was used to calculate approximate percentages of nirS, nirK, and nrfA functional genes relative to overall bacterial abundance in our samples (Table A5).

Statistical Analysis

We used three-way analysis of variance (ANOVA) analyses to compare gene abundance and biogeochemical measurements by site, season, and habitat. We used
multiple linear regressions to assess relationships between gene abundance, water column and sediment characteristic measurements, and N transformation rates. Multiple linear regressions were completed using a forward stepping method. All biogeochemical and gene abundance data were included in the initial models to explain variation in net N\textsubscript{2} flux, DNF, and DNP. For the gene abundance models, net N\textsubscript{2} flux, DNF, and DNP were not included as we would not expect rate measures to control functional gene abundance. Finally, principal components analysis (PCA) was used to analyze relationships between samples and overall patterns in our entire matrix of data. ANOVAs and regressions were carried out in R using R Commander, while PCA was completed using the package FactoMineR. Models were tested for normality using the Shapiro-Wilk normality test, examining the normal probability plots, and checking plots of residuals. If needed, data were log transformed to meet the assumptions of normality.

**Results**

**Water Chemistry and Chlorophyll a**

Most measurements for water chemistry showed higher values at North Meadow relative to Cuba Island (Table 5). The only exception to this pattern was NO\textsubscript{3} concentration in July, when North Meadow was 1 \mu M NO\textsubscript{3} L\textsuperscript{-1} and Cuba island was 2 \mu M NO\textsubscript{3} - N L\textsuperscript{-1}. For both Cuba and North sampling sites, NH\textsubscript{4}\textsuperscript{+} concentrations were highest in summer, while the highest values for chlorophyll-\(a\) were in summer (Table 5). Both sites also had the highest measures for water column SRP and NO\textsubscript{3} concentrations in the fall. At Cuba, NO\textsubscript{2} was below detection in all three sampling seasons, but North showed variation in NO\textsubscript{2} concentrations across seasons, with the highest measurements in the fall.
Table 5. Average physiochemical measures for WLISS estuary sampling sites. Chla= Chlorophyll a, SRP= soluble reactive phosphorus, NO$_3^-$ = water column nitrate, NO$_2^-$ = water column nitrite, NH$_4^+$ = water column ammonium. All measures reported in µM (mmol N or P L$^{-1}$) and chl a units are mg L$^{-1}$.

<table>
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<tr>
<th>Environmental Characteristic</th>
<th>Cuba</th>
<th>North</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>July</td>
<td>Nov</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>SRP</td>
<td>1</td>
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<td>3</td>
</tr>
<tr>
<td>Chla</td>
<td>15</td>
<td>6</td>
</tr>
</tbody>
</table>

Gene Abundance

Abundance of nirS, nirK, nrfA, and 16s genes were determined via qPCR. Abundance ranged from $7.6 \times 10^4$ - $7.3 \times 10^7$ copies gram$^{-1}$ sediment nirS, $5.3 \times 10^4$ - $4.9 \times 10^6$ copies gram$^{-1}$ sediment nirK, $2.1 \times 10^2$ - $4.6 \times 10^5$ copies gram$^{-1}$ sediment nrfA, and $2.8 \times 10^7$ - $6.7 \times 10^9$ copies gram$^{-1}$ sediment 16s. These values were comparable to those found in Jamaica Bay, a nearby estuary (Lindemann et al., unpublished data) and to values reported for San Francisco Bay Estuary, Chesapeake Bay, and the Colne Estuary (Mosier and Francis 2010, Bulow et al. 2008, Smith et al. 2007).

In each season, the pattern for gene abundance among habitat types was variable. In July at Cuba Island, the abundance of nirS and nirK in the marsh was significantly greater than subtidal sediment (Figure 6, 7). In July at North Meadow, nirS abundance during July was greater in intertidal zone than the marsh or subtidal habitats. November samples from Cuba Island showed a similar pattern for nirS and nirK, where the marsh had significantly more gene copies than the subtidal and intertidal zones (Figure 6, 7). At North Meadow in November, intertidal and marsh samples had significantly more copies of nirS than subtidal samples (Figure 6). February samples from Cuba Island and North Meadow showed no significant differences in nirS or nirK abundance across habitats.
Figure 6. qPCR results of absolute abundance and relative abundance (%) of nirS (as function of 16S abundance) in bacterial DNA from WLlSS at the reference (Cuba) and impacted (North) sites. (A) nirS copies for Cuba. (B) nirS copies for North. (C) nirS relative abundance for Cuba. (D) nirS relative abundance for North. One-way ANOVAs with Tukey’s HSD carried out for abundances in each month. Different letters indicate significant differences among habitat types in each sampling period. Letters a-c= July, h-j= November, and x-z= February.
Figure 7. qPCR results of absolute abundance and relative abundance (%) of \textit{nirK} (as function of 16S abundance) in bacterial DNA from WLISS at the reference (Cuba) and impacted (North) sites. (A) \textit{nirK} copies for Cuba. (B) \textit{nirK} copies for North. (C) \textit{nirK} relative abundance for Cuba. (D) \textit{nirK} relative abundance for North. One-way ANOVAs with Tukey’s HSD carried out for abundances in each month. Different letters indicate significant differences among habitat types in each sampling period. Letters a-c= July, h-j= November, and x-z= February.
Figure 8. qPCR results of absolute abundance and relative abundance, (%) of *nrfA* (as function of 16S abundance) in bacterial DNA from WLISS at the reference (Cuba) and impacted (North) sites. (A) Gene copies *nrfA* for Cuba. (B) Gene copies *nrfA* for North. (C) *nrfA* relative abundance for Cuba. (D) *nrfA* relative abundance for North. One-way ANOVAs with Tukey’s HSD carried out for abundances in each month. Different letters indicate significant differences among habitat types in each sampling period. Letters a-c= July, h-j= November, and x-z= February.
In contrast to nirS and nirK, nrfA and 16S gene abundance was less variable among habitat types. For example, there were no significant differences in nrfA abundance among habitat types for Cuba Island in any season, and no differences among habitats at North Meadow in July or February (Figure 8). In November at North Meadow, the abundance of nrfA was significantly higher in subtidal and intertidal than marsh sites. The abundance of 16S genes was not significantly different across habitat types in any season with one exception. In July at North Meadow, marsh sediments had fewer copies of 16S than subtidal or intertidal sediments.

Relative Gene Abundance

Relative gene abundance was calculated by dividing functional gene abundance by 16S abundance, and multiplying by 100. In July, the relative nirS and nirK abundance was significantly greater in the marsh than in subtidal sediments at Cuba Island and North Meadow (Figure 6). In November, relative abundance of nirS was significantly greater in marsh than subtidal and intertidal habitats for Cuba Island, while no significant
differences for *nirS* percentage were found across habitats in February. The relative abundance of *nirS* at North was not significantly different across habitats in November, but marsh and intertidal samples had significantly more *nirS* copies than subtidal samples in February (Figure 6).

