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Linking structure and function of microbial communities responsible for sedimentary nitrogen processes in North Carolina estuaries

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Linking Structure and Function of Microbial Communities Responsible for Sedimentary Nitrogen Processes in North Carolina Estuaries

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by

Jessica A. Lisa

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APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

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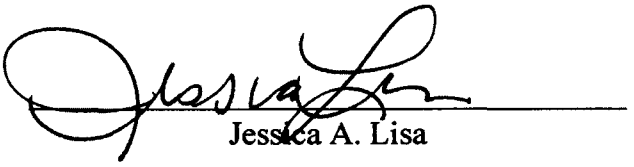

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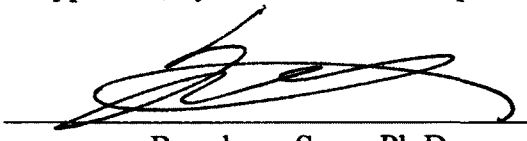

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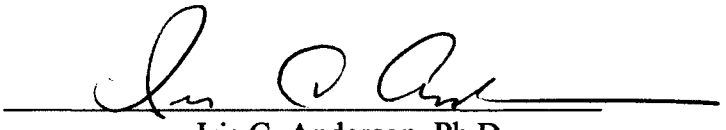

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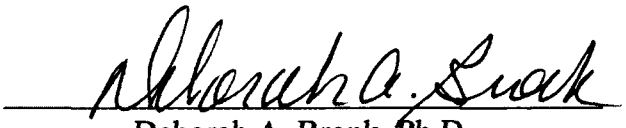

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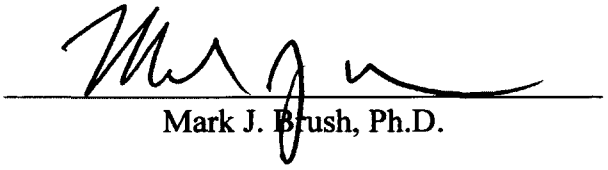

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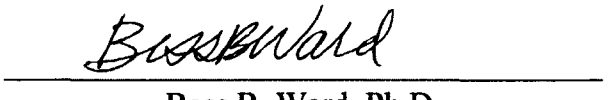

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DEDICATION

This dissertation was written in loving memory of my papa, Ernesto, and is dedicated to my grandmother, Anne, for her unconditional love and support throughout my life. Mom and Dad, I would have dedicated this to you for the same reasons but I had to secure my position as favorite grandchild.

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ABSTRACT

Estuaries and coastal areas are highly diverse and productive ecosystems that serve as critical habitats for many marine organisms. Anthropogenic activities and changes in coastal land use have resulted in nutrient over-enrichment, posing a threat to the ecological integrity of estuaries. Nitrogen is often the limiting nutrient and its dynamics are central in determining coastal ecosystem health. The cycling and removal of nitrogen is primarily microbially mediated and the structure of these microbial communities is tightly interlaced with ecosystem function. Recycling processes nitrification and dissimilatory nitrate reduction to ammonium (DNRA) serve as a conduit for loss when coupled to the removal processes of denitrification and anaerobic ammonium oxidation (anammox). These processes occur simultaneously in estuarine sediments and are influenced by a suite of environmental factors. Understanding these interactions within the complex microbial communities, how microbial community structure responds to environmental changes, and how this relates to ecosystem function is a challenging but important goal in microbial ecology and particularly relevant in today's changing world, as climate change and sea level rise are eminent threats to coastal ecosystems. This study identified geomorphological, hydrological, and biogeochemical features, including water residence time, sediment %organics, nutrient availability, salinity, and hydrogen sulfide, that are important in controlling the community structures of nitrogen cycling microbes and their activities. Quantitative criteria were established to identify and assess the role of nitrogen removal hotspots in estuarine sediments. Specific groups of denitrifying and anammox bacteria with a higher nitrogen removal capacity were identified, suggesting the community composition is important in ecosystem function. Additionally, niche differentiation of nitrifying microbes was unveiled by linking their physiology to specific environmental parameters. Examination of microbial community structure and its response to environmental conditions combined with measurements of biogeochemical rates enabled the linkage of nitrogen cycling members with particular functions, a theme addressed throughout this dissertation.

Linking Structure and Function of Microbial Communities Responsible for Sedimentary Nitrogen Processes in North Carolina Estuaries

CHAPTER 1

Introduction to the Dissertation

Microbial communities are the "engines" of globally important biogeochemical processes, such as the nitrogen cycle, and are vital to ecosystem function (Fuhrman 2009). Understanding what controls their distribution in the environment has important implications on the processes they mediate and the systems in which they are found (Arrigo 2005). Within a microbial community exists a large and complex diversity of phylogenetically distinct members that vary in distribution and abundance of organisms. Shifts in microbial community structure in response to environmental variables are often observed in natural systems (Zumft 1997, Braker et al. 2000, Bernhard et al. 2005, Jones & Hallin 2010, Bollmann et al. 2014, Wei et al. 2015). These shifts in community structure are accompanied by changes in metabolic capabilities (Tiedje 1988, Cavigelli & Robertson 2000, Philippot & Hallin 2005, Petersen et al. 2012, Liu, Mao, et al. 2013). Examination of microbial community structure and its response to environmental conditions combined with measurements of biogeochemical rates enable the linkage of groups of organisms or individual members with particular functions, a challenging but important goal in microbial ecology and particularly relevant in today's changing world (Fuhrman 2009).

Estuaries and coastal areas are highly diverse and productive ecosystems that serve as critical habitats for many marine organisms (Costanza et al. 1997). Anthropogenic activities, human alteration of the nitrogen cycle, and changes in coastal land use have resulted in nutrient over-enrichment, which poses a threat to the ecological integrity of estuaries (Nixon 1995, Howarth & Marino 2006). Nitrogen is often the nutrient limiting net primary production in estuarine and coastal systems and the dynamics are central in determining the levels and patterns of primary productivity

(Ryther & Dunstan 1971, Howarth 1988, Paerl 2009). Excessive nitrogen has been implicated in major perturbations in these coastal ecosystems and is of growing concern to water-quality. It is important to note that the potential for co-limitation of primary production also exists, often in the form of phosphorous or silicon in coastal systems (Paerl 2009).

The addition of limiting nutrients can dramatically alter an ecosystem (Vitousek et al. 1997). Eutrophication is one of the best-documented consequences of excess nitrogen in coastal and estuarine systems. This increase in the rate of supply of organic matter to an ecosystem is most commonly fueled by excess nitrogen that supports an increase in primary productivity in estuaries and coastal systems (Nixon 1995). As a result of persistent algal blooms and subsequent die off, an accumulation of particulate organic matter may lead to localized deficiencies in oxygen. These hypoxic and anoxic events are increasing world wide and often lead to habitat degradation and loss, changes in the distributions and trophic interactions of organisms, as well as significant losses of fish and shellfish resources in coastal areas (Vitousek et al. 1997, Breitburg et al. 2009). Ecologically important places such as coastal and estuarine systems tend to suffer as a result of hypoxia due to the timing of the low oxygen events, which typically occur during summer, when growth and predator energy demands are high (Diaz & Rosenberg 2008).

Nitrogen enters the environment through anthropogenic activities including fossil fuel combustion and fertilizer production as well as biological nitrogen fixation (Figure 1). Several microbially mediated processes are responsible for recycling of fixed nitrogen and include regeneration nitrification, and dissimilatory nitrate reduction to

ammonium (DNRA). These recycling pathways allow fixed nitrogen to remain in the system for biological use, but also serve as a link for loss via removal processes (Henrikson & Kemp 1988). Two microbial nitrogen removal processes, denitrification and anaerobic ammonium oxidation (anammox), may play a mitigating role in the intensity and duration of estuarine eutrophication in response to high nitrogen loading.

Nitrogen recycling and removal processes occur simultaneously in estuarine sediments and may be influenced by the heterogeneous distribution of biogeochemical factors such as temperature, salinity, dissolved oxygen (DO), nitrate (NO_3^-), ammonium (NH_4^+), organic carbon and hydrogen sulfide (H_2S), as well as ecosystem-scale controls such as hydrological and geomorphological features of the system (Cornwell et al. 1999, Dalsgaard et al. 2005, Seitzinger et al. 2006, Joye & Anderson 2008). Biological factors such as benthic microalgae (BMA) are responsible for nitrogen turnover in estuarine systems and are known to influence nitrification and denitrification through competition for dissolved nitrogen species and production of oxygen (O_2; An & Joye 2001, Tobias et al. 2003, Hardison et al. 2013). The role of BMA as a stimulator or inhibitor is dependent on the distribution of nitrogen in the system. Multiple biotic and abiotic factors influence microbial processes, and the communities that mediate nitrogen cycling are continually changing in response to the environment. Therefore, examining structural response of microbial communities to changing environmental condition may provide a better understanding in the complexity of sedimentary nitrogen cycling in estuaries. The individual processes and microorganisms that mediate the processes are discussed in the chapters of this dissertation with a focus on the role of the microbial community in sedimentary nitrogen cycling processes.

The overall objective of this dissertation is to examine the linkage between the structure and function of microbial communities responsible for sedimentary nitrogen processes in the New River Estuary and Cape Fear River Estuary, North Carolina, USA. This dissertation is separated into five main chapters that address specific components of nitrogen cycling in microbial communities and their respective activities in estuarine sediments.

- In Chapter 2, I examined how the geomorphological features and water residence time contribute to the structure and function of anammox communities of the New River Estuary.
- In Chapter 3, I quantitatively identified nitrogen removal hotspots at the sediment-water interface throughout the New River Estuary using newly proposed criteria for determining biogeochemical hotspots.
- In Chapter 4, I employed *nir*S microarrays to investigate the community composition of denitrifiers at several sites in the New River Estuary, including identified hotspots, to examine the relationship between the composition and activity of the denitrifying community.
- In Chapter 5, I examined the effects of short (tidal) and long (seasonal) term variations in the environment on abundance and activities of sediment communities involved in nitrification, denitrification, anammox and DNRA in the Cape Fear River Estuary.
- And finally, in Chapter 6, I investigated niche differentiation of nitrifying prokaryotes based on next generation sequencing analysis of 16S rDNA and rRNA in the sediment communities of Cape Fear River Estuary.

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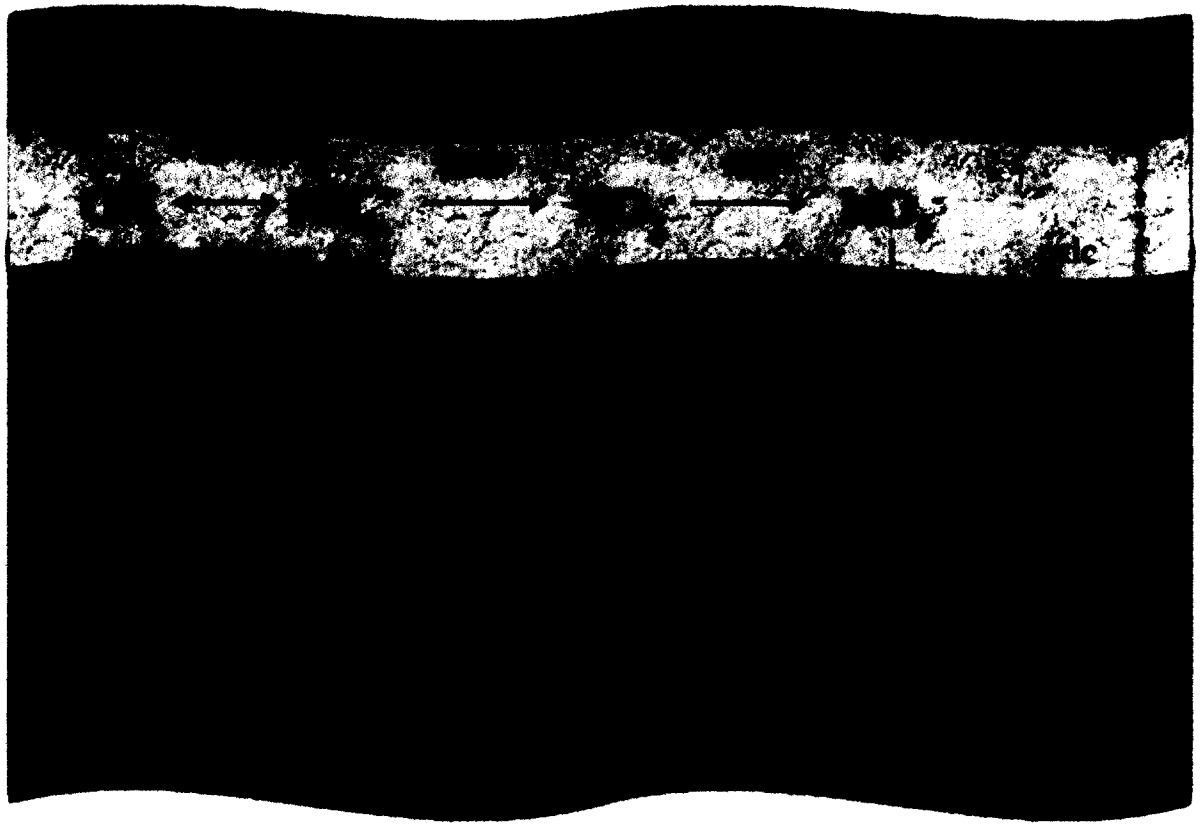
Figure 1. The Sedimentary Nitrogen Cycle. This schematic of sedimentary nitrogen cycling shows the overlying bottom water in blue, oxic sediments in the light sand color, anoxic sediments in the dark sand color. The genes encoding for the enzymes that mediate nitrogen cycling are shown in burgundy and include: nitrogenase (*nrf*A), ammonia monooxygenase (*amo*A), cytochrome C nitrite reductase (*nrf*A), nitrite reductase (*nir*S), nitrous oxide reductase (*nos*Z), and hydrazine oxidoreductase (*hzo*). Pathways, in dark gray, are as follows: nitrogen fixation, regeneration, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), denitrification, and anaerobic ammonium oxidation (anammox).