Relative abundance of *nirK* was greatest in marsh samples for both November and February collection dates in Cuba Island, while no significant habitat differences for *nirK* abundance were found in November or February at North Meadow (Figure 7). No significant differences among habitat were found in July, November, or February for *nrfA* relative abundance at Cuba (Figure 8). At North, the only significant difference in relative abundance of *nrfA* was in July, where marsh sediments had significantly more *nrfA* copies than subtidal or intertidal sediments (Figure 8).

N Cycling Fluxes and Bacterial Abundance Affected by Season and Habitat

There was no difference in any of the gene abundance measurements between the study sites, and most measurements of N fluxes were also identical between sites (Table 6). The exceptions were that net N₂ flux, sediment C:N, *nirK* abundance, and 16S abundance were higher at the impacted site. In contrast to site, season affected most fluxes and gene abundance measurements including net N₂ flux, DNP, SOD, NH₄⁺ flux, and abundance of *nirS*, *nirK*, *nrfA*, and 16S genes (Table 6). Habitat also had a significant effect on many measurements including net N₂ flux, DNP, SOD, SRP flux, NH₄⁺ flux, sediment organic matter, C:N, and abundance of *nirS*, *nrfA*, and 16S genes (Table 6). The interaction effect of habitat and season was significant for SOD, SRP flux, NH₄⁺ flux, and abundance of *nirS*, *nirK*, and *nrfA*, which indicates that the patterns among habitats were different across seasons. SRP flux, NH₄⁺ flux, %OM, C:N, and *nirS*, *nirK*, and 16S abundance all showed a significant interaction effect for habitat and site. This indicates
the patterns among habitats were different at the two sites. Season and site also showed significant interaction effects for net N$_2$ flux, DNP, and sediment C:N. No significant three way interactions were detected (Table 6).

Table 6. Three-way ANOVA p-values for biogeochemistry and microbial gene abundance measurements at North Meadow and Cuba Island sites from marsh, intertidal, and subtidal habitats during summer, fall, and winter sampling seasons. DNP= denitrification potential, DNF= denitrification, %OM= percent organic matter, SOD= sediment oxygen demand, Chla= Chlorophyll a. Bolded values represent those values considered statistically significant at the level of p<0.05.

<table>
<thead>
<tr>
<th>Gas Fluxes</th>
<th>Habitat</th>
<th>Season</th>
<th>Site</th>
<th>Habitat x Season</th>
<th>Habitat x Site</th>
<th>Season x Site</th>
<th>Habitat x Season x Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net N$_2$</td>
<td>-0.001</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>0.920</td>
<td>0.750</td>
<td>-0.001</td>
<td>0.065</td>
</tr>
<tr>
<td>DNF</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.315</td>
<td>0.362</td>
<td>0.522</td>
<td>0.038</td>
<td>0.050</td>
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<tr>
<td>DNP</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.034</td>
<td>0.052</td>
<td>0.584</td>
<td>0.021</td>
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<tr>
<td>SOD</td>
<td>0.050</td>
<td>&lt;0.001</td>
<td>0.894</td>
<td>0.030</td>
<td>0.268</td>
<td>0.439</td>
<td>0.056</td>
</tr>
</tbody>
</table>

| Solute Fluxes | | | | | | | |
|---------------| | | | | | | |
| SRP flux      | 0.006  | 0.813 | 0.086| 0.044            | 0.030          | 0.891         | 0.061                  |
| NO$_3$ flux   | 0.123  | 0.327 | 0.866| 0.392            | 0.925          | 0.324         | 0.785                  |
| NH$_4^+$ flux | <0.001 | 0.065 | 0.153| <0.001           | <0.001        | 0.356         | 0.499                  |

| Sediment characteristics | | | | | | | |
|--------------------------| | | | | | | |
| %OM                      | <0.001 | 0.225 | 0.859| 0.422            | 0.041          | 0.937         | 0.329                  |
| C:N                      | <0.001 | 0.445 | 0.001| 0.415            | <0.001        | <0.001        | 0.067                  |

| Gene abundance | | | | | | | |
|----------------| | | | | | | |
| nirS           | 0.029  | 0.028 | 0.399| 0.023            | 0.001          | 0.224         | 0.089                  |
| nirK           | 0.068  | 0.001 | 0.004| 0.001            | <0.001        | 0.062         | 0.094                  |
| nrfA           | 0.001  | <0.001 | 0.555| 0.015            | 0.262          | 0.376         | 0.368                  |
| %nirS          | <0.001 | 0.905 | 0.840| 0.029            | 0.272          | 0.069         | 0.191                  |
| %nirK          | <0.001 | 0.003 | 0.251| 0.001            | 0.181          | 0.070         | 0.579                  |
| %nrfA          | 0.065  | <0.001 | 0.294| 0.018            | 0.083          | 0.027         | 0.160                  |
| 16S            | 0.023  | 0.006 | 0.009| 0.175            | 0.002          | 0.231         | 0.098                  |

Factors Controlling N Cycling Fluxes and Sediment Bacteria

We employed multiple linear regression (MLR) to explore environmental factors controlling net N$_2$ flux, DNF, DNP, and abundance of nirS, nirK, nrfA, and 16S (Table 7). The model generated for net N$_2$ flux accounted for 67% of the variation in the data, where (Table 7) significant factors included NO$_3$ flux, SOD, nirS abundance, and NH$_4^+$ flux. All factors, excluding nirS abundance, had a negative relationship with net N$_2$ flux. This is because rates when nutrient fluxes and SOD are negative values, results indicate net uptake (i.e., more NO$_3$ uptake into sediment with more N$_2$ flux out of sediment is a
negative relationship). For DNF, 53% of the variation was explained by MLR. Significant variables showing a positive relationship with DNF included NO$_2^-$ and NO$_3^-$ concentrations, chlorophyll $a$, SRP flux, and abundance of nirS and nirK. In this model, NH$_4^+$ flux was negatively related to DNF, while other metrics had a positive relationship (Table 7). Finally, 52% of the variation in DNP was accounted for by combined abundance of nirS/nirK, SOD, SRP, and %OM (Table 7). In this model, SOD had a negative relationship with DNP, while combined nirS/nirK abundance, SRP, and %OM were positively related to DNP.