CHAPTER 2

Impacts of freshwater flushing on anammox community structure and activities in the New River Estuary, USA

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ABSTRACT

Anaerobic ammonium oxidation (anammox) and denitrification are two microbial nitrogen removal processes that may play an important role in controlling the intensity and duration of estuarine and coastal eutrophication. Sediment communities in the New River Estuary, North Carolina were investigated to determine dynamics of anammox activity and community structure in conjunction with environmental conditions. ^{15}N tracer incubation experiments with sediment slurries were used to measure anammox and denitrification rates and estimate anammox contribution to total N_2 production. Molecular analyses targeting the hydrazine oxidoreductase (*hzo*) gene were conducted to examine structure of anammox communities and to quantify abundance of anammox bacteria in sediments. Potential anammox rates ranged from 0.02 to 1.4 nmol N_2 g^{-1} h^{-1}, with highest potential activities observed during winter and spring when the estuary received large doses of nitrogen from the watershed. Anammox contributed up to 14.1% of total N_2 production in upstream estuarine sediments and abundance ranged from 1.55 x 10^2 to 2.59 x 10^5 *hzo* gene copies g^{-1} sediment. Both activities and abundance of anammox communities were correlated with percent sediment organics (%organics) and the porewater concentrations of hydrogen sulfide (H_2S). Based on Hzo sequence analysis, anammox bacteria related to *Candidatus* "Jettenia spp." were widespread in estuarine sediments, which may be attributed to freshwater flushing and associated changes in environmental parameters as well as the geomorphology of the estuary. This is the first study to describe a dominance of *Candidatus* "Jettenia spp." in relation to %organics and hydrogen sulfide in an estuarine ecosystem driven by meteorological forcing.

INTRODUCTION

Estuaries and coastal areas are highly productive ecosystems that serve as critical habitats for many marine organisms (Costanza et al. 1997). Anthropogenic activities and changes in coastal land use have resulted in excess nitrogen loading and eutrophication, which pose a threat to the ecological integrity of estuaries (Nixon 1995, Howarth et al. 2000). Two microbial nitrogen removal processes, anaerobic ammonium oxidation (anammox) and denitrification may play a mitigating role in the intensity and duration of estuarine eutrophication in response to high nitrogen loading. Both microbial processes occur simultaneously in estuarine sediments and are influenced by biogeochemical factors as well as ecosystem-scale controls such as hydrological and geomorphological features of the system (Cornwell et al. 1999, Dalsgaard et al. 2005, Seitzinger et al. 2006).

Denitrification is an anaerobic respiratory process that reduces two molecules of nitrate (NO_3^-) to gaseous N_2 and mainly occurs in the sediments and in the water column at the oxic-anoxic interface (Tuominen et al. 1998). A phylogenetically diverse group of organisms is capable of this respiratory process (Zumft 1997). Denitrification rates are generally influenced by labile organic carbon and NO_3^- availability, which can vary spatially and temporally within an estuary (Cornwell et al. 1999, Seitzinger et al. 2006).

Anaerobic ammonium oxidation (anammox), a more recently discovered pathway to N_2 production, is a process that couples the reduction of one molecule of nitrite (NO_2^-) with the oxidation of one molecule of ammonium (NH_4^+) to produce N_2 under anaerobic conditions (van de Graaf et al. 1995). Anammox, shown to occur in estuarine systems around the world, also exhibits spatial and temporal variations related to biological and

geochemical parameters such as temperature, (Rysgaard et al. 2004), salinity (Rich et al. 2008), organic carbon content (Trimmer et al. 2003) and substrate availability (Risgaard-Petersen & Meyer 2004, Trimmer et al. 2005, Rich et al. 2008, Nicholls & Trimmer 2009). Higher potential anammox rates and contribution to total N_2 production (%anammox) have been consistently observed in the oligohaline reaches of estuaries relative to higher salinities (Trimmer et al. 2003, Meyer et al. 2005, Koop-Jakobsen & Giblin 2009, Minjeaud et al. 2009).

Investigations of anammox bacterial communities in estuarine sediments revealed spatial variation in community structure in relation to an estuarine salinity gradient. *Candidatus* "Scalindua spp." dominated throughout the estuarine systems (Risgaard-Petersen & Meyer 2004, Tal et al. 2005, Amano et al. 2007, Rich et al. 2008, Dang et al. 2010, Hirsch et al. 2011) while *Candidatus* "Brocadia", *Candidatus* "Kuenenia", *Candidatus* "Anammoxoglobous" and *Candidatus* "Jettenia" were primarily detected in fresh to oligohaline sediment communities (Zhang et al. 2007, Dale et al. 2009, Li et al. 2010, Amano et al. 2011, Hirsch et al. 2011).

It has been suggested that variability in anammox activities and contribution to local and regional N loss are probably due to abundance, composition, and distribution of anammox bacteria (Dang et al. 2010), which are in turn constrained by environmental parameters such as salinity and substrate availability. If this is the case, shifts in anammox community structure and activities should covary with environmental parameters. However, the majority of studies exploring anammox in estuaries reported either community structure based on 16S rRNA and hydrazine oxidoreductase gene (*hzo*) analyses (Tal et al. 2005, Amano et al. 2007, Rich et al. 2008, Dale et al. 2009, Dang et

al. 2010, Hirsch et al. 2011) or rate measurements using ^{15}N isotope incubation methods (Thamdrup & Dalsgaard 2002, Trimmer et al. 2003, Risgaard-Petersen & Meyer 2004, Meyer et al. 2005, Koop-Jakobsen & Giblin 2009, Nicholls & Trimmer 2009). Thus the coupling between community structure and activity rates of anammox communities along with spatial and temporal gradients in estuarine ecosystems has not been directly addressed.

Further, geomorphological characteristics and water residence time of a system influence nutrient cycling and availability for biological use (Ensign & Doyle 2006) as well as community composition (Crump et al. 2004). Systems governed by periodic anoxic events lead to the spatial separation of the coupled aerobic nitrification and anaerobic denitrification processes and result in a strong temporal link between the two processes (Seitzinger et al. 2006). In these systems, NO_3^- is supplied primarily through advection of high nutrient waters and denitrification occurs continuously. Findings by Kana et al. (Kana et al. 1998) suggest that sediment denitrifying microbial populations remain poised to use NO_3^- and short-term temporal changes in NO_3^- concentrations in the overlying water control denitrification rates in these communities.

The degree of meteorological forcing such as freshwater flushing has also been shown to govern biological activities in estuarine systems (Peierls et al. 2012, Anderson et al. in press, Hall et al. in press). During moderate hydrologic forcing and high new nutrient loads, a system trends towards higher productivity and increased biomass. Excessively higher hydrologic forcing can overshadow nutrient availability however, by increasing freshwater discharge and thereby reducing residence time, primary productivity and biomass, exerting a top down control on community structure. As a

result, it is important to consider different estuarine systems and potential effects that meteorological forcing may have on microbial community structure and activities. This is especially true with slow growing organisms such as anammox bacteria, which may adapt differently to environments that experience periods of stagnation followed by pulses of high nutrient freshwater.

Studies conducted on anammox activities and community structure in estuarine systems have captured the dynamics of anammox in various estuarine settings (Meyer et al. 2005, Rich et al. 2008, Nicholls & Trimmer 2009), mangrove sediments (Meyer et al. 2005), coastal marine sediments (Rysgaard et al. 2004, Engström et al. 2005, Dalsgaard et al. 2005), and anoxic marine systems (Jensen et al. 2008). Episodic inflow events have been shown to cause a switch from denitrification to anammox as the main form of nitrogen loss in the Baltic Sea (Hannig et al. 2007), however limited studies have been conducted in shallow estuarine systems that experience these dramatic shifts in environmental conditions.

The goal of this study was to investigate linkages between structure and activity of anammox communities corresponding to spatial variations of environmental conditions and episodic flushing events in the New River Estuary (NRE), North Carolina, USA. A four-fold approach was used to define these linkages: 1) Full characterization of environmental parameters previously associated with anammox rates and community structure were conducted; 2) ^{15}N tracer incubation experiments with sediment slurries were used to measure the potential rates of anammox and denitrification with respect to environmental conditions; 3) Molecular analyses of *hzo* genes were conducted to examine composition and abundance of anammox communities in sediments; and 4)

Multi-regression and multidimensional statistical methods were employed to assess the nature and extent of covariance among anammox community structure, activities of anammox communities (in absolute terms and relative to denitrification), and environmental parameters in the NRE.

MATERIALS AND METHODS

Site Description

The New River Estuary (NRE), Onslow Bay, North Carolina, USA, is a coastal plain estuary consisting of a series of shallow (< 5m deep), broad lagoons connected by narrow channels. High concentrations of humic materials and tannic acids (Dame et al. 2000, Dafner et al. 2007) characterize the upper estuary and the lower estuary is bordered by intertidal wetlands. The NRE watershed encompasses a 1436 km^2 area draining mostly forest and agricultural lands in the upper regions of the watershed (Burkholder et al. 1997, Mallin et al. 2005). Within the watershed are over 138 registered swine facilities with 150,000 animals as well as poultry farms containing over one million birds. The City of Jacksonville located in Wilson Bay and The United States Marine Corps Base at Camp Lejeune are also within the watershed. Barrier islands at the mouth of the NRE restrict tidal exchange and are likely responsible for the relatively long (64 day) mean flushing time in this estuary (Ensign et al. 2004). The North Carolina Division of Environmental Management has classified the NRE as "nutrient sensitive" since 1998, with nitrogen being the limiting nutrient of pelagic and benthic productivity in this eutrophic coastal system (Mallin et al. 2000). Shifts in seasonal wet and dry periods and episodic storm activity further impact nutrient loads and water residence

times, leading to pulsed events that alter biological structure and function of this estuary as a benthic filter (Peierls et al. 2012, Anderson et al. in press, Hall et al. in press),

Seasonal Sampling of the NRE

Sampling was conducted seasonally (summer, fall, winter, spring) during 2009-2010 along nutrient and salinity gradients from the headwaters to the mouth of the estuary to capture intermittent flushing events. Summer (June 2009) and fall (September 2009) sampling events were conducted under normal conditions with rates of <1 and <5 $m^3 s^{-1}$ freshwater discharge, respectively (Peierls et al. 2012). Winter sampling (December 2009) followed several pulsed discharge events ranging from <5 to >25 $m^3 s^{-1}$ freshwater discharge, leading to elevated levels of new nutrients, likely from land based sources, that remained in the system for several months. Spring sampling (April 2010) occurred at the tail end of these winter/spring episodic events during what was considered a relatively wet year, when freshwater discharge declined to <1 $m^3 s^{-1}$.

Seven sites were examined and included upper estuary sites AA2 (34.76N, 77.45W), Jax (34.73N, 77.43W), M53 (34.72N, 77.43W), mid-estuary sites M47 (34.68N, 77.39W), M39 (34.64N, 77.36W), and lower estuary sites M31 (34.59N, 77.40W), M15 (34.55N, 77.35W) (Figure 1). All samples and measurements were collected in the channel west of the indicated channel markers.

Environmental Parameter Measurements

Environmental parameters including water column depth, temperature, salinity and dissolved oxygen (DO) were measured in surface and bottom waters (when site was

>1 m depth) at the time of sampling using a 6820 multi parameter YSI datasonde (YSI Incorporated, Yellow Springs, OH). Surface and bottom waters were 0.7μm filtered and stored on ice prior to nutrient analysis. Ammonium and NO_3^- concentrations were measured spectrophotometrically on a Bran Luebbe segmented flow nutrient auto analyzer using phenol hypochlorite and Cd-reduction/azo dye methods, respectively following modified standard EPA methods (Long & Martin 1997).

Sediment samples were collected using a petite ponar grab (Wildco, Buffalo NY). The top 2 centimeters of sediment were collected and used to pack full 50 ml centrifuge tubes (BD Biosciences Franklin Lakes, NJ) and stored on ice. In the laboratory, porewater was extracted by centrifugation and analyzed for ferrous iron (Fe^{2+}; Stookey 1970) and hydrogen sulfide (H_2S; Cline 1969). Porewater $NO_3^- + NO_2^-$ (NO_x^-) was measured using Vanadium (III) reduction and chemiluminescent detection (Braman & Hendrix 1989). Sediment percent organic content was determined by loss on ignition (LOI) of dried sediments (500°C for 4 hours). Sediment NH_4^+ (free plus extractable) was measured by phenol hypochlorite following KCl extraction using a 1:1 ratio of KCl to sediments (Mackin & Aller 1984). Benthic chlorophyll *a* analysis on 2 cm thick sediment plugs was conducted as described by Whitney and Darley (1979). Mean sediment grain size was determined after organic material was dissolved with 35% H_2O_2 and using LS 200 Beckman Coulter Particle Sizer (Bechman Coulter, Brea, CA). Sediments from each site were stored at -80°C for molecular analysis.

^{15}N Tracer Incubations

Sediment slurry incubation experiments with ^{15}N tracer were conducted to measure potential rates of denitrification and anammox using a modified method of Dale et al. (2009). Eight sediment slurries containing two grams of homogenized sediment and porewater were pre-incubated in helium purged Exetainer tubes (Labco Limited, High Wycombe, Buckinghamshire, England) in the dark overnight to remove residual NO_x^-. After pre-incubation, two of the Exetainers were sacrificed to measure residual NO_x^- in sediment porewater (Braman & Hendrix 1989). The residual concentration of NO_x^- was used to correct the mole fraction ^{15}N enrichment of the added $^{15}NO_3^-$ (Song & Tobias 2011) in subsequent rate calculations for anammox and denitrification. Remaining Exetainer tubes with sediment slurries were again purged with helium, amended with 200 nmoles $^{15}NO_3^-$ and 200 nmoles $^{14}NH_4^+$ and placed in the dark during incubations. Time series incubations were carried out in duplicates and the activities stopped by the addition of saturated $ZnCl_2$. Production of $^{29}N_2$, and $^{30}N_2$ was measured on an Isotopic Ratio Mass Spectrometer (Delta V Plus, Thermo Fisher Scientific, Waltham, MA) and used to calculate the rate of anammox and denitrification following the method of Thamdrup and Dalsgaard (2002) as modified by Song and Tobias (2011). Percent anammox (%anammox) was estimated based on the rates of anammox and total N_2 production in each sample.

DNA Extraction

Sediment DNA was extracted using PowerSoil DNA Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA) following the manufacture's protocol with two modifications, 1) the

amount of wet sediment was increased to 0.6 g and 2) Thermo Savant Fast Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad, CA) was used for cell disruption.