We also used MLR to determine significant environmental factors that influence nirS, nirK, nrfA, and 16S abundance. For nirS abundance, 56% of the variation could be explained by NO$_x$ flux and %OM (Table 7). Sediment OM was positively related to nirS abundance, while NO$_x$ flux showed a negative relationship with nirS abundance. MLR showed 58% of the variation in nirK abundance was accounted for by NO$_x$ flux, chlorophyll $a$, SRP flux, and NH$_4^+$ concentration (Table 7). Here, nirK abundance was negatively related to NO$_x$ flux and NH$_4^+$ concentration, and positively related to chlorophyll $a$ and SRP flux. The MLR model for nrfA accounted for 60% of the variation in gene abundance (Table 7). Significant factors were temperature and concentrations of SRP, NO$_2^-$, NO$_3^-$, and NH$_4^+$. Temperature, NO$_2^-$, and NH$_4^+$ concentrations were positively related to nrfA abundance while all the other significant factors showed negative relationships. The MLR model generated for 16S abundance explained the least amount of variance for any of the 4 genes examined (29%; Table 7). Significant factors included NH$_4^+$ flux, N-fixation, sediment OM, and chlorophyll $a$. Abundance of 16S showed a negative relationship with N-fixation, but positive relationships with sediment OM, NH$_4^+$ flux, and chlorophyll $a$. 
Table 7. Forward-stepping multiple regression results for denitrification and gene abundance data. Direction of relationship between independent and dependent variable shown in final step of each model. Final $R^2$ for each regression highlighted in bold. All models checked for normality with Shapiro-Wilk test. NO$_x$ flux= flux of NO$_3^-$ and NO$_2^-$ combined, NH$_4^+$ = water column ammonium, SRP flux= soluble reactive phosphorus flux, DNF= denitrification, Chla= Chlorophyll $a$, %OM= percent organic matter, C:N= carbon to nitrogen ratio, SOD= sediment oxygen demand, N fix= nitrogen fixation.

<table>
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<th>Dependent Variable</th>
<th>Independent Variable(s)</th>
<th>$R^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>Net N$_2$ Flux</td>
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<td>NO$_x$ Flux, Chla, SRP flux</td>
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<td>&lt;0.001</td>
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<td></td>
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<td></td>
<td>nrfA</td>
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<td>&lt;0.001</td>
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<tr>
<td></td>
<td>Temp, SRP</td>
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<td>&lt;0.001</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td></td>
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<td>NH$_4^+$ flux</td>
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<td>0.041</td>
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<td></td>
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<td>0.008</td>
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<td></td>
<td>NH$_4^+$ flux(+), N fix(-), %OM(+), Chla (+)</td>
<td>0.29</td>
<td>0.003</td>
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</table>
Principal Components Analysis

Underlying patterns related to similarities among sample data based on gene abundance, nutrient fluxes, and water column chemistry were explored using Principal Components Analysis (PCA). The analysis included data on functional gene abundance, \( \text{N}_2 \) flux, \( \text{NH}_4^+ \) flux, \( \text{NO}_x^- \) flux, chlorophyll \( a \), sediment C:N, sediment OM, DNP, DNF, N-fixation, and SOD (Figure 10A). The first principal component (PC1) of the analysis explained 30.85% of the variation in the data and the second principal component (PC2) explained 17.74% of the variation, resulting in a total explanation of variation in the data set of 48.59% (Figure 10A).
**Figure 10.** PCA results for WLISS. (A) Factor map for variables included in the PCA from the WLISS set. (B) PCA factor map for individual WLISS samples from the reference (Cuba) and impacted (North) sites taken during July, November, and February (n=53). Ellipses drawn to show month of sampling.
PC1 had strong, positive correlations with nirS and nirK abundance, DNP, DNF, and net N₂ flux (Figure 10A). PC2 showed positive correlations with temperature, chlorophyll a, and nrfA abundance, while SOD and NO₃⁻ concentration showed strong, negative relationships with PC2 (Figure 10A). Overall, PC 1 represented a gradient of gene abundance and flux measurements related to denitrification. PC2 represented a mixed gradient of water column chemistry, nrfA abundance, and SOD.

The samples cluster loosely by month of sample collection and habitat type on the PCA (Figure 10B). Samples from Cuba Island and North Meadow collected in July form a large cluster positioned high on PC1 and spanning into the larger values on PC2. Here, there is overlap between the Cuba and North samples, with those samples collected from marsh habitats separating out and scoring high on PC1 and PC2. This aligns with the high denitrification gene abundance, DNP, DNF, and chlorophyll a observed in these samples.

Samples collected in November formed two clusters; one consisting of samples from Cuba Island and one composed of North Meadow samples. This reflects the slightly lower DNP and DNF measurements observed in November Cuba Island samples. The second November cluster, consisting of North Meadow samples, scored negatively on PC2 and relatively high on PC1. For this cluster, samples generally had higher denitrification gene abundance, DNP, DNF, and NO₃⁻ concentrations than the samples in the Cuba Island cluster. The fourth cluster, composed of Cuba Island and North Meadow samples from February, scored negatively on both PC1 and PC2. This reflects the lower abundance of denitrifying bacteria, decreased levels of DNP, DNF, and lower temperatures at both sites. November was the only sampling date when sediment samples from the two sites were distinct on the PCA (Figure 10B).
Discussion

Variation in Gene Abundance by Site

A primary hypothesis of this study was that denitrifier gene abundance and denitrification rates would be higher at North Meadow relative to Cuba Island, attributed to the wastewater treatment plant shutdown near North Meadow after Hurricane Sandy. In contrast to our expectations, there was no site effect on measurements related to denitrification, including nirS abundance, DNP, and DNF. There was a site effect for nirK abundance, but nirK was less abundant than nirS and the interaction of habitat and site was also significant (i.e., the pattern among habitats was different at each site). The lack of a site effect may be attributed to a number of factors, including bacterial community resistance and resilience, the duration of exposure to untreated sewage, and functional redundancy of denitrification genes.

The resistance and resilience of bacterial communities following disturbance has received increased research attention due to technological advancements in measuring microbial diversity and interest to include microbes in the study of global change biology (e.g., climate change and eutrophication). The resistance of a bacterial community refers to its ability to withstand disturbance, while the ability of the bacterial community to recover is defined as resilience (Wertz et al. 2007). Results from several recent studies suggest high resistance and/or resilience of microbial communities which carry out denitrification. For example, Wertz et al. (2007), found that a heat disturbance (i.e., 42°C lasting 24 hours) reduced denitrification activity 3 hours after the disturbance, but there was no effect after one month (Wertz et al. 2007). Furthermore, the disturbance had no effect on the abundance of denitrifying bacteria. Instead, the lowered denitrification activity may have been caused by physiological effects including inactivation of
denitrification enzymes or cell damage. In a soil microcosm study, the addition of silver nitrate (AgNO$_3$) decreased nirK denitrifier abundance, but increased denitrifier diversity (Throback et al. 2007). Individual denitrifiers were not resistant to silver contamination, but increased diversity suggests that the community may be resilient to this disturbance. In our study, it is possible there was an immediate, but short-lived, response to the wastewater treatment plant shutdown (i.e., in November 2012) that was not detected by our sampling design, the start of which was constrained by grant review and administration processes. This timing would explain the lack of differences detected between North Meadow and Cuba Island samples in regards to denitrification gene abundance and activity.