Quantitative PCR Amplification of hzo Genes

Quantitative PCR (qPCR) of *hzo* genes was carried out to measure the abundance of anammox bacteria in the NRE sediments following the method of Long et al. (2013). PCR mixture contained the HZAOQPCR1F and HZOQPCR1R primers, sediment DNA (3 ng/μL) and the Go-Taq qPCR Master Mix (Promega Corporation, Madison, WI). PCR specificity was monitored by analysis of dissociation curves. The R^2 values for the standard curves were >0.996 and detection limit of *hzo* qPCR was 78 *hzo* gene copies per sample.

PCR amplification, Cloning of PCR products, and Sequencing

In order to examine the composition of anammox communities exposed to different freshwater discharge rates, nested PCR of the *hzo* genes was conducted with winter and summer samples using the Go-Taq Master Mix (Promega Corporation, Madison, WI) under the following PCR conditions: 95°C for 5:00 min, 94°C for 1:00 min, 55°C for 1:00 min, 72°C for 1:00 min, 35 times, 95°C for 1:00 min, 55°C for 1:00 min, 72°C for 10:00 min. The initial PCR reaction was conducted with the primer HZO4F and HZO1R (Hirsch et al. 2011) to amplify a 1037 bp region of the *hzo* gene. A second, nested reaction was conducted using initial PCR reaction mixture as a template and the primers hzocl1 F1 and hzocl1 R2 (Schmid et al. 2008) to generate 470 bp fragments.

The nested PCR products were purified using UltraClean GelSpin DNA Purification Kit (Mo-Bio, Carlsbad, CA) and cloned using Perfect Prep Cloning Kit (5 Prime, Gaithersburg, MD). At least 24 clones were picked for *hzo* gene library constructions. Sequencing was conducted with the Big Dye Terminator v1.1 and a 3130x/ Genetic Analyzer sequencing machine (Applied Biosystems, Carlsbad Ca).

Phylogenetic Analysis of hzo Genes

The *hzo* gene sequences were assembled and edited using the SeqMan program (DNASTAR Lasergene) and compared to *hzo* gene sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/). The *hzo* gene sequences were translated to amino acid sequences using Transeq (European Bioinformatics Institute http://www.ebi.ac.uk/Tools/emboss/transeq/index.html). Translated Hzo sequences were aligned with reference sequences using Mega 5.1. Phylogenetic tree was constructed using the Neighbor Joining method with Poisson model. Representative Hzo sequences were selected within the sequences sharing 100% identity. The number of sequences is denoted in parentheses of a representative sequence in the phylogenetic tree. Bootstrap analysis of 1000 repetitions was used to test the confidence of phylogenetic reconstruction with 50% support threshold. Hzo protein similarity was determined using pairwise distance computation in MEGA 5.1. The *hzo* gene sequences are available at the GenBank with the accession numbers KF192638 to KF192691.

Statistical Analysis

Several statistical evaluations of the data were done. First, Two-Way Analysis of Variance was conducted to identify significant differences among anammox activities by location within the estuary and season using R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing). Correlation analyses were also conducted to identify relationships between anammox rates, anammox gene abundance and community structure with environmental parameters also using R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing). A weighted principal components analysis (PCA) using UniFrac (http://bmf2.colorado.edu/unifrac/imdex.psp) was conducted to examine anammox community structure based on sequence dissimilarities between sites in the estuary (Lozupone & Knight 2005). UniFrac was also used to determine significant differences in community structure using cluster analysis that was corrected for the number of pairwise comparisons using the Bonferroni correction. Non-metric multidimensional scaling (NMDS) was then performed to explore relationships between anammox community structure and environmental parameters in the NRE using the function meta-MDS in the vegan package in R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing).

RESULTS

Environmental Characteristics in the New River Estuary

Bottom water temperature was generally uniform throughout the estuary during each season ranging from 13.5 ± 2.5°C in the winter to 27.7 ± 0.6°C in summer (Table 1). Salinity in the upper and mid reaches of the estuary varied by a factor of ten depending upon river discharge (Table 1). Highest salinities were encountered in fall and spring. Winter sampling was coincident with large freshwater inputs from the watershed that completely freshened the uppermost station AA2. Elevated levels of bottom water NO_3^- and NH_4^+ were observed throughout the estuary during winter sampling, coincident with the winter freshening event (Table 1). Highest bottom water NO_3^- and NH_4^+ concentrations were at upper and mid estuary winter sites, reaching 68.61 μM and 14.89 μM, respectively. Bottom water hypoxia, where DO levels were below 5 mg/L, was observed throughout the estuary during summer and at upper estuary stations during fall and spring (Table 1).

Iron (Fe^{2+}) concentration in porewater varied between sites and seasons with highest observed concentration of Fe^{2+} exceeding 70 μM at the lower estuary M31 station during the winter (Table 2). Hydrogen sulfide (H_2S) concentrations ranged from 0.2 to 1100 μM, with elevated concentrations consistently in the upper and mid estuary (Table 2). Porewater NO_x^- was greater throughout the estuary during the spring, however the highest overall concentration was observed in winter at Jax (Table 2).

Sediment %organics were highest at the upper and mid-estuary sites during all seasons sampled. High organic content was coincident with the highest porewater H_2S concentrations in these sites (Table 3). Extractable sediment NH_4^+ concentrations were

below 0.5 μmol NH_4^+ g^{-1} wet sediment in summer and spring while NH_4^+ concentrations in the winter exceeded 1μmol NH_4^+ g^{-1} wet sediment at four sites (Table 3). Benthic chlorophyll *a* was highest in winter at mid-estuary sites with values ranging from13 to 24 μg chlorophyll *a* g^{-1} sediment (Table 3). Sediment grain size increased from fine sand and silt in the upper estuary to fine-medium grained sands towards the mouth of the estuary (Table 3).

In summary, the low salinity upper estuary sites (AA2, Jax and M53) were generally lower in DO and higher in NO_3^- and NH_4^+ than middle and lower sites. These sites contained medium silt to fine sand sediments that were moderately rich in organic content and variable in ferrous iron and sulfide. Mid estuary sites (M47 and M39) were representative of a transitional salinity and DO gradient, while elevated in H_2S, NH_4^+ and NO_3^- concentrations. Lower estuary sites (M31 and M15) were highest in salinity and DO, and lower in NO_3^- concentrations. They contained medium silt to sandy sediments with higher ferrous iron and chlorophyll *a* content. Overall, spatial variations in salinity and dissolved oxygen were observed consistently throughout the estuary. Temporal variance among sites occurred but did not show smooth seasonal cyclic pattern at any given site. Instead, seasonal trends were superseded by pulses of nutrients that were observed and elevated throughout a major portion of the estuary. The occurrence of these pulses were evident in bottom water NO_3^- and NH_4^+, H_2S porewater and sediment NH_4^+ concentrations, where trends of elevated levels were observed throughout large portions of the estuary following periods of high freshwater discharge events.

Anammox and Denitrification Activities and their Correlation to Environmental Parameters in the New River Estuary

Production of $^{30}N_2$ (denitrification) and $^{29}N_2$ (anammox) was immediately observed in the sediment incubations and the two processes were positively and significantly correlated with each other ($p = 0.013$, $r = 0.624$). Both N_2 producing processes were detected at all sites throughout the estuary during all four seasons. Potential denitrification rates ranged from 0.4 to 31 nmol $N_2\ g^{-1}\ h^{-1}$, while anammox rates were lower at 0.02 to 1.4 nmol $N_2\ g^{-1}\ h^{-1}$ (Table 4). Overall average of %anammox for the NRE was 5.2% and ranged from 1.8% to 14.1%, the highest contribution of anammox occurred in the AA2 sediment community during spring (Table 4).

For all seasons, the distribution of denitrification and anammox rates as well as %anammox along the estuary was variable. There was however, a spatial trend of higher activities in the upper estuary relative to lower activities towards the mouth of the estuary ($p = 0236$, $p = 0.0018$ and $p = 0.031$ respectively; Figure 2). Seasonal fluctuations in anammox and denitrification rates were also observed in the NRE with significant differences observed during spring relative to the other seasons ($p = 0.021$, $p = 0.00453$ and $p = 0.0153$ for denitrification, anammox and %anammox respectively).

Potential denitrification rates were positively correlated with H_2S ($p = 0.002$, $r = 0.5139$) and %organics ($p = 0.009$, $r = 0.5936$) and extractable sediment NH_4^+ ($p = 0.002$, $r = 0.2635$). Anammox rates also significantly correlated with increasing levels of porewater H_2S ($p = 0.009$, $r = 0.5681$) and sediment %organics ($p = 0.005$, $r = 0.6829$), but were negatively correlated with salinity ($p = 0.004$, $r = -0.3956$), while %anammox showed a negative correlation with DO levels ($p = 0.004$, $r = -0.5364$). This is the first

reporting of a significant positive correlation among anammox activity with porewater sulfide in sediments.

Anammox Bacterial Abundance and its Correlation to Environmental Parameters in the New River Estuary

Based on *hzo* gene qPCR, spatial and temporal abundance of anammox bacteria were compared in the sediment samples collected in summer and winter. Abundance of *hzo* genes ranged from 1.55×10^2 to 2.59×10^5 copies g^{-1} sediment (Figure 3 and Table 5). These values fell within the lower ranges of previously published values for *hzo* gene abundance in the environment (Dang et al. 2010, Hong et al. 2011). The lowest anammox bacterial abundance was found in the summer at M15 corresponding to relatively lower activities. Seasonal variation in anammox abundance was observed with highest values observed during summer at AA2, Jax, M47 and M39, and during winter at M53, M31 and M15.

Anammox bacterial abundance was significantly and positively correlated with anammox activities ($p = 0.033$, $r = 0.4336$), porewater H_2S concentrations ($p = 0.0456$, $r = 0.5412$) and sediment %organics ($p = 0.033$, $r = 0.5715$). Independent positive relationships between the co-varying estuarine conditions porewater H_2S and sediment % organics with *hzo* gene abundance and potential anammox N_2 production in the NRE may provide support demonstrating that environmental conditions influence anammox abundance which in turn influences potential anammox activities. This association was explored further in anammox community composition based on sequence analysis of *hzo* genes.

Spatial and Temporal Variation of Anammox Community Composition in the New River Estuary

Nested PCR amplified 470 bp fragments of *hzo* genes in the summer and winter samples. Eighty-seven sequences of the *hzo* genes were obtained and translated to amino acid sequences prior to phylogenetic analysis. All Hzo sequences showed high similarity (> 69.7% amino acid similarity) to the translated *hzo* genes in *hzo* cluster 1 (Schmid et al. 2008). In general, Hzo sequences found throughout the NRE formed two clusters designated "Jettenia" and "Scalindua" clusters, dominated by "Jettenia-like" anammox.

A discernible spatial pattern of anammox sequences was not observed in the NRE. Most sequences detected in the upper and mid estuary sites as well as the M15 winter sample were assigned to the Jettenia cluster as they showed >88.9% Hzo amino acid sequence similarity with the *Ca.* "Jettenia" enrichment culture clone ANAHZOf (Figure 4). A combined group of sequences from the upper and mid estuary site samples collected in summer and winter, as well as lower estuary (M15) winter sequences, closely associated with the Hzo sequences detected in North Carolina aquifers, freshwater sites of Cape Fear River Estuary (Hirsch et al. 2011) and the AnaHZO3 sequence from an activated sludge reactor (Quan et al. 2008). One sequence related to the Hzo sequence of a Kuenenia enrichment culture was found in the M53 summer sample. Hzo sequences from M31 winter and M15 summer samples formed the Scalindua cluster, with >88.5% similarity to the Hzo sequence of *Ca.* "Scalindua sp.", Mai Po Mangrove (Li et al. 2010), South China Sea (Hong et al. 2011), and Jiaozhou Bay (Dang et al. 2010)

A sharp change of anammox community composition was observed at the M15 site and corresponded to changes in environmental conditions in the NRE. The M15

sediment sequences clustered with the Scalindua Hzo sequences during the summer when the estuary was experiencing low-flow conditions. During the winter sampling, a higher diversity of phylotypes was seen at this site, all closely related to *Ca* "Jettenia spp". During the winter, the estuary experienced high freshwater inputs that led to elevated concentrations of NO_3^- throughout the estuary (Table 2). These nutrient data in conjunction with the Hzo sequences suggest that a winter flushing of the NRE brought anammox bacteria present at upper estuary sites, throughout the estuary to the most saline site, during which potential activities increased suggesting the presence of Jettenia in these sediments led to greater nitrate removal. The *hzo* gene abundance and potential anammox activities at sites where *Ca* "Jettenia spp." dominated were positively correlated ($r=0.635$) although the observed relationship was not significant ($p > 0.05$).

Overall, non-Scalindua organisms were shown to be the dominant anammox bacterial group in the NRE. The Hzo sequences typically associated with Jettenia like sequences found in fresh and oligohaline environments were widely distributed throughout the NRE. Additionally, anammox communities in the lower estuary site, M15, showed unique seasonal changes from Scalindua to Jettenia like organisms that have not yet been observed in other studies.

Determination of Environmental Factors that Influence Anammox Community Composition

Anammox Hzo sequences were further analyzed to determine the environmental parameters influencing community composition. The Unifrac analysis enabled comparison of phylogenetic differences in anammox communities detected in the NRE

through the application of principal coordinates analysis (PCA). PCA explained 90.83% of the variation observed in NRE anammox communities (data not shown). PC 1 (x-axis) explained 82.27% of the variation in communities while PC 2 (y-axis) accounted for 8.56% of the variation in communities. Communities appeared to form two distinct clusters that were not significantly different from one another following the Bonferroni correction ($p>0.05$), likely due to the highly conserved *hzo* gene sequences between anammox groups. However, the PCA clearly demonstrated a difference among the sites that were dominated by Jettenia like and Scalindua like organisms.

Non-metric multidimensional scaling analysis (MDS) was applied to the community structure matrix generated by Unifrac and compared with environmental parameters (Figure 5). This enabled the visualization of the influence of environmental parameters on the anammox communities in the NRE. The strongest relationship between the anammox community structure and environmental parameters was observed with salinity ($p = 0.054$, $R^2 = 0.6274$). Relationships between anammox community composition and environmental parameters were not deemed to be significant, likely due to a lower sample number for the sequence analysis in this study. However, weak relationships between salinity, porewater H_2S, and sediment %organics with anammox community composition are consistent with observations from anammox activity and abundance analyses.

DISCUSSION

Potential activities of denitrification and anammox and %anammox observed in the NRE were dynamic but consistently higher at fresh to oligohaline sites and especially elevated during the spring. Rates and %anammox observed in this study were on the same order of magnitude as those reported in other shallow coastal ecosystems that also describe upstream rate maxima (Thamdrup & Dalsgaard 2002, Trimmer et al. 2003, Risgaard-Petersen & Meyer 2004, Meyer et al. 2005, Rich et al. 2008, Koop-Jakobsen & Giblin 2009, Nicholls & Trimmer 2009, Dale et al. 2009). In these studies, spatial differences were attributed to availability of NO_3^- and NO_2^- in the suboxic zone of sediments with higher activity measurements in upper estuary sites (Trimmer et al. 2003, Risgaard-Petersen & Meyer 2004, Meyer et al. 2005, Rich et al. 2008, Koop-Jakobsen & Giblin 2009, Nicholls & Trimmer 2009). Anammox and denitrification in NRE also showed temporal variability in the NRE with higher activities in spring, consistent with other aquatic studies (Risgaard-Petersen & Meyer 2004, Hietanen & Kuparinen 2007, Minjeaud et al. 2009).

In this study, the positive relationship of anammox activities and abundance with sediment %organics and porewater H_2S demonstrates an interplay among environmental conditions, bacterial community structure and activities. Hu et al. (2011) reported a dominance of Brocadia and Kuenenia in in the Qiantang River, China and also demonstrated a positive correlation between sediment organic carbon and anammox bacteria, where organic carbon significantly influences anammox bacterial diversity. Dang et al. (2010) also showed sediment organic material was among the most influential environmental parameters related to the distribution of anammox bacteria in the

hypernutrified Jiaozhou Bay, China. The positive correlation of sediment %organics with anammox activities and abundance, in particular to Jettenia-like organisms, might indicate that anammox bacteria can use organic matter as an electron donor or carbon source. This proposal is supported by previous studies where anammox bacteria were able to anaerobically remove ammonia in the presence of organic matter in bioreactors (Guven et al. 2005, Sabumon 2007). The ability of anammox bacteria to use organic acids such as acetate and propionate as supplementary carbon sources has been demonstrated in *Ca* "K. stuttgartiensis", *Ca* "A. propionicus", and *Ca.* "B. fulgida" enrichment cultures (Strous et al. 2006, Kartal 2008). This study provides further support that anammox bacteria may be capable of capitalizing on organic carbon, which may be of particular interest in the future when examining anammox bacteria in organic rich estuarine systems.

An alternative explanation to the relationship of anammox with sediment %organics includes the positive association with porewater H_2S and points to a coupling of the nitrogen and sulfur cycles in the New River Estuary. Micromolar concentrations of H_2S were initially reported to inhibit activity of anammox (Jensen et al. 2008) possibly due to direct inhibition (Dalsgaard et al. 2003). However a more recent study examining the influence of H_2S by Wenk et al. (2013) suggests otherwise. Several modes of fixed nitrogen removal were examined in the water column of an alpine lake. Anammox activities were shown to coincide with sulfide-dependent denitrification and were even enhanced with the addition of H_2S in incubations. It has been suggested that NH_4^+ for the anammox process may be provided by sulfate (SO_4^{2-}) reduction coupled to organic matter oxidation, as was observed in Chilean waters where an active coupling of

the sulfur and nitrogen cycles was observed in a NO_3^- rich, oxygen free zone (Canfield et al. 2010). This coupling was proposed to generate up to 22% of the NH_4^+ necessary to support observed activities of anammox in this oxygen depleted-system.

Although not explicitly examined in this study, anammox communities in the NRE may be supported through remineralization of organic matter by sulfate reducing communities in these sulfide rich sediments. Elevated levels of H_2S were observed in the mesohaline reaches of the estuary at the mid-estuary stations concurring with a rich supply of sediment %organics and higher potential activities. A coupling between anammox and other nitrogen cycling processes, in particular denitrification and dissimilatory nitrate reduction to ammonia (DNRA) might also explain the correlation between anammox activities, H_2S and %organics. Under the incubation conditions used in this study, intermediates produced during heterotrophic denitrification driven by carbon availability or chemolithotrophic denitrification by H_2S observed in some aquatic systems (Cardoso et al. 2006) may provide the substrate that supports anammox via NO_2^- production. Likewise, hydrogen sulfide or organic matter could provide reducing power for the reduction of NO_3^- to NH_4^+ via DNRA and would also cause a dependency of anammox on another respiratory processes for substrate. This dependency of anammox on denitrification or DNRA for substrate might be driving the observed correlation of anammox with %organics and H_2S observed in the NRE.

The positive correlation between denitrification and porewater H_2S still suggests that a coupling between the nitrogen and sulfur cycles might exist in the NRE as denitrifying bacteria are capable of using reduced sulfur species as electron donors for the reduction of NO_3^-. Given the fact that anammox activities were observed in the presence

of high levels of H_2S and organic matter, the results suggest that these environmental parameters do not inhibit anammox or denitrification as previously thought. Additionally, DNRA is an important mechanism for retaining N within saline systems such as estuaries (Koike & Hattori 1978, Tobias et al. 2001, Giblin et al. 2013) and it may be of interest in future studies to examine the relationship between DNRA and anammox in estuarine sediments.

Studies of anammox bacterial communities in estuarine sediments generally report the dominance of Scalindua-like organisms as described in the Chesapeake Bay, USA (Rich et al. 2008), Yodo River Estuary, Japan (Amano et al. 2007), Jiaozhou Bay, China (Dang et al. 2010), and the CFRE, USA (Dale et al. 2009, Hirsch et al. 2011). While Scalindua-like groups were detected in the lower reaches of the NRE, *Ca.* "Scalindua spp." were not the dominant anammox bacteria in the NRE. Jettenia-like anammox dominated 71% of the clones obtained seasonally throughout the NRE. This is the first time that an estuarine environment was shown to have a dominance of Jettenia-like anammox. Previous studies of anammox activity measurements in batch incubations containing *Ca* "Brocadia anammoxidans" or *Ca* "Kuenenia stuttgartiensis" resulted in activities four times higher than *Ca* "Scalindua spp." enrichments under similar conditions (Schmid et al. 2003). The presence of *Ca* "Jettenia spp." and the increase in activities at M15 coinciding with the shift away from *Ca* "Scalindua spp." at this site may provide some evidence to support the differences in nitrogen removal efficiency of anammox groups in a natural system. This notion may have implications for nitrate removal in estuaries that tend to favor one group over another, particularly in organic rich, high nutrient systems such as the NRE.

Finally, as mentioned earlier, the degree of meteorological forcing has been shown to govern biological activities in estuarine systems (Peierls et al. 2012). In this study, a dramatic increase of freshwater discharge during the winter sampling event supported bottom up effects on anammox communities in the NRE. During this freshening event, anammox community structure in the downstream site M15 shifted to closely resemble upstream areas of the estuary. Two possible mechanisms may be responsible for this occurrence; the advection of upstream anammox organisms to the lower estuary or changes in environmental conditions as a result of the long term freshening of the estuary throughout the winter enabled small population of *Ca* "Jettenia spp." anammox to become dominant. It is still unclear how to determine if dispersal or environmental conditions affect composition of microbial communities due to the covariance of changing environmental conditions during rainfall events responsible for advection of microbes (Crump et al. 2007). Thus, the species sorting perspective can be used to provide an explanation for the presence and dominance of *Ca* "Jettenia spp." throughout the NRE. The species sorting perspective emphasizes the control of environmental conditions on community structure and dispersal (Leibold et al. 2004, Crump et al. 2007). This is important because it sets the community from which the dominant assemblage will develop. In the case of the NRE, *Ca* "Jettenia spp." may have been transported throughout the estuary and favorable environmental conditions allowed for the proliferation of the bacteria. Increased substrate availability and a shift in community structure allowed for higher anammox activity at this site and further support the linkage between environmental parameters, anammox community composition and activities in an estuarine system.

CONCLUSIONS

Anammox and denitrification activities were detected throughout the 7 sites during all seasons and denitrification was the dominant N_2 producing pathway in the NRE. Anammox contributed up to 14% of total N_2 production and activities were positively correlated with abundance of the *hzo* genes, both of which were positively correlated with porewater H_2S concentrations and %organics. These correlations can be explained by the ability of anammox bacteria to use organic material as a carbon substrate or through the coupling of the nitrogen, sulfur and carbon cycles. Phylogenetic analysis of Hzo sequences revealed that Jettenia-like organisms were dominant throughout the NRE. A freshening of the estuary during the winter distributed upstream anammox communities to downstream sties and increased anammox activities. This study provides evidence that an episodic flushing event may have influenced the widespread distribution of Jettenia-like organisms, and together with the geochemical conditions of the system governed anammox bacterial community structure and activities in a temperate estuarine ecosystem.

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Table 1. Environmental parameters of New River Estuary bottom water. Missing data points not determined at the time of sampling are indicated by "n.d.".

Site	Temperature (°C)				Salinity				Nitrate (μM)				Ammonium (μM)				Dissolved Oxygen (mg/L)			
	June	Sept	Dec	April	June	Sept	Dec	April	June	Sept	Dec	April	June	Sept	Dec	April	June	Sept	Dec	April
AA2	28.8	24.0	n.d	21.2	2.2	9.3	0.1	9.1	0.4	n.d	68.6	0.4	9.1	n.d	2.5	7.8	0.2	0.9	6.4	0.6
Jax	27.7	25.1	n.d	21.2	12.3	13.6	5.7	17.8	0.6	n.d	13.3	0.3	2.8	n.d	14.9	0.8	3.0	2.2	7.0	4.1
M53	26.9	24.5	n.d	21.1	13.2	14.1	6.6	18.6	n.d	n.d	9.0	0.2	n.d	n.d	14.7	1.0	4.0	4.7	7.0	3.4
M47	27.3	22.7	11.8	19.9	18.8	13.3	5.2	16.4	0.7	n.d	13.8	0.4	1.3	n.d	14.8	1.2	4.4	7.5	10.2	7.9
M39	27.2	23.4	11.7	20.0	20.8	16.6	10.2	22.0	1.1	n.d	9.3	0.3	1.9	n.d	9.3	0.9	3.0	7.0	9.5	7.9
M31	27.8	23.8	n.d	19.2	23.3	20.5	12.3	27.0	0.2	n.d	6.4	0.5	0.5	n.d	9.4	1.5	n.d	6.2	7.4	7.6
M15	28.1	23.1	17.0	17.8	31.0	29.3	29.8	33.6	0.7	n.d	0.7	0.6	5.4	n.d	1.1	1.3	n.d	7.1	6.5	8.2

Table 2. New River Estuary sediment porewater characteristics. Missing data points not determined at the time of sampling are indicated by "n.d.".

Site	Ferrous Iron (μM)				Hydrogen Sulfide (μM)				Nitrate + Nitrite (μM)			
	June	Sept	Dec	April	June	Sept	Dec	April	June	Sept	Dec	April
AA2	1.92	n.d	5.49	59.00	11.7	n.d	16.4	0.9	0.37	n.d	0.21	0.26
Jax	9.84	n.d	1.71	3.03	973.4	n.d	800.7	486.9	0.58	0.07	2.37	0.23
M53	6.99	n.d	1.20	4.28	544.1	n.d	1100.4	829.9	0.58	0.00	0.16	0.34
M47	0.67	n.d	1.36	1.46	773.9	n.d	398.6	249.9	0.16	0.02	0.07	0.54
M39	2.43	n.d	1.11	1.56	734.3	n.d	667.1	54.3	0.01	0.28	0.11	0.91
M31	4.35	n.d	20.62	70.79	13.8	n.d	5.3	3.2	0.03	0.06	0.07	0.33
M15	1.56	n.d	3.72	1.55	0.7	n.d	1.1	0.2	0.33	0.00	2.15	0.72

Table 3. New River Estuary sediment characteristics. Missing data points not determined at the time of sampling are indicated by "n.d.".

Site	Organics (%)				Ammonium (μmol (g sed)$^{-1}$)				Benthic Chlorophyll a (μg chl a (g sed)$^{-1}$)				Sediment Grain Size (μm)			
	June	Sept	Dec	April	June	Sept	Dec	April	June	Sept	Dec	April	June	Sept	Dec	April
AA2	3.21	n.d	2.96	15.57	0.14	n.d	0.20	0.06	0.00	1.83	0.00	7.75	35.8	121.9	344.1	23.5
Jax	14.71	n.d	22.96	17.98	0.36	n.d	1.24	0.21	5.98	3.54	4.68	4.34	35.7	75.7	54.7	105.7
M53	17.17	n.d	18.46	20.69	0.42	n.d	1.22	0.33	5.17	0.00	14.54	0.00	24.2	37.7	30.3	17.9
M47	14.66	n.d	17.62	18.95	0.50	n.d	1.04	0.28	4.60	3.38	13.48	5.43	19.4	30.8	58.6	38.2
M39	11.66	n.d	11.46	12.29	0.45	n.d	0.47	0.24	4.25	0.00	25.41	8.32	29.1	34.8	30.6	35.8
M31	9.21	n.d	9.51	10.19	0.19	n.d	1.21	0.20	4.92	6.46	17.35	8.97	28.3	41.9	33.9	36.0
M15	0.34	n.d	0.13	0.33	0.12	n.d	0.13	0.04	4.03	4.98	7.84	5.36	181.8	244.8	251.4	291.0

Table 4. Potential N_2 production in the New River Estuary. ^{15}N isotope pairing experiments were conducted to measure (A) denitrification, (B) anammox, and (C) percent anammox.