Previous studies have found denitrifier communities show both resistance and resilience following a variety of nutrient enrichment events. For example, the structure of the denitrifying bacterial community was not affected by increased nutrient levels in salt marsh sediments, indicating that denitrifying communities were resistant to nutrient enrichment (Bowen et al. 2011). Denitrification gene abundance in agricultural soils (measured by nosZ) was not affected by enrichment with organic C and N fertilizers, conventional fertilizers, or low-input nutrients (Kong et al. 2011). Zhou (et al. 2011) found that in agricultural soils treated with clean water, reclaimed water, or wastewater, the only detectable differences in nirS and nirK abundance were between the clean water and wastewater treatments. In a 50 year study of the effects of fertilization on soil, Hallin (et al. 2009) found that nirK abundance was higher in plots fertilized with sewage sludge than control plots, while nirS abundance was lower in the sewage sludge treated plots than the controls. These data show that the two types of denitrification genes may facilitate resistance of denitrification to changing conditions and disturbance (Hallin et al.
Overall, the high resistance and resilience of denitrification genes could explain the lack of site on gene abundance and denitrification rates.

Functional redundancy of the nirS and nirK genes in denitrifiers is likely to be important to the resilience and resistance of the denitrifying bacterial communities because it reduces possible negative effects of environmental disturbance on biodiversity (Griffiths and Philippot 2012). For example, denitrifying bacteria were more resistant than nitrite oxidizers to heat disturbance, despite a reduction in biodiversity in both the nitrite oxidizing and denitrifying groups. Wertz et al. 2007, attributed this resistance to the functional redundancy of nirS and nirK. The bacterial community of denitrifiers in the WLISS estuary at the North Meadow site may also be resistant to nutrient enrichment due to continuous exposure to wastewater effluent, or periodic exposure to untreated wastewater from combined sewer overflows. This type of previous exposure could condition the denitrifying community to be resistant to nutrient enrichment, and thereby become adapted to withstand disturbances such as experienced after Hurricane Sandy (Griffiths and Philippot 2012).

Environmental Factors Controlling Denitrification Rate and Gene Abundance in WLISS

Despite the lack of a clear effect of site on response variables related to denitrification, there was significant variation by season and habitat for all measurements, which present a unique opportunity to document which environmental factors control denitrification and 16S gene abundance throughout the WLISS. As expected, multiple linear regression showed that DNF and DNP were both positively related to nirS and nirK gene abundance. Our results are consistent with recent studies which have found similar relationships between nirS and/or nirK abundance and some measure of denitrification
rate. In a wetland, both nirS and nirK abundance were positively related to denitrification measured via \( ^{15}\text{N} \) isotope tracing (Yi et al. 2013). A positive relationship between nirS abundance and denitrification rate was found in United Kingdom estuary (Dong et al. 2009). The abundance of nirS and nirK were also related to denitrification potential and \( \text{N}_2 \) flux in an agricultural pasture (Chronakova et al. 2009).

Many studies have explored the relationship between denitrification rates and the abundance of denitrification genes, but it appears the method used to measure denitrification rate may affect the strength of the relationship. Two of the most common methods for measuring denitrification in aquatic ecosystems are the acetylene block method and direct \( \text{N}_2 \) measurements using MIMS (with or without \( ^{15}\text{N} \) isotopes). We found positive relationships between functional gene abundance and denitrification rate measures from MIMS in the WLISS estuary, however, gene abundance was unrelated to DNP measured with the acetylene block method in nearby Jamaica Bay (S. Lindemann, unpublished data). The direct \( \text{N}_2 \) measurements are preferred in marine ecosystems where coupled nitrification-denitrification may be significant (Cornwell et al. 1999), while acetylene block is valuable for measuring denitrification potential (i.e., the capacity of the existing microbial community to carry out denitrification), and measurements of N or C limitation of DNP (Hoellein and Zanorch 2014). Similar patterns conducted in a wide variety of environments can be found in the literature. For example, Yi et al. (2013) found that losses of \( ^{15}\text{N} \) due to denitrification were positively related to both nirS and nirK abundance in a wetland while Song et al. (2010) found no relationship between denitrification potential measured via acetylene block and nirS abundance in a wetland. Chronakova et al. (2009) found nirS and nirK abundance to be positively correlated to \( \text{N}_2 \) flux in an agricultural pasture, but other studies carried out in agricultural fields using
acetylene block find no relationship or very weak correlations between \textit{nir} abundances and DNP (Dandie et al. 2008, Attard et al. 2011, Enwall et al. 2010).

In addition to the abundance of denitrification genes abundance, denitrification measurements were related to other factors we expected to be important, including NO$_x$- concentrations, NH$_4^+$ and NO$_3^-$ flux, SOD, and chlorophyll \textit{a}. Other studies have found positive relationships between denitrification and NO$_x$- concentrations, as both NO$_3^-$ and NO$_2^-$ are important chemical substrates for microbial denitrification (Payne 1981, Zumft 1997, Mulholland et al. 2008). In addition, denitrification was positively related to net uptake of NH$_4^+$ and NO$_3^-$, which suggests direct denitrification and coupled nitrification-denitrification contributed to N$_2$ production. The relationship between chlorophyll \textit{a} and SOD with denitrification suggests both metrics may be proxies for direct drivers of denitrification: carbon availability and anoxic conditions in sediment. Denitrification rate was not related to sediment organic matter (although \textit{nirS} and 16s gene abundance each were; Table 7), but the relationship between denitrification and chlorophyll \textit{a} may indicate that water column phytoplankton which settle on the sediment surface may represent a high quality C source for denitrification. In a similar fashion, negative flux values for SOD indicated that increased O$_2$ consumption promoted conditions for increased denitrification, likely by creating anoxic microsites.

Regression analysis also revealed factors linked to the abundance of \textit{nirS} and \textit{nirK} functional genes, including NO$_x$- flux, sediment organic matter, chlorophyll \textit{a}, and two unexpected factors, SRP flux (positive relationship) and water column NH$_4^+$ concentrations (negative relationship). Organic matter and NO$_x$- uptake (and to a lesser extent, chlorophyll \textit{a}) reflect chemical substrates needed for denitrification. These results are consistent with recent studies (Knapp et al. 2009), including a nearby coastal site,
Jamaica Bay (S. Lindeman, unpublished data). The positive relationship between nirK abundance and SRP flux is similar to the results of a study in spruce forest soil which found a positive relationship between P availability and nirK abundance, but no relationship between P and nirS (Barta et al. 2010). These results suggest that nirK denitrifiers may be more sensitive to P availability (Barta et al. 2010). The negative relationship between nirK abundance and water column NH$_4^+$ concentration is unclear, but may also be related to nutrient sensitivity or competition with N metabolic pathways which produce NH$_4^+$ (i.e., mineralization and DNRA).