A

Site	Potential Denitrification Rates (nmoles $^{30}N_2$ g^{-1} wet sed hr^{-1})							
	June	S.E.	Sept	S.E.	Dec	S.E.	April	S.E.
AA2	4.29	0.43	11.43	0.07	5.06	0.10	4.66	0.11
JAX	9.92	0.91	6.14	4.10	16.20	2.12	12.13	0.95
M53	7.27	0.30	1.79	0.06	14.13	0.15	30.98	3.00
M47	9.91	3.00	3.37	0.45	0.89	0.20	9.37	0.31
M39	4.63	1.35	3.40	0.47	16.08	0.74	21.29	7.11
M31	3.10	0.01	4.15	0.03	11.21	1.67	5.41	0.27
M15	0.45	0.05	2.16	0.27	1.77	0.13	0.36	0.10

B

Site	Potential Anammox Rates (nmoles $^{29}N_2$ g^{-1} wet sed hr^{-1})							
	June	S.E.	Sept	S.E.	Dec	S.E.	April	S.E.
AA2	0.35	0.02	0.86	0.03	0.55	0.00	0.76	0.05
JAX	0.80	0.09	0.26	0.17	0.89	0.14	1.05	0.18
M53	0.48	0.00	0.07	0.00	0.70	0.06	1.40	0.41
M47	0.68	0.16	0.08	0.01	0.02	0.00	0.61	0.03
M39	0.15	0.05	0.07	0.01	0.55	0.10	0.50	0.22
M31	0.08	0.00	0.07	0.00	0.42	0.08	0.31	0.02
M15	0.03	0.00	0.10	0.01	0.11	0.01	0.02	0.00

C

Site	Anammox (%)							
	June	S.E.	Sept	S.E.	Dec	S.E.	April	S.E.
AA2	7.61	0.23	7.02	0.18	9.78	0.20	14.08	1.01
JAX	7.43	0.13	4.11	0.07	5.20	0.13	7.96	0.65
M53	6.16	0.20	3.91	0.04	4.74	0.37	4.29	0.81
M47	6.51	0.41	2.26	0.01	1.98	0.38	6.11	0.07
M39	3.10	0.14	2.14	0.02	3.30	0.41	2.27	0.24
M31	2.49	0.04	1.66	0.01	3.62	0.10	5.41	0.12
M15	7.26	0.19	4.41	0.06	4.75	0.30	6.29	1.34

Table 5. Abundance of anammox bacteria in New River Estuary sediments. Quantitative-PCR assays targeting *hzo* genes were conducted on summer and winter samples. Standard error (SE) for triplicate samples is shown.

Site	*hzo* (copy number g^{-1} wet sed)			
	June	SE	Dec	SE
AA2	2.40E+05	7.12E+04	6.57E+04	1.17E+04
Jax	2.54E+05	5.94E+04	2.09E+05	3.27E+04
M53	1.20E+05	1.56E+04	1.69E+05	7.46E+04
M47	2.45E+05	7.85E+04	1.92E+05	3.32E+04
M39	2.59E+05	9.30E+04	2.16E+05	3.61E+04
M31	1.22E+05	6.10E+04	2.42E+05	6.65E+03
M15	1.55E+02	2.94E+00	7.48E+03	3.43E+03

Figure 1. Sampling sites in the New River Estuary, NC, USA. Seven sites were examined and included upper estuary sites (AA2, Jax and M53), mid-estuary sites (M47 and M39), and lower estuary sites (M31 and M15).

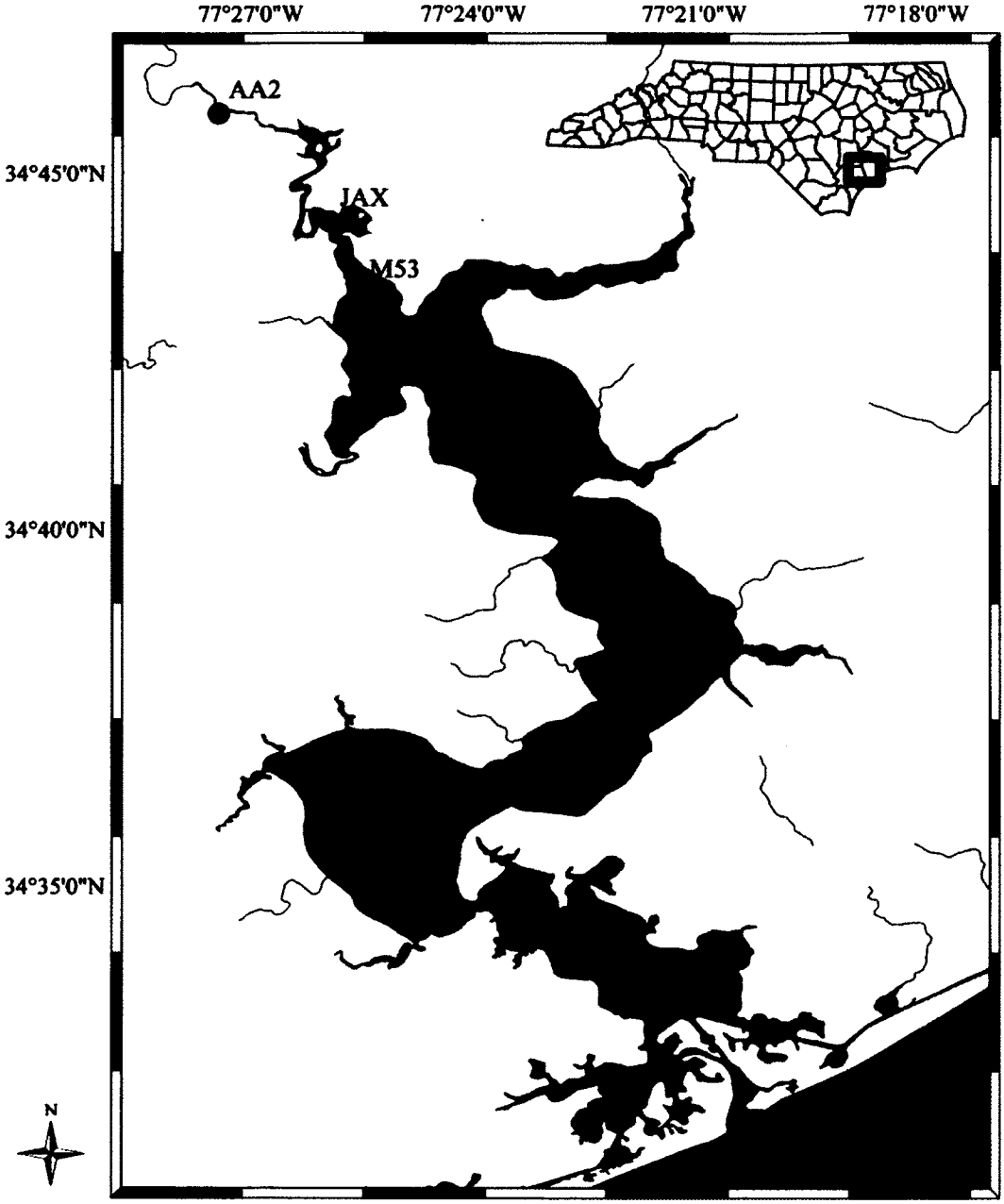

77°27'0"W
77°24'0"W
77°21'0"W
77°18'0"W
34°45'0"N
34°40'0"N
34°35'0"N
AA2
JAX
M53
N

Figure 2. Seasonal comparison of rates of denitrification and anammox, and %anammox in the New River Estuary. Potential N_2 production of (a) denitrification, (b) anammox and (c) %anammox. Samples are oriented from headwaters to mouth of the estuary on the x-axis. Error bars represent standard error.

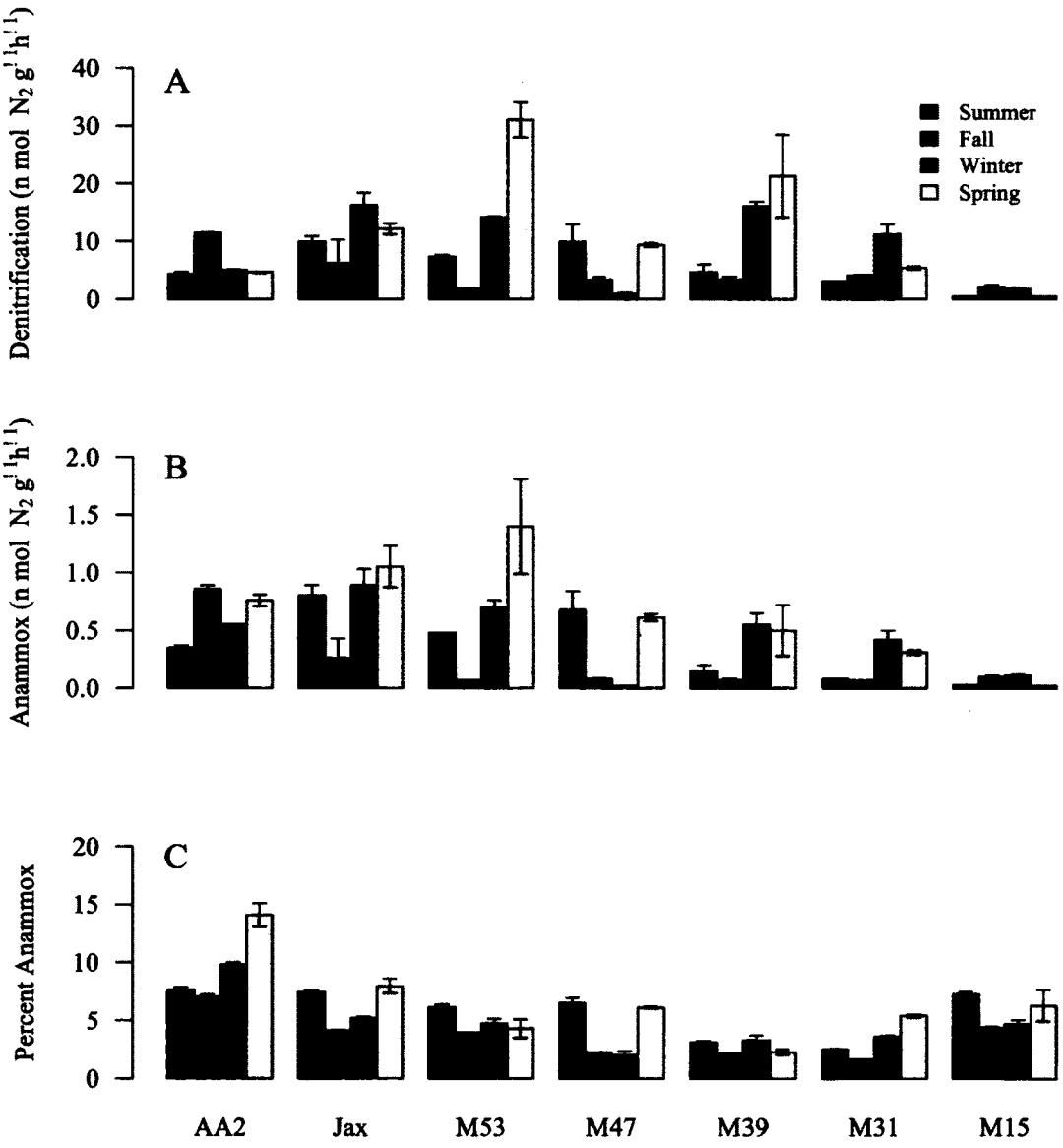

A
B
C
Summer
Fall
Winter
Spring
Denitrification (n mol N_2 $g^{-1}h^{-1}$)
Anammox (n mol N_2 $g^{-1}h^{-1}$)
Percent Anammox
40
30
20
10
0
2.0
1.5
1.0
0.5
0.0
20
15
10
5
0
AA2
Jax
M53
M47
M39
M31
M15

Figure 3. Quantification of *hzo* genes in New River Estuary sediment communities using quantitative-PCR assays. Assays were conducted on summer and winter samples indicated by black and dark gray bars, respectively. Error bars represent standard error.