*nrfA* gene abundance was related to a different set of environmental factors than the denitrification genes. The positive relationships between *nrfA* gene abundance and water column NO$_2^-$ and NH$_4^+$ concentrations are logical because NO$_2^-$ is an intermediate product of DNRA and NH$_4^+$ is the end product. The abundance of *nrfA* was also negatively related to water column SRP and NO$_3^-$ concentrations. We expected *nrfA* abundance to be negatively related to NO$_3^-$ concentrations because bacteria capable of DNRA are thought to be out-competed by denitrifiers in high NO$_3^-$ conditions (Morrissey et al. 2013, Rutting et al. 2011). The abundance of *nrfA* was also positively related to temperature, which accounted for the most variation in *nrfA*. This suggests that bacteria that carry out DNRA in WLISS are susceptible to changes in temperature, or controlled by other environmental factors which we did not measure directly that change in concert with temperature.

Total bacterial abundance was estimated by quantifying the abundance of the 16S rRNA gene and positively related to organic matter and chlorophyll *a*. We expected to find the positive relationship between bacterial abundance and sediment organic matter because a large portion of the sediment bacterial community was likely to be
heterotrophic. However, we did not predict that 16S abundance would be positively related to NH$_4^+$ flux and negatively related to N fixation. The positive relationship with NH$_4^+$ flux out of the sediment (i.e. net NH$_4^+$ mineralization) also suggests a role for heterotrophic decomposition of organic matter. The underlying reason for the relationship between 16S abundance and N-fixation is unclear and merits further study.

Variation in Gene Abundance by Habitat

We expected denitrifier abundance would be highest in marsh habitats at each site, because salt marsh plants can stimulate denitrification (Koop-Jakobsen and Giblin 2010), and marsh sediments have been shown to have greater denitrification rates than intertidal or subtidal sediments (Piehler and Smyth 2011). The patterns of nirS and nirK relative abundance show each was highest in marsh sediments from Cuba Island in each month (Figure 6C, 7C), and in marsh sediments at North Meadow at each month (except nirK in February Figure 7D). Although these patterns were not always significant, the effect is correlated with the summer and fall periods when salt marsh plants are more active. This effect was visualized in the PCA where marsh samples were separated from subtidal and intertidal samples in July and November, but all habitats were grouped together in February. Salt marsh plants can increase denitrification by root exudates from the rhizomes which serve as a labile C source. In addition, O$_2$ transport in salt marsh roots creates anoxic/oxic microsites which can stimulate coupled nitrification-denitrification (Koop-Jakobsen and Giblin 2010).

Although subtidal sediments are continuously exposed to water, they are not introduced to the same organic matter and N sources as marsh or intertidal sediments, and may therefore have lower denitrification gene abundance than intertidal or marsh habitats. This is reflected in our data which show in the majority of subtidal samples had
lower nirS and nirK gene copies than intertidal and marsh sediments. These data are consistent with Piehler and Smyth (2011), which showed rates of denitrification in subtidal flats were lower than those in intertidal zones and salt marshes in the Bouge Sound estuary, North Carolina. Structured habitats like salt marshes are likely to have more nutrient processing including denitrification due to the creation of organic material via photosynthesis and exposure to terrestrial N sources (Piehler and Smyth 2011). The close proximity of intertidal zones to structured marsh habitats increases the amount of organic matter and terrestrial N received from marshes in comparison to subtidal habitats (Piehler and Smyth 2011).

Contrary to our expectations, nrfA abundance was relatively uniform across seasons and habitat types (except marsh at North Meadow in July; Figure 8D) and related to temperature rather than NO₃⁻ concentrations and sediment organic matter. In other coastal ecosystems, nrfA has been found to vary seasonally, with the highest abundance detected in summer (Giblin et al. 2013). The relationship between nrfA and temperature may be attributed to highly reduced sediment conditions during summer months in which sediment oxygen demand is high (Ferron et al. 2009, Gardener and McCarthy 2009). The strong relationship between nrfA abundance and temperature may also be related to seasonal variation in N uptake by plants. Extensive plant growth during summer months leads to more N uptake. These conditions may suppress the growth of denitrifying bacteria and be more favorable to sulfur oxidizing, DNRA bacteria that can store NO₃⁻ in vacuoles (Sayama 2001). Finally, abundance of DNRA bacteria has been shown to be unaffected by enrichments of NO₃⁻ and organic matter (Morissey et al. 2013), and therefore DNRA may not be affected by high water column nutrients delivered by either WWTP failures (i.e., after Hurricane Sandy) or from treated WWTP effluent.
Variation in Denitrification Gene Abundance by Season

Our final hypothesis was that functional gene abundance, DNF, and DNP would be greatest in the July followed by November and February due to increased nutrient and organic matter availability during summer. However, the general trend was that nirS and nirK gene abundance were higher in July and November than in February, and was similar to results for DNP and DNF. PCA confirmed that season affected the clustering of the WLISS data. Interestingly, PCA also showed that the only sampling period in which there was distinct separation between the two sites was during November. The largest difference in water column NO$_3^-$ was detected between North Meadow and Cuba Island during November. This difference may have been caused by some undocumented nutrient loading event associated with the wastewater treatment plant and caused a more detectable difference between the two sites. Biological and chemical conditions that vary by season have also shown to affect denitrifying bacteria abundance elsewhere (Jung et al. 2013, Levy-Booth and Windor 2010).

Conclusion

This study explored the impacts of a wastewater treatment plant shutdown caused by Hurricane Sandy, a major storm event, by combining physiochemical measurements, biogeochemistry, and with microbial gene abundance. Overall, the abundance of bacterial genes which carry out denitrification and DNRA were largely identical at the site impacted by the release of raw sewage relative to the un-impacted site. However, variation between sites, habitats, and seasons allowed us to better understand the underlying factors controlling in microbial gene abundance and N cycling rates and better predict the response of denitrification to future disturbances. Understanding how extreme weather events impact surrounding environments is of interest to managers, researchers,
and citizens in coastal environments. Such studies will become of greater importance as extreme weather events become more common due to effects of global climate change.
CHAPTER FOUR
CONCLUDING COMPARATIVE ANALYSIS OF JAMAICA BAY AND THE
WESTERN LONG ISLAND SOUTH SHORE ESTUARY

We employed qPCR to analyze bacterial communities capable of denitrification and dissimilatory reduction of nitrate (NO$_3^-$) to ammonium (NH$_4^+$; DNRA) in two urban coastal environments: Jamaica Bay and the Western Long Island South Shore Estuary (WLISS). Both coastal environments are heavily impacted by anthropogenic activities from urban land-use in and around New York City, NY (NYC) (Hoellein and Zarnoch 2014). The objectives of this conclusion chapter were to combine results from the 6 sites across 2 ecosystems to (1) compare gene abundance among locations, (2) quantify relationships between gene abundance and environmental controlling factors including sediment organic matter, chlorophyll a, and NO$_3^-$ concentrations across the 6 sites, (3) determine if gene abundance is related to denitrification rates among sites, and (4) compare our results to literature values which have examined the relationship between gene abundance and denitrification across ecosystems and using various methods to measure denitrification.

To understand how the overall abundance of bacterial genes for denitrification and DNRA compared between Jamaica Bay and WLISS, we calculated the mean functional gene abundance, averaged across fall and winter at each site (Figure 11). Gene abundance data from the summer at WLISS was not included because summer data were not available for Jamaica Bay. In addition, we omitted data from marsh habitats at
WLISS because sites at Jamaica Bay were on the border between subtidal and intertidal. Overall, sites from Jamaica Bay and WL ISS had similar nirS gene abundance, although North Meadow in WL ISS had significantly higher nirS abundance than Floyd Bennett Field and Spring Creek in Jamaica Bay (Figure 11). For nirK abundance, Mott’s Basin from Jamaica Bay had significantly more gene copies than the two WL ISS sites (Figure 11). Both North Meadow at WL ISS and Wildlife Refuge at Jamaica Bay had significantly higher nrfA abundance than Mott’s Basin and Spring Creek (Figure 11).

Denitrification gene abundance is driven by availability of organic C, redox conditions, and NO3⁻ availability. In our studies, we did not measure redox, but we directly measured of sediment organic matter, water column NO3⁻, sediment carbon to nitrogen ratio (C:N), and water column chlorophyll a (which may indirectly indicate sediment C availability and water quality (Lensen 2006, Boyer et al. 2009)) Sediment organic matter was highest in the WL ISS at North Meadow, while chlorophyll a was highest at Mott’s Basin in Jamaica Bay (Figure 12A, B). Water column NO3⁻ was
significantly higher in the Jamaica Bay sites compared to WLISS (Figure 12C). Other chapters previously used multiple linear regressions for Jamaica Bay and WLISS data. Chapters 2 and 3 of this thesis show that nirS abundance was positively related to sediment organic matter when sites in each estuary were considered individually. Combined data from both Jamaica Bay and WLISS also showed nirS abundance was positively related to organic matter (Figure 13A). These data support previous research that identified organic matter as an important factor related to nirS abundance elsewhere (Barett et al. 2013, Kandler et al. 2006, Levy-Booth and Winder 2010). The findings add to previous studies by identifying organic matter abundance as an influential factor for the abundance of nirS denitrifying bacteria in urban, coastal environments.

![Figure 12](image.png)

Figure 12. Mean organic matter, chlorophyll $a$, and NO$_3^-$ for Jamaica Bay and WLISS. (A) Mean (±SE) percent organic matter for fall and winter in JBAY and WLISS. (B) Mean (±SE) chlorophyll $a$ for fall and winter sampling dates. (C) Mean (±SE) NO$_3^-$ for fall and winter sampling dates. Bars that share a letter are not significantly different.
In contrast to nirS, nirK abundance was not related to organic matter in both Jamaica Bay and WLISS. However, both data from both sites showed a positive relationship between nirK abundance and water column chlorophyll a, which were each highest at Mott’s Basin (Figure 12, Figure 13). Our combined regression analyses of Jamaica Bay and WLISS data also indicated that chlorophyll a was positively related to nirK abundance (Figure 13B). These comparisons and the results of our statistical analyses indicate that chlorophyll a is a significant factor that influences the abundance of nirK denitrifiers in these urban, coastal habitats. Papaspyrou et al. (2014) also found chlorophyll a to be positively related to nirS in the hyper-nutrified Colne Estuary.

We predicted nitrite reductase gene abundance would be positively related to water column NO$_3^-$ concentrations, however, when each site was considered independently, we did not find strong relationships between nirS/K or nrfA gene abundance and water column NO$_3^-$ concentrations. When data for Jamaica Bay and WLISS were combined, nirK and water column NO$_3^-$ concentrations showed a significant, although relatively weak ($R^2= 0.05$) correlation (Figure 13C). This pattern has been found in other environments including arctic and forested habitats and may be related to some type of habitat-specific threshold for denitrification (Kandler et al. 2006, Levy-Booth and Winder 2010). In addition, denitrifiers rely on a combination of NO$_3^-$ sources including water column, porewater NO$_3^-$ and NO$_3^-$ produced by nitrification (although our previous research suggests the latter was minimal; Hoellein and Zarnoch 2014, Hoellein unpublished data).
Abundance of nrfA at both sites was lower than nirS and nirK abundance, which suggests the potential for DNRA is lower than denitrification (Dong et al. 2009). Overall, the average proportion of nrfA abundance relative to nirS/K abundance (i.e., nrfA/(nirS + nirK)) among all sites was approximately 1%. These results are of interest due to their implications in eutrophication in these urban, coastal environments. Lower potential for DNRA than denitrification suggests a higher likelihood of NO\textsubscript{3} removal rather than return of NH\textsubscript{4}\textsuperscript{+} back to the ecosystem. This is beneficial because these eutrophic
environments may promote N loss over N recycling, at least in the context of dissimilatory NO\textsubscript{3} cycling. The data also show that nrf\textsubscript{A} abundance variable among sites, but the pattern differed from that of nir\textsubscript{S} or nir\textsubscript{K} (Figure 11). In the Jamaica Bay study (Chapter 2), nrf\textsubscript{A} abundance was positively related to sediment C:N. When the data for Jamaica Bay and WLISS was combined, nrf\textsubscript{A} abundance was also positively related to C:N (Figure 12D). This finding is in line with the hypothesis that under conditions of abundant sediment C and low N (i.e. high C:N), bacteria capable of DNRA can use NO\textsubscript{3}\textsuperscript{-} more efficiently than denitrifiers (Tiedje 1988, Burgin and Hamilton 2007).

Our final objective was to explore the relationship between denitrifying functional gene abundance and denitrification rates in the literature. Because denitrifying bacteria are responsible for carrying out denitrification, there should be a positive relationship between the abundance of denitrifying bacteria and denitrification rates. To explore trends among published studies, we assembled a table of research from the literature that report results from comparing gene abundance to denitrification rate across ecosystem types and using different methods for measuring denitrification (Table 8).
Table 8. Comparison of denitrification studies carried out in various environments. The table lists overall results related to denitrification functional gene abundance and whether or not these data were related to denitrification rates. The methods used by each study to obtain denitrification rate are listed as well as the habitat the study was conducted in.