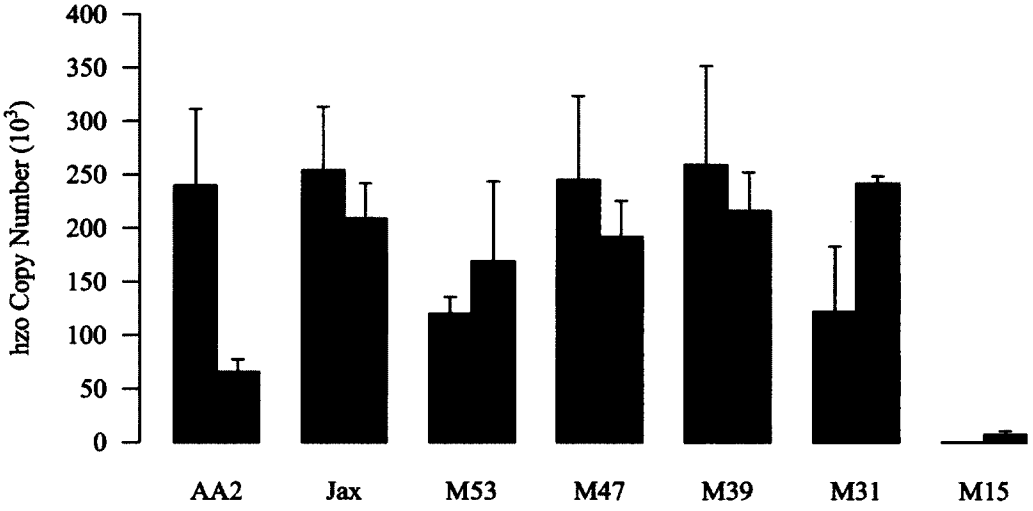
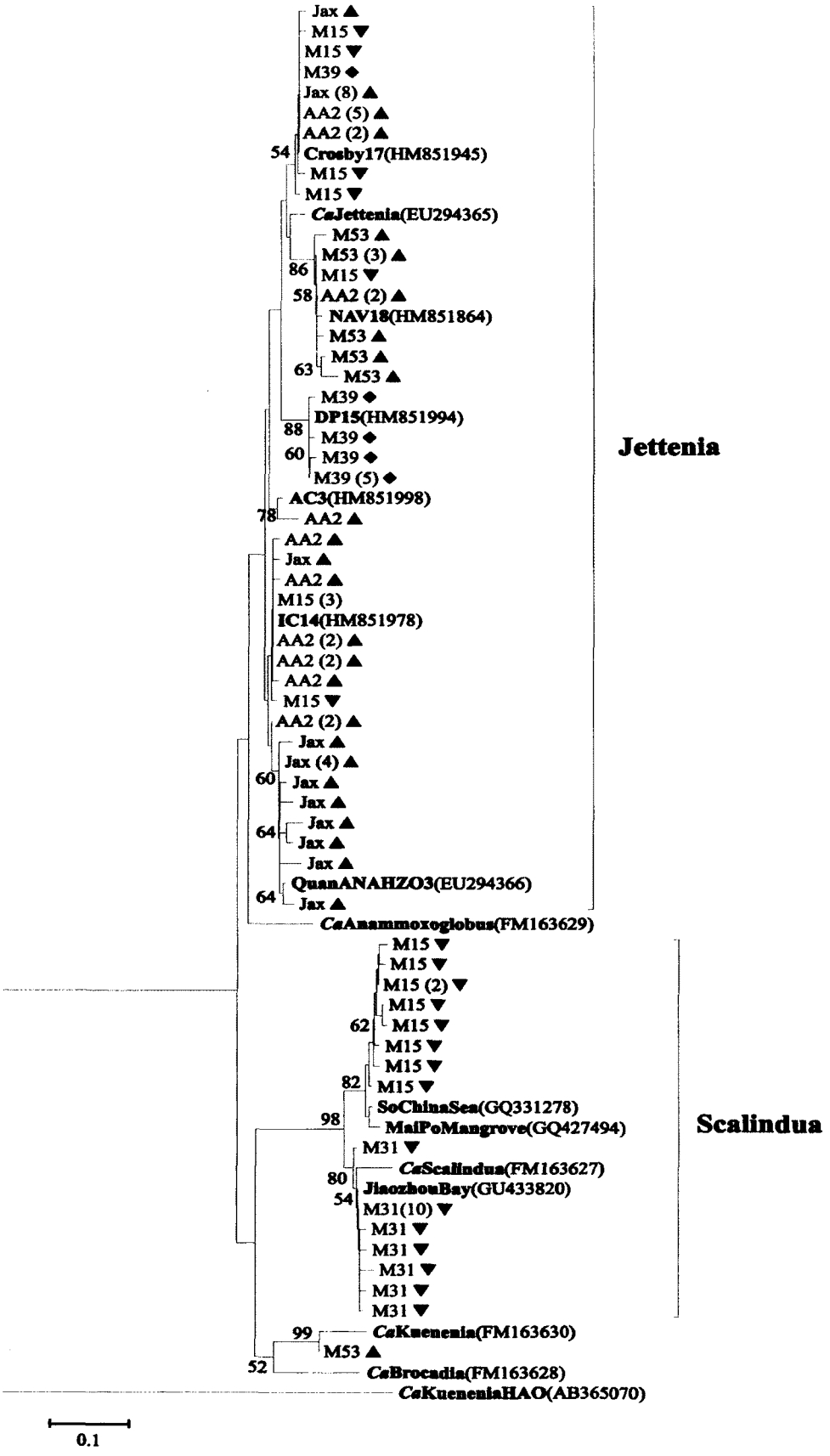

400
350
300
250
200
150
100
50
0
hzo Copy Number (10^3)
AA2
Jax
M53
M47
M39
M31
M15

Figure 4. Phylogenetic tree of translated *hzo* gene sequences depicting seasonal variation of anammox communities in New River Estuary sediments. Neighbor-joining trees were constructed and Bootstrap analysis with 1000 replicates was used to estimate confidence. The outgroup sequence is the translated *Ca.* "Kunenia sp." *hao* gene sequence. Bootstrap values of >50% (from 1,000 replicates). Clusters are marked by brackets and labeled according to *Candidatus* species most closely related. Sequences with 100% identity detected more than one time are indicated by the number of times detected in parentheses. New River sites are labeled according to the site names and a symbol denoting location in the estuary; an upward facing triangle indicates upper estuary sites (AA2, Jax and M53), a diamond is used for mid-estuary sites (M47 and M39), and a downward facing triangle represents downstream sites (M31 and M15). Labels are also colored by season; summer (black) and winter (dark gray).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M15 ▼
M15 ▼
M39 ◆
Jax (8) ▲
AA2 (5) ▲
AA2 (2) ▲
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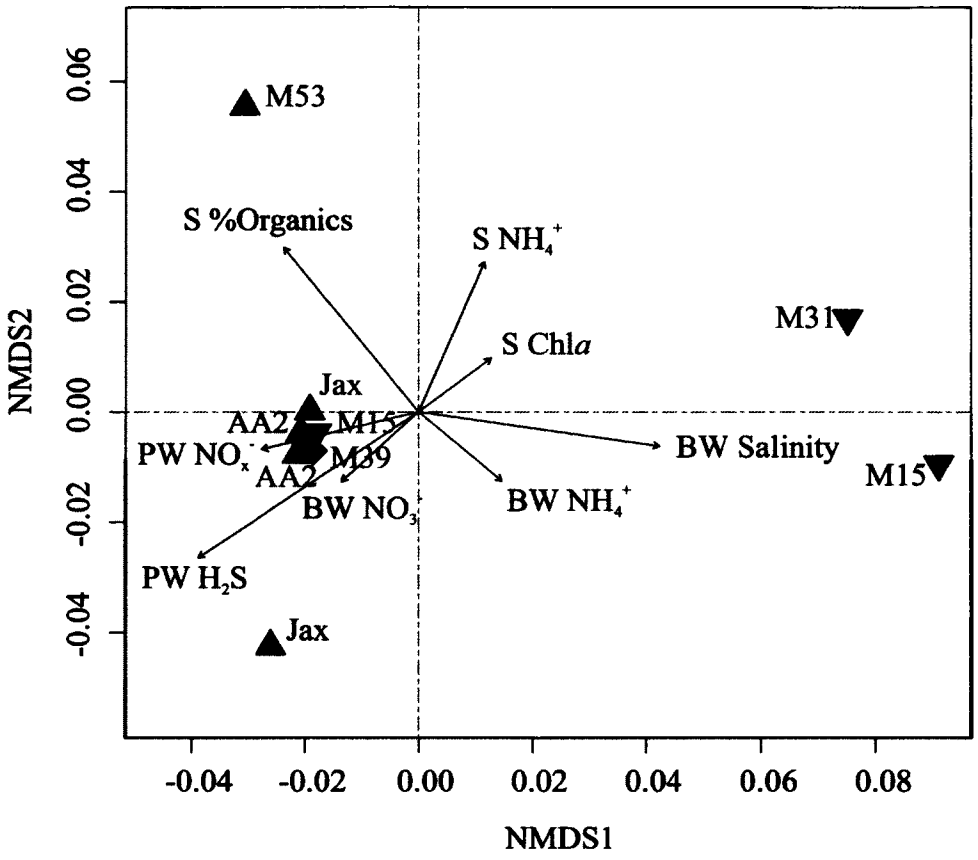
M15 ▼
M15 ▼
CsJettenia(EU294365)
M53 ▲
M53 (3) ▲
86
M15 ▼
58
AA2 (2) ▲
NAV18(HM851864)
M53 ▲
M53 ▲
63
M53 ▲
M39 ◆
DP15(HM851994)
88
M39 ◆
60
M39 ◆
M39 (5) ◆
AC3(HM851998)
78
AA2 ▲
AA2 ▲
Jax ▲
AA2 ▲
M15 (3)
IC14(HM851978)
AA2 (2) ▲
AA2 (2) ▲
AA2 ▲
M15 ▼
AA2 (2) ▲
Jax ▲
Jax (4) ▲
60
Jax ▲
Jax ▲
64
Jax ▲
Jax ▲
Jax ▲
QuanANAHZO3(EU294366)
64
Jax ▲
CsAnammoxoglobus(FM163629)
Jettenia
M15 ▼
M15 ▼
M15 (2) ▼
M15 ▼
62
M15 ▼
M15 ▼
82
M15 ▼
SoChinaSea(GQ331278)
98
MaiPoMangrove(GQ427494)
M31 ▼
CsScalindua(FM163627)
80
JiaozhouBay(GU433820)
54
M31(10) ▼
M31 ▼
M31 ▼
M31 ▼
M31 ▼
M31 ▼
Scalindua
99
CsKuenenia(FM163630)
M53 ▲
52
CsBrocadia(FM163628)
CsKueneniaHAO(AB365070)
0.1

Figure 5. Non-metric multidimensional scaling analyses comparing anammox community structure with environmental parameters. Anammox community structure was based on Hzo sequence dissimilarities. Analyzed environmental parameters include bottom water (BW), salinity (psu), NO_3^- (μM), NH_4^+ (μM); porewater (PW) H_2S (μM), NO_x^- (μM); and sediment (S) %organics, extractable sediment NH_4^+ (μmol NH_4^+ g^{-1} wet sediment), benthic Chlorophyll *a* (μg chl a g sed^{-1}).


M53
S %Organics
S NH_4^+
M31
S Chl*a*
Jax
AA2
M15
PW NO_x^-
M39
AA2
BW Salinity
M15
BW NO_3^-
BW NH_4^+
PW H_2S
Jax
NMDS2
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.04
-0.02
0.00
0.02
0.04
0.06
0.08
NMDS1

CHAPTER 3

Denitrification and anammox hotspots in the New River Estuary, North Carolina, USA

ABSTRACT

Biogeochemical hotspots are characterized by a few sites that exhibit extremely high reaction rates relative to surrounding area and often account for a high percentage of the overall reaction rates in an ecosystem. However, criteria for quantitatively identifying biogeochemical hotspots have not been well established. Further, the underlying mechanisms of hotspots have primarily been described in terms of environmental conditions with little attention paid to the microbial community structure. The objectives of this study were to establish quantitative criteria for determining biogeochemical hotspots, use those criteria to identify denitrification and anammox hotspots in an estuarine system, and determine the underlying microbial and environmental factors responsible for the production of such anonymously high activities. We used ^{15}N isotope pairing incubation experiments to measure rates of denitrification and anammox in The New River Estuary, NC, a shallow, microtidal estuarine system. The hotspots were quantitatively defined as statistical outliers, which were identified using Tukey's method of outlier detection. Denitrification hotspots accounted for 35.6% total denitrification while comprising only 7.3% of the sampling sites. Anammox hotspots made up 10.6% of the sampling sites and accounted for 60.9% of anammox N_2 production. Quantification of the nitrous oxide reductase (*nosZ* Clades I and II) and hydrazine oxidoreductase (*hzo*) genes, conducted using real time PCR assays, revealed higher than predicted activities at hotspots given the functional gene abundance. Elevated substrate availability at lower salinities and the absence of inhibitors were largely associated with hotspots. This is the first time that a quantitative definition of biogeochemical hotspots was put forth and used to determine the importance of anammox and denitrification hotspots in nitrogen removal capacity at the estuarine ecosystem level. Despite the low area coverage, denitrification and anammox hotspots are major nitrogen removal components and may play a significant role in mitigating the intensity and duration of eutrophication in the estuary.

INTRODUCTION

When complementary or missing environmental factors converge, areas of anomalously high biogeochemical activities occur, often referred to as "hotspots" (McClain et al. 2003, Burt et al. 2010). Biogeochemical hotspots were originally characterized as "a few samples that exhibit extremely high rates" and "skew sample frequency distributions" (Parkin 1987). More recently, McClain et al. (2003) described hotspots as "patches that show disproportionately high reaction rates relative to the surrounding matrix". However, a clear definition of a biogeochemical hotspot is unavailable, as are criteria used to identify hotspots in a given system. Statistical outliers are observations far away from other data points that can be indicative of the inherent variability of biogeochemical activities within a natural system; therefore, tests to identify statistical outliers can be used to quantitatively identify biogeochemical hotspots.

Biogeochemical hotspots often account for a high percentage of the overall fluxes in a system and in the case of denitrification and anammox, make a significant contribution to the overall nitrogen loss in an ecosystem (Groffman 2012). Denitrification and anammox are two microbial nitrogen removal processes that can control the intensity and duration of estuarine eutrophication in response to high nitrogen loading (Costanza et al. 1997, Seitzinger et al. 2006). Both microbial processes occur simultaneously in estuarine sediments and are influenced by a suite of environmental factors that include oxygen, nitrogen availability, organic carbon and hydrogen sulfide (Cornwell et al. 1999, Dalsgaard et al. 2005, Seitzinger et al. 2006). These environmental factors exhibit complex interactions and vary spatially as well as temporally within a system, which may lead to "hotspots" and "hot moments" regulating the magnitude of N_2 producing activities

in a particular place at any given moment. For example, in the terrestrial environment, 0.4 to 0.8% of the area may account for 25 to 85% of the overall denitrification N_2 flux in soils (Lowrance et al. 1984, Peterjohn & Correll 1984).

The underlying mechanisms responsible for biogeochemical hotspots are often portrayed in terms of environmental stimulation of activities, with little mention of the microbial communities responsible for mediating the processes. Denitrification hotspots in terrestrial systems were associated with organic carbon availability (Parkin 1987). Groffman et al. (2009) describes aquatic systems as a general area for denitrification hotspots due to the high substrate availability, elevated organic matter and suboxic conditions. Similarly, transitional areas between two adjacent ecosystems have long been regarded as ideal locations for biogeochemical hotspots due to the convergence of complimentary or missing reactants at these ecotones (McClain et al. 2003, Groffman et al. 2009, Zhu et al. 2013). As such, anammox hotspots were observed at the land-freshwater interface of a lake riparian zone (Zhu et al. 2013). An increase in anammox bacterial abundance and a decrease in overall biodiversity accompanied the elevated anammox activities at the land-freshwater interface. Specific anammox cell activity was also highest at hotspots suggesting that microbial abundance alone was not responsible for elevated anammox activities. This highlights the importance of the role that microbial communities, in conjunction with environmental parameters, may play in the occurrence of hotspots.

The goals of this study were to: 1) Provide quantitative definition of biogeochemical hotspots based on activity measurements; 2) Identify hotspots of denitrification and anammox using this newly established criteria; 3) Estimate the

importance of hotspots in fixed nitrogen removal in an estuary; and 4) Determine the biological and environmental parameters influencing activities of denitrification and anammox in hotspots. We conducted sediment sampling from 12 transects distributed throughout the estuary and encompassing a total of 62 sites. These sites were revisited during summer and winter in the New River Estuary (NRE), North Carolina, USA. Biogeochemical and molecular techniques were used in conjunction with environmental monitoring to gain a complete picture of the environmental parameters driving hotspot activities and the role community structure may play.