<table>
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<th>Results</th>
<th>Denitrification Method</th>
<th>Habitat</th>
<th>Author(s)</th>
</tr>
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<td>$^{15}$N loss from denitrification positively related to nirS and nirK gene abundance</td>
<td>$^{15}$N Tracer</td>
<td>Wetland</td>
<td>Yi et al. (2013)</td>
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<td>Positive correlation between nirS/K gene abundance and DNP as well flux</td>
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<td>Estuary</td>
<td>Dong et al. (2009)</td>
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<td>nirS positively related to DNP; nirS positively related combined N$_2$ and N$_2$O</td>
<td>$^{15}$N Tracer and acetylene block</td>
<td>Agricultural Grassland</td>
<td>Cuhel et al. (2010)</td>
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<td>Positive correlations found for nirS and nirK abundance with DNP</td>
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<td>Forest Soil</td>
<td>Peterson et al. (2012)</td>
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<td>Arctic Soil</td>
<td>Banerjee and Siciliano (2012)</td>
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<td>nirS and nirK abundance positively related to DNP and DNF from gas fluxes</td>
<td>$^{15}$N Tracer</td>
<td>Estuary</td>
<td>Lindemann et al. (unpublished)</td>
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<td>Agricultural field</td>
<td>Attard et al. (2011)</td>
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<td>Abundance of nirS and nirK weakly correlated to DNP</td>
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<td>Baudoin et al. (2009)</td>
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<td>Tropical Soil</td>
<td>Djigal et al. (2010)</td>
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</tbody>
</table>
Our synthesis of literature values confirm that results for studies comparing denitrification gene abundance with denitrification rates are mixed (Table 8). Some results support the hypothesis that there will be a relationship between denitrifier functional gene abundance and rate measures, while others find no relationship (Table 8). One main distinction between these studies with conflicting results includes the method used to measure denitrification rate. Many of the studies that have found a positive relationship (i.e., using regression) or correlation between nirS and/or nirK functional gene abundance and denitrification rate have employed $^{15}$N isotope tracing methods. For example, Yi (et al. 2013) found that $^{15}$N losses due to denitrification in a wetland were positively related to the abundance of both nirS and nirK. Dong et al. (2009) used $^{15}$N isotopic methods to show a significant relationship between denitrification rate and nirS abundance in sediment from the Colne Estuary, United Kingdom. Results from our WLISS study were consistent with these conclusions. In WLISS, nirS and nirK abundance were significantly and positively related to two measures of denitrification: denitrification potential (DNP) and denitrification (DNF). These denitrification rate measurements were measured via $^{15}$N isotope methods and membrane inlet mass spectrometry (MIMS) (Figure 14).
A number of previous studies finding no relationship between \textit{nirS} and/or \textit{nirK} gene abundance and denitrification rates have employed the acetylene block method (Table 8). Unlike \textsuperscript{15}N tracer methods, extra N and C is often provided in the flasks (as NaNO\textsubscript{3} and glucose), which are made anaerobic by flushing with N\textsubscript{2} or helium. These steps create conditions ideal for denitrification to occur and measure the potential of the microbial community in the sediment to produce N\textsubscript{2}. Acetylene is added which inhibits the final step in denitrification, and the accumulation of N\textsubscript{2}O is measured on a gas chromatograph. Therefore, acetylene block has been criticized for not represent environmental conditions under which denitrification takes place naturally, nor does it include the final enzymatic step in denitrification. Although, exceptions are made for locations with high water column NO\textsubscript{3}\textsuperscript{-}, methodological artifacts may account for the lack.
of relationship found between gene abundance and denitrification rate when employing this method across variety of environments including agricultural soils, wetlands, and tropical soils (Dandie et al. 2008, Djigal et al. 2010, Song et al. 2010). It has also been suggested that abundance of denitrification functional genes may not be well suited to predict small changes in potential denitrification rates, but rather, the gene abundance data can be better utilized for large scale environmental studies (Peterson et al. 2012).

**Conclusion**

Our studies of denitrification gene abundance were carried out in urban, coastal environments and showed a number of explanatory variables related to nitrite reductase functional gene abundance and denitrification. In general, organic matter was an important factor to both denitrification gene abundance and denitrification rate, while $\text{NO}_3^-$ was less important than initially expected. We also found a consistent relationship between chlorophyll $a$ and nirK abundance, which has only recently been found in studies of aquatic habitats (Abell et al. 2009, Papaspyrou et al. 2014). Overall, we found that using a combination of functional gene data, several methods for measuring denitrification rates, and multiple measurements of water column and sediment physiochemistry data allowed us to uncover a variety of seasonal and environmental patterns related to denitrification in urban, coastal environments. These patterns are of interest due to their implications related to coastal ecosystem health and can be used for development of environmental management practices and models which maximize ecosystem health in urban aquatic environments.
APPENDIX A

SUPPLEMENTARY INFORMATION
### Table A1. Primers used for quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Amplicon</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>cd3aF</td>
<td>GTSAACGTAAGGARACSGG</td>
<td>425 bp</td>
<td>Throback et al. 2004</td>
</tr>
<tr>
<td>(nirS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rc3d</td>
<td>GASTTCGRTGSGTCTTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nirS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1aCu</td>
<td>ATCATGGTSGCTGCCGCG</td>
<td>475 bp</td>
<td>Throback et al. 2004</td>
</tr>
<tr>
<td>(nirK)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3Cu</td>
<td>GCCTCGATCAGRTTGTTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nirK)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>341F</td>
<td>CCTACGGAGGGCAGCAG</td>
<td>193 bp</td>
<td>Muyzer et al. 1996</td>
</tr>
<tr>
<td>(16S)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>534R</td>
<td>ATTACCGGGCTGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(16S)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nrfA6F</td>
<td>GAYTGCCAYATGCCRAAAGT</td>
<td>222 bp</td>
<td>Takeuchi 2006</td>
</tr>
<tr>
<td>(nrfA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nrfA6R</td>
<td>GCBKCTTTTYGCTTCRAAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nrfA)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table A2. PCR cloning results