MATERIALS AND METHODS

Seasonal Sampling of the NRE

Sampling was conducted during June (summer) and December (winter) of 2009 along a salinity gradient from the headwaters to the mouth of the New River Estuary (NRE), NC, USA (Figure 1A). Twelve markers were established at AA, JAX, M53, M50, M47, M43, M39, M36, M31, 172, M18, M15. Transect sampling across each marker, from the most western to the most eastern banks, was conducted at a total of 62 sites (Table 1). All sites were visited during both summer and winter with one exception (39 WB during winter due to live fire training at the military base).

Environmental Parameter Measurements

Environmental parameters including water column depth, temperature, salinity and dissolved oxygen were measured in surface and bottom waters (BW) at the time of sampling using a 6820 multi parameter YSI data sound (YSI Incorporated, Yellow

Springs, OH; Table 2). Samples were filtered through both 2.7 μm and 0.7μm glass microfiber filters (respectively GF/D and GF/F Whatman Ltd., Piscataway, NJ 08854 USA) and stored on ice for nutrient analysis. Bottom water ammonium and nitrate concentrations were measured colormetrically on the Bran Luebbe segmented flow nutrient autoanalyzer using phenol hypochlorite and Cd-reduction/azo dye methods, respectively, following standard Environmental Protection Agency operating procedures (Long & Martin 1997).

Sediment (S) samples were collected using a petite ponar grab (Wildeco, Buffalo NY). The top 2 cm of sediment was collected in 50 mL falcon tubes (BD Biosciences Franklin Lakes, NJ) and stored in ice water to minimize metabolic activity. Upon arrival to the laboratory, pore water was immediately extracted by centrifugation. Ferrous iron (Fe^{2+}) concentration in porewater was measured using the method of Stookey (1970) (Table 3). Hydrogen sulfide (H_2S) concentration in porewater was determined using the spectrophotometric method as described by Cline (1969). Porewater combined nitrate and nitrite (NO_x^-) was measured reductively using Vanadium (III) and chemiluminescent detection developed by Braman & Hendrix (1989).

Sediment percent organics (% organics) in was calculated by loss on ignition after sediments were baked at 500°C for 4 hours (Table 4). Total ammonium (NH_4^+) in sediments was measured after KCl extraction following Mackin & Aller (1984). Benthic chlorophyll *a* analysis was conducted with 1 gram of surface sediment as described by Whitney & Darley (1979). Mean sediment grain size was determined after organic material was dissolved with 35% H_2O_2 and using LS 200 Beckman Coulter Particle Sizer. In addition, sediments from each site were stored at -80°C for molecular analyses.

^{15}N Tracer Incubations

Anaerobic sediment slurry incubation experiments with ^{15}N tracer were conducted to measure potential rates of denitrification and anammox as described by Dale et al. (2009). Two grams of homogenized wet sediment was distributed into Exetainer tubes, purged with helium and incubated in the dark overnight to remove residual nitrate (NO_3^-). The following day, the Exertainer tubes with sediment slurries were again purged with helium, amended with 100 nmoles $^{15}NO_3^-$ and 100 nmoles $^{14}NH_4^+$ per g of wet sediment, and placed in the dark at room temperature. Time series incubations were carried out in duplicate and the activity stopped by the addition of saturated $ZnCl_2$. Ambient NO_x^- was measured after overnight incubation as described above to correct for mole fraction ^{15}N enrichment. $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ production was measured on a Isotopic Ratio Mass Spectrometer (Delta V, Thermo Fisher Scientific) and converted to mass of nmoles N g^{-1} wet sediment hr^{-1} (Table 5) following the method of Thamdrup & Dalsgaard (2002) as modified by Song & Tobias (2011).

Quantitative PCR Assays of Functional Genes

A subset of the sites (64 samples in total) was examined for molecular analyses. Sediment DNA was extracted from select sites using PowerSoil DNA Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA) following the manufacture's protocol with the following modifications: 1) Wet sediment (0.6 g) was used for the extraction; 2) Thermo Savant Fast Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad CA) was used for cell disruption.

Thirty-two samples were analyzed for anammox and denitrifier gene abundance during each season (summer and winter). Real time PCR assays of the nitrous oxide reductase (*nosZ* Clades I and II) and hydrazine oxidoreductase (*hzo*) genes (Table 6). Assays were carried out in a volume of 20uL containing 9.0 to 15 ng of template DNA and SYBR green using Go-Taq qPCR Master Mix (Promega Corporation, Madison, WI). No-template and 8 positive controls were run for each assay in triplicates. Thermal cycling, fluorescent data collection, and data analysis were carried out using ABI Prism 7500 Real Time PCR System Sequence Detection System (Applied Biosystems, Carlsbad, CA). PCR specificity was monitored by analysis of dissociation curves. PCR efficiency was monitored and remained above 89% on average. The R^2 values for the standard curves were >0.987 for all runs.

Primers nosZ2F and nosZ2R targeting *nosZ* gene encoding for the catalytic subunit of nitrous oxide reductase Clade I designed by Henry et al. (2006) were used to quantify the abundance of denitrifying bacteria capable of N_2 production in NRE sediments. Thermal cycling conditions were as follows: an initial cycle of 95°C for 10 m; followed by 40 cycles of 95°C for 15 s, 55°C for 45 s, 72°C for 35 s, 80°C for 35 s (data acquisition step); and a final dissociation step of 95°C for 15 s, 55°C for 1 m, 95°C for 15 s, 60°C for 15 s.

The primers nosZ-II-F (50-CTI GGI CCI YTK CAY AC-30) and nosZ-II-R (50-GCI GAR CAR AAI TCB GTR C-30) were used to amplify the *nosZ* gene within Clade II according to the conditions described by Jones et al. (2013) with modifications. Briefly, thermal cycling conditions were as follows: an initial cycle of 95°C for 10 m; followed by 40 cycles of 95°C for 15 s, 53°C for 1 m, 72°C for 1 m, 80°C for 45 s (data

acquisition step); and a final dissociation step of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s.

Primers hzo1F and hzo1R, targeting *hzo* gene encoding for the hydrazine oxidoreductase designed by Long et al. (2013) were used to quantify the abundance of anammox bacteria in NRE sediments. Thermal cycling conditions for the hzo1 PCR were as follows: an initial cycle of 95°C for 10 m; 50 cycles of 95°C for 15 s, 53°C for 45 s, 72°C for 35 s, 75°C for 35 s (data acquisition step); and a dissociation step of 95°C for 15 s, 53°C for 1 m, 95°C for 15 s, 60°C for 15 s. qPCR assays were run for 7 of the 12 transects in duplicates.

Determination of Hotspots

Data were examined for normality using the Shapiro-Wilk Normality Test and did not meet the assumptions of a normal distribution. Log transformed data also did not meet the assumptions of statistical inference for parametric tests. Data were not log-transformed in order to retain sensitivity to variability in data that are lost during the transformation (Parkin 1987). Non-parametric tests were conducted. Kruskal-Wallis Rank Sum Test was conducted to determine temporal differences between denitrification and anammox activities and environmental parameters. Significant differences between summer and winter activities were not observed and the data for both seasons were pooled for downstream analysis. Due to the robust nature of the analyses, $\alpha < 0.05$ was retained to delineate significant relationships between all response and explanatory variables.

Denitrification and anammox hotspots were identified using Tukey's Boxplot Method for outlier detection. Observations beyond whiskers (Q3 + 1.5 x Inter Quartile

Range (IQR)), identified as outliers, were deemed to represent anomalously high activities and thus considered biogeochemical "hotspots". Using this quantitative definition we were able to identify sites where biogeochemical activities were abnormally high relative to the surrounding matrix or estuary, also fulfilling the definition put forth by McClain et al. (2003). Sites within the whiskers (Q1 - 1.5 x IQR and Q3 + 1.5 x IQR) were deemed "moderate spots" or "modspots". All statistical tests were performed using R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing).

Exploration of Microbial and Environmental Factors Responsible for Hotspots

The approach for identifying the factors responsible for hotspots considered both the microbial as well as environmental features of hotspots. Hotspots were first separated from modspots. Regression analysis between activities and functional gene abundance was initially run for modspots to determine the relationship between microbial community abundance and capacity for N_2 production. Hotspots were then added to the graph to identify where they fell with respect to the expected activities given the functional gene abundance.

The method of Clarke & Ainsworth (1993) linking multivariate community structure data to environmental parameters (BIOENV) was used to identify the best set of environmental parameters to explain the dissimilarities in the biological features of the community structure, in this case, gene abundance and activities using The R Vegan 2.0 Package (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing). In the function BIOENV, a community dissimilarity matrix is calculated for the biological data and Euclidian distances are calculated for subsets of environmental data.

Correlations between community dissimilarities and environmental distances are found and the best results are saved.

RESULTS

Denitrification and Anammox Hotspots in the NRE

The newly described criteria for quantitative determination of biogeochemical hotspots outlined in this paper consider outliers in measured N_2 production, hotspots. Using Tukey's boxplot method of outlier detection, activities greater than 16.7 and 0.77 nmol g^{-1} wet sed hr^{-1} for denitrification and anammox, respectively, were considered outliers (Figure 2). A total of nine denitrification and 13 anammox hotspots were detected throughout the NRE, which were evenly distributed during both summer and winter sampling events (denoted in red in Figure 1 B-E). Although there was some variation between denitrification and anammox hotspots, spatial overlap in the location of hotspots for both N_2 producing pathways was observed, primarily restricted to the upper reaches of the estuary. Reoccurrence of hotspots was observed seasonally at JAX for both N_2 pathways during summer and winter.

The overall means ± standard deviations of denitrification and anammox activities were 7.02 ± 11.00 and 0.41 ± 1.19 nmoles N g^{-1} wet sediment hr^{-1} when hotspots were included (Table 7). Mean denitrification activities at the hotspot sites were significantly different than modspots, decreasing from 33.87 ± 26.98 to 4.88 ± 4.01 nmoles N g^{-1} wet sediment hr^{-1} once these anomalously high activities were removed. Anammox hotspots also skewed the data and once removed showed a similar magnitude of activity reduction, with mean activities reduced to 0.18 ± 0.18 nmoles N g^{-1} wet sediment hr^{-1}; anammox

hotspot mean activity rose to 2.35 ± 3.07 nmoles N g^{-1} wet sediment hr^{-1}. Medians were much closer to means following the removal of denitrification and anammox hotspots, demonstrating a less skewed distribution of activities.

Denitrification hotspots made up 7.3% of the sites sampled and contributed to 35.6% of the total measured activities (Table 8). Anammox hotspots occurred at 10.6% of the sites but accounted for 60.9% of total measured anammox activities.

Microbial features of Hotspots and Modspots

Gene abundance for *nosZ* Clade I ranged from $2.75x10^6$ to $2.82x10^8$ copy number g^{-1} wet sediment (Table 2). Gene abundance for *nosZ* Clade II and *hzo* were several orders of magnitude lower and showed greater variability among samples, ranging from $8.33x10^3$ to $7.1x10^6$ and $3.74x10^2$ to $1.30x10^6$, respectively (Table 2).

We explored relationships between overall potential activities and respective gene abundance separately for a subsample of hot and modspots. This molecular approach was used to explore the microbial community characteristics for denitrifying and anammox hotspots and modspots. Regressions for modspots were plotted using potential activities and functional gene abundance (Figure 3; Table 9). At these sites, positive relationships were observed between denitrification and total *nosZ* gene abundance ($R^2 = 0.13$, $p = 0.007$). The *nosZ* genes in Clades I and II were also analyzed separately. Total *nosZ* genes were comprised mostly of genes from *nosZ* Clade I denitrifiers and strongly reflected patterns for Clade I gene abundance as seen in the regression analysis ($R^2 = 0.13$, $p = 0.007$). The *nosZ* Clade II lacked a significant relationship between gene abundance and potential denitrification ($R^2 = 0.06$, $p = 0.079$). Potential anammox

activities were also positively correlated with *hzo* gene abundance ($R^2 = 0.10$, $p = 0.021$). Low R^2 values indicate high variability within the data. Despite this variability, hotspots clearly fell above the predicted level of activity given the measured gene abundance when added to the graphs.

Environmental features of Hotspots and Modspots

The final task was to identify environmental factors that may explain the presence of hotspots. BIOENV, conducted separately for denitrification and anammox hotspots and modspots, revealed several parameters that explained elevated activities for each N_2 producing pathway (Table 10). Comparison of the variability within the biological and environmental features within each data set generated seven combinations of environmental variables that best explained the biological. The best model for denitrification hotspots contained three parameters that were not statistically significant: salinity, bottom water NO_3^- and porewater H_2S ($r = 0.412$, $p = 0.146$). Elevated sediment %organics were best correlated with denitrification modspots ($r = 0.25$, $p = 0.008$). The most determining variables for anammox hotspots and modspots were bottom water NH_4^+ under low salinity conditions ($r = 0.476$, $p = 0.019$ and $r = 0.274$, $p = 0.001$ respectively). Correlation analyses for environmental parameters were conducted to determine the direction of the relationships identified in the BIOENV analyses.

DISCUSSION

Denitrification and Anammox in the NRE

Denitrification rates and *nosZ* Clade I gene copy numbers were consistent with those observed in other temperate estuaries (Dalsgaard et al. 2005, Henry et al. 2006, Seitzinger et al. 2006, Smith et al. 2007, Dong et al. 2009, Henderson et al. 2010). The *nosZ* Clade II gene abundance was several orders of magnitude lower than Clade I. Due to the greater abundance of *nosZ* Clade I, total *nosZ* gene abundance was driven by Clade I. This suggests that similar environmental factors may regulate the majority of denitrifiers, regardless of the minor differences between the two clades.

Anammox and *hzo* gene copy number were consistently several orders of magnitude lower than denitrification and *nosZ* copy number, but within the range of activities (Dalsgaard et al. 2005, Nicholls & Trimmer 2009) and gene abundance (Dang et al. 2010, Lisa et al. 2014) reported in estuarine systems. Anammox bacteria are generally outcompeted in NO_2^- uptake since they are vastly outnumbered by heterotrophic NO_3^- and NO_2^- reducing communities in sediments (Trimmer et al. 2005). Interestingly, *hzo* gene abundance comprises 0.21% of total *nosZ* gene abundance, yet anammox still contributes up to % of nitrogen removal in the NRE. This infers that anammox bacteria themselves may be considered biogeochemical hotspots.