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Gene cloned</th>
<th>PCR Primers Used</th>
<th>BLAST result (M13 primers used for sequencing)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. stutzeri</em></td>
<td><em>nirS</em></td>
<td>nirS1F/6R Braker</td>
<td>CP002881.1 <em>Pseudomonas stutzeri</em> Tfp pilus assembly protein</td>
</tr>
<tr>
<td>ATCC 17588</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pf5 ATCC BAA-477</td>
<td><em>nirK</em></td>
<td>nirK1F/5R Braker</td>
<td>CP0000076.1 <em>Pseudomonas protogens</em> Pf-5 nitrite reductase, copper-containing</td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td><em>nirS</em></td>
<td>Cd3aF/Rc3d Throback</td>
<td>CP002881.1 <em>Pseudomonas stutzeri</em> ATCC 17588 nitrite reductase</td>
</tr>
<tr>
<td>ATCC 17588</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pf5 ATCC BAA-477</td>
<td><em>nirK</em></td>
<td>F1aCu/R3Cu Throback</td>
<td>CP0000076.1 <em>Pseudomonas protogens</em> Pf-5 nitrite reductase, copper-containing</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>nrfA</em></td>
<td>nrfA6F/nrfA6R Takeuchi</td>
<td>HG738867.1 <em>Escherichia coli</em> str. K-12 substr. MC4100 complete genome, cytochrome c552 nrfA</td>
</tr>
<tr>
<td>MC4100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table A3. Conditions used during qPCR of different genes of interest

<table>
<thead>
<tr>
<th>Primers</th>
<th>Thermal Conditions</th>
<th>Average Efficiency</th>
<th>Average R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>nirS</td>
<td>95°C 10 minutes, 1 cycle</td>
<td>91%</td>
<td>0.99</td>
</tr>
<tr>
<td>Cd3aF</td>
<td>95°C 30s, 60°C 30s, 72°C 60s, 80°C 15s, 40 cycles</td>
<td>95°C 15s, 65-95°C (+0.2°C) 1 cycle</td>
<td></td>
</tr>
<tr>
<td>Rc3d</td>
<td>95°C 15s, 65-95°C (+0.2°C) 1 cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nirK</td>
<td>95°C, 10 minutes, 1 cycle</td>
<td>92%</td>
<td>0.99</td>
</tr>
<tr>
<td>F1aCu</td>
<td>95°C 30s, 60°C 30s, 72°C 60s, 80°C 15s, 40 cycles</td>
<td>95°C 15s, 65-95°C (+0.2°C) 1 cycle</td>
<td></td>
</tr>
<tr>
<td>R3Cu</td>
<td>95°C 15s, 65-95°C (+0.2°C) 1 cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16s</td>
<td>95°C 10min, 1 cycle</td>
<td>93%</td>
<td>0.99</td>
</tr>
<tr>
<td>rRNA</td>
<td>95°C 15s, 60°C 30s, 72°C 30s, 80°C 15s, 40 cycles</td>
<td>95°C 15s, 65-95°C (+0.2°C), 1 cycle</td>
<td></td>
</tr>
<tr>
<td>341F</td>
<td>95°C 15s, 65-95°C (+0.2°C), 1 cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>534R</td>
<td>95°C 15s, 65-95°C (+0.2°C), 1 cycle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nrfA</td>
<td>95°C 10min, 1 cycle</td>
<td>82%</td>
<td>0.99</td>
</tr>
<tr>
<td>nrfA6F</td>
<td>95°C 15, 54.5°C 30s, 72°C 60s, 76°C 10s, 50 cycles</td>
<td>95°C 15s, 65-95°C (+0.2°C), 1 cycle</td>
<td></td>
</tr>
<tr>
<td>nrfA6R</td>
<td>95°C 15s, 65-95°C (+0.2°C), 1 cycle</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table A4. Factor loadings as correlation coefficients for each variable included in Jamaica Bay PCA analysis.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>nirS</td>
<td>0.355</td>
<td>0.050</td>
<td>0.080</td>
</tr>
<tr>
<td>nirK</td>
<td>0.365</td>
<td>-0.059</td>
<td>0.095</td>
</tr>
<tr>
<td>16s</td>
<td>0.295</td>
<td>0.089</td>
<td>0.051</td>
</tr>
<tr>
<td>nrfA</td>
<td>0.138</td>
<td>0.095</td>
<td>0.426</td>
</tr>
<tr>
<td>OM</td>
<td>0.207</td>
<td>0.317</td>
<td>0.077</td>
</tr>
<tr>
<td>C.N</td>
<td>-0.061</td>
<td>0.163</td>
<td>0.064</td>
</tr>
<tr>
<td>NH₄Min</td>
<td>-0.149</td>
<td>-0.265</td>
<td>0.414</td>
</tr>
<tr>
<td>NIT</td>
<td>-0.097</td>
<td>0.142</td>
<td>0.603</td>
</tr>
<tr>
<td>DNP</td>
<td>0.171</td>
<td>-0.038</td>
<td>-0.485</td>
</tr>
<tr>
<td>TEMP</td>
<td>0.310</td>
<td>-0.391</td>
<td>0.033</td>
</tr>
<tr>
<td>SRP</td>
<td>0.315</td>
<td>-0.233</td>
<td>0.008</td>
</tr>
<tr>
<td>NO₃</td>
<td>0.034</td>
<td>0.545</td>
<td>-0.090</td>
</tr>
<tr>
<td>NO₂</td>
<td>0.315</td>
<td>0.305</td>
<td>0.045</td>
</tr>
<tr>
<td>Chla</td>
<td>0.322</td>
<td>-0.313</td>
<td>0.077</td>
</tr>
<tr>
<td>NH₄</td>
<td>0.355</td>
<td>0.217</td>
<td>0.015</td>
</tr>
</tbody>
</table>
Figure A1. Example of \textit{nirS} standard curve (efficiency= 91\%)

\begin{align*}
y &= -3.5582x + 36.736 \\
R^2 &= 0.994
\end{align*}

Figure A2. Example of \textit{nirK} standard curve (efficiency= 95\%)

\begin{align*}
y &= -3.4455x + 37.812 \\
R^2 &= 0.9961
\end{align*}
Figure A3. Example of 16s rRNA standard curve (efficiency= 92%)

Figure A4. Example of nrfA standard curve (efficiency= 83%)
Figure A5. Example of \( nirS \) melt curve

Figure A6. Example of \( nirK \) melt curve
Figure A7. Example of 16s melt curve

Figure A8. Example of nrfA melt curve
REFERENCE LIST


Kennedy, V., and Breisch, L. (1981). Maryland’s Oysters, Research and Management (University of Maryland, College Park, MD, USA: Maryland Sea Grant).


VITA

Samantha Lindemann of Montevideo, Minnesota, graduated from the University of Minnesota Duluth in 2011 with a Bachelor of Science degree in Cell and Molecular Biology. After working at Era Laboratories as an aquatic toxicology technician for one year, she began the Master of Science program at Loyola University Chicago in 2012 where she joined the labs of Dr. Domenic Castignetti and Dr. Timothy Hoellein.