Microbial and Environmental Features of Denitrification and Anammox Modspots

Increases in the number of individuals within the respective functional guilds partly explain increases in potential N_2 production at modspots in this study of the NRE as well as in other coastal systems (Bernhard et al. 2007, Caffrey et al. 2007, Dong et al.

2009, Petersen et al. 2012, Song et al. 2014). Since the microbial communities at these NRE modspots are operating under the non-limiting conditions in the ^{15}N tracer incubations, we can conclude that the addition of missing reactants or increased substrate will likely not result in any higher rate measurements at modspots. Activities at modspots appear to be restricted by microbial community abundance at these sites. This is likely a result of the existing microbial community structure, suggesting that changes in environmental conditions will result in changes in activities only if the existing microbial communities increase in number at these sites.

In addition to the increase in functional gene abundance, the presence of elevated sediment %organics supports both denitrification and anammox modspots. This correlation of organic matter with denitrification is not a surprising finding given the heterotrophic nature of the process (Zumft 1997, Seitzinger et al. 2006). Although generally an autotrophic process, anammox bacteria can also use organic matter as an electron donor or carbon source (Guven et al. 2005, Strous et al. 2006, Sabumon 2007, Kartal 2008).

Microbial and Environmental Features of Denitrification and Anammox Hotspots

The positive relationship between biogeochemical activities and functional gene abundance does not always hold true in environmental studies (Bernhard & Bollmann 2010), as was observed at NRE denitrification and anammox hotspots. Hotspots for both processes revealed substantial increases in rates with little change in functional gene abundance. Although the phylogeny of microbial communities was not examined in this study, the observed lower gene abundance relative to potential denitrification and

anammox activities suggests that the microbial communities at hotspots may contain members that have differences in substrate affinity or metabolic activity. Denitrifiers perform differently based on the individual organisms within the community and the overall community's affinity for substrate and uptake constants/capacity (Philippot & Hallin 2005, Bowles et al. 2012). Similarly, '*Candidatus* Brocadia anammoxidans' or '*Candidatus* Kuenenia stuttgartiensis' were reported to have higher specific activity than '*Candidatus* Scalindua spp.' (Schmid et al. 2003). We hypothesize that the presence of denitrifiers and anammox bacteria with higher substrate affinity or greater metabolic activity leads to have the significantly higher activities relative to gene abundance observed at hotspots when the environmental conditions are optimal.

Denitrification and anammox activities decreased with increasing salinity and hotspots were restricted to the upper estuary, lower salinity sites. Elevated substrate availability in conjunction with lower salinity and lower levels of H_2S in the upper portion of the estuary appear to support the creation of denitrification hotspots. Sulfide has been shown to inhibit NO and N_2O reduction and halt the denitrification process in some denitrifying bacteria (Sorensen et al. 1980). Thus, lower concentrations of H_2S combined with higher NO_3^- concentrations allow for higher N_2 production at denitrification hotspots. Bottom water NH_4^+ at lower salinity were the environmental features strongly associated with both anammox modspots and hotspots suggesting the convergence of missing reactants and increased substrate alone does not completely explain elevated anammox activities at hotspots.

Further, it appears that higher denitrification activities and denitrification hotspots co-vary with anammox hotspots. Overlap between hotspots of both denitrification and

anammox is clear and in the cases where there is no overlap, high denitrification activities coincide with anammox hotspots. Nitrite reduction is the rate-limiting step in denitrification as dissolved NO_2^- is converted into a gaseous form; therefore, NO_2^- often accumulates due to the faster reduction of NO_3^- relative to the reduction of NO_2^- (Almeida et al. 1995). Often anammox bacteria depend on denitrifiers for leakage of NO_2^- (Trimmer et al. 2003) and anammox microbial communities present at NRE hotspots might be capitalizing on this leakage.

CONCLUSIONS

Biogeochemical hotspots were identified as statistical outliers using our newly established quantitative method of hotspot determination. We found denitrification and anammox hotspots accounted for a small percentage of the total area in a system but had the potential to contribute significantly to enhanced N removal. The presence and interactions of denitrifiers and anammox bacteria with higher substrate affinity under ideal environmental conditions may allow for the elevated activities observed at hotspots. This is the first study conducted on the estuarine ecosystem level that quantitatively defines biogeochemical hotspots, highlights the occurrence of denitrification and anammox hotspots in an estuarine system, and proposes biological and environmental mechanisms governing these areas of elevated activities.

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Table 1. Sampling site coordinates in the New River Estuary. Each site was visited during the summer and winter. ID indicates the location in the transect across the NRE at the designated site: the first letter denotes East ("E") or West ("W") , the second letter specifies Channel ("C") or Bank ("B") and the third letter ("A", "B", "C" or "D") specifies the bank location in the broader shallow portions of the estuary.

Site	ID	Degrees	
		Latitude	Longitude
AA1	EC	34.76056	-77.44944
AA2	WC	34.76389	-77.45722
JAX	WB	34.73806	-77.43278
	C	34.73861	-77.43222
	EBC	34.73944	-77.43028
	EBB	34.74083	-77.42889
	EBA	34.74194	-77.42750
M53	WB	34.72167	-77.42833
	WC	34.72194	-77.42556
	EC	34.72333	-77.42361
	EB	34.72528	-77.42056
M50	WBA	34.69917	-77.41083
	WBB	34.70167	-77.41056
	WC	34.70528	-77.40972
	C	34.70694	-77.40944
	EC	34.71000	-77.40900
	EBD	34.71222	-77.40833
	EBC	34.71611	-77.40667
	EBB	34.72306	-77.40278
	EBA	34.72861	-77.40111
M47	WBA	34.68250	-77.40611
	WBB	34.68444	-77.40306
	WC	34.68583	-77.39889
	EC	34.68750	-77.39583
	EBB	34.69139	-77.38889
	EBA	34.69944	-77.37722
M43	WB	34.66445	-77.38000
	WC	34.66583	-77.37750
	EC	34.66694	-77.37500
	EBC	34.66889	-77.37278
	EBB	34.67083	-77.37056
	EBA	34.67278	-77.36806

Site	ID	Degrees	
		Latitude	Longitude
M39	WB	34.64222	-77.37083
	WC	34.64278	-77.36722
	EC	34.64361	-77.36389
	EBC	34.64444	-77.36028
	EBB	34.64667	-77.35361
	EBA	34.64917	-77.34417
M36	WB	34.62417	-77.37472
	WC	34.62278	-77.37333
	EC	34.62139	-77.37139
	EBC	34.61972	-77.36944
	EBB	34.61750	-77.36611
	EBA	34.61444	-77.36167
M31	WBA	34.60694	-77.43389
	WBB	34.60667	-77.42972
	WBC	34.60500	-77.42333
	WC	34.59972	-77.40944
	EC	34.59861	-77.40500
	EB	34.59778	-77.40083
172	WB	34.57778	-77.39694
	WC	34.57972	-77.39667
	EC	34.58000	-77.39694
	EB	34.58111	-77.39833
M18	WBA	34.56444	-77.38111
	WBB	34.56806	-77.37806
	WC	34.57111	-77.37694
	EC	34.57250	-77.37694
	EBB	34.57833	-77.37556
	EBA	34.58306	-77.37472
M15	WB	34.55917	-77.35750
	WC	34.55861	-77.35806
	EC	34.56000	-77.35722
	EB	34.56083	-77.35611

Table 2. Bottom water parameters in the New River Estuary. ID indicates the location in the transect across the NRE at the designated site: the first letter denotes East ("E") or West ("W"), the second letter specifies Channel ("C") or Bank ("B") and the third letter ("A", "B", "C" or "D") specifies the bank location in the broader shallow portions of the estuary.

		Temperature (°C)		Salinity		Nitrate (μM)		Ammonium (μM)		DO (mg/L)	
Site	ID	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
AA1	EC	27.3	n.d.	4.5	0.1	1.50	27.03	5.58	4.57	0.65	6.38
AA2	WC	28.8	n.d.	2.2	0.1	0.40	68.61	9.05	2.54	0.15	6.40
JAX	WB	27.0	n.d.	8.2	0.6	0.14	45.45	0.61	4.71	8.10	7.28
	C	27.7	n.d.	12.3	5.7	0.64	13.30	2.75	14.89	3.02	7.02
	EBC	27.6	n.d.	10.9	6.0	0.07	5.29	0.57	11.87	5.80	7.27
	EBB	27.5	n.d.	10.9	1.3	0.42	39.83	1.43	33.29	5.75	6.19
	EBA	27.8	n.d.	10.9	1.1	0.47	40.81	4.98	9.26	6.10	7.26
M53	WB	26.7	n.d.	11.5	3.2	0.31	10.62	1.05	7.40	8.49	8.19
	WC	26.9	n.d.	13.2	6.6	n.d.	9.00	n.d.	14.66	4.02	7.03
	EC	26.9	n.d.	13.6	6.5	n.d.	8.95	n.d.	12.85	3.99	7.69
	EB	27.6	n.d.	12.8	6.2	0.12	10.81	0.37	19.62	5.20	6.34
M50	WBA	26.9	n.d.	13.4	3.2	1.68	14.81	1.39	9.26	6.80	8.54
	WBB	26.6	n.d.	14.2	5.4	0.19	7.57	0.90	33.79	6.02	7.33
	WC	26.9	n.d.	14.5	5.3	0.35	8.32	0.59	10.59	5.50	8.59
	C	26.8	n.d.	14.5	6.7	0.16	9.68	0.63	13.98	6.22	7.98
	EC	27.2	n.d.	15.3	6.3	0.28	6.15	1.15	13.05	4.70	6.28
	EBD	26.8	n.d.	14.5	7.6	0.11	7.81	0.22	26.68	6.30	6.44
	EBC	26.9	n.d.	14.5	7.4	0.26	4.11	0.52	14.23	5.98	6.83
	EBB	26.9	n.d.	14.3	7.3	0.74	10.30	0.98	14.85	6.48	7.43
	EBA	27.0	n.d.	13.8	6.0	0.26	5.48	0.86	32.04	6.04	7.76
M47	WBA	31.5	12.7	13.1	3.8	1.05	13.78	1.56	9.39	5.90	10.72
	WBB	27.2	12.0	13.7	4.4	1.73	18.47	0.62	12.83	4.81	10.41
	WC	27.3	11.8	18.8	5.2	0.70	13.78	1.27	14.76	4.43	10.19
	EC	27.4	12.1	19.9	6.8	0.10	12.55	0.22	18.02	2.30	10.14
	EBB	27.4	12.0	18.0	7.1	0.09	18.12	0.20	14.08	3.02	10.00
	EBA	30.1	12.9	14.2	4.7	0.09	18.85	0.30	13.56	5.23	10.50
M43	WB	29.1	12.3	14.8	5.6	0.10	8.88	0.31	5.13	5.80	10.54
	WC	27.4	11.7	22.4	9.7	0.17	18.89	0.17	11.74	2.04	9.40
	EC	27.3	11.8	21.9	9.7	0.79	18.88	0.48	14.20	2.20	9.64
	EBC	27.4	11.6	21.7	9.0	0.16	17.23	0.22	10.94	2.73	9.86
	EBB	27.5	11.8	20.5	8.0	n.d.	6.94	n.d.	8.53	3.80	10.00
	EBA	29.2	12.6	17.6	5.9	0.08	20.13	0.19	17.25	6.10	10.80

		Temperature (°C)		Salinity		Nitrate (μM)		Ammonium (μM)		DO (mg/L)	
Site	ID	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
M39	WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	WC	27.2	11.7	20.8	10.2	1.05	9.26	1.92	9.28	2.95	9.49
	EC	27.5	11.8	23.5	10.3	0.47	7.89	0.63	24.63	2.06	9.43
	EBC	27.4	12.2	22.8	10.8	0.10	9.80	0.12	10.61	2.99	10.04
	EBB	27.4	11.8	20.6	10.7	1.03	9.04	1.07	15.49	4.43	9.41
	EBA	28.1	13.0	18.4	6.1	0.19	11.14	0.31	10.60	5.80	10.43
M36	WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	WC	27.4	12.4	24.2	14.5	0.33	8.17	2.90	10.56	1.20	9.45
	EC	27.5	12.2	23.2	15.0	0.19	6.92	1.29	15.39	3.32	9.77
	EBC	27.5	12.4	24.0	16.1	0.19	4.05	4.95	12.53	1.50	9.12
	EBB	27.4	12.3	23.5	15.0	1.17	9.53	3.19	12.01	2.56	9.53
	EBA	27.4	13.6	19.6	9.6	0.34	10.49	8.99	9.91	3.18	11.02
M31	WBA	28.6	n.d.	22.9	9.0	0.54	8.31	0.37	16.12	n.d.	8.26
	WBB	28.1	n.d.	24.8	10.3	0.19	8.12	0.33	11.46	n.d.	8.50
	WBC	28.0	n.d.	24.0	10.6	0.52	3.91	2.01	7.66	n.d.	7.79
	WC	27.8	n.d.	23.3	12.3	0.17	6.44	0.51	9.41	n.d.	7.39
	EC	27.9	n.d.	23.9	13.6	0.14	4.46	1.87	8.28	n.d.	8.38
	EB	28.6	n.d.	23.3	11.6	0.30	3.61	1.47	7.68	n.d.	9.13
172	WB	28.7	15.0	25.3	17.2	0.16	5.85	1.31	9.23	n.d.	7.72
	WC	27.5	15.0	27.2	26.9	0.37	2.36	1.03	4.76	n.d.	6.43
	EC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EB	28.6	17.0	25.0	20.3	0.23	3.26	1.44	6.24	n.d.	7.29
M18	WBA	28.8	11.0	32.1	22.0	0.13	3.23	0.40	6.58	n.d.	7.40
	WBB	28.4	13.0	26.6	29.2	0.19	1.33	1.17	3.17	n.d.	6.69
	WC	27.3	11.0	28.5	29.0	0.50	1.64	0.43	2.88	n.d.	6.73
	EC	27.5	13.0	28.1	29.2	0.23	1.03	1.07	2.54	n.d.	6.37
	EBB	28.6	15.0	30.5	27.4	0.24	1.63	0.85	3.34	n.d.	5.49
	EBA	30.2	11.0	30.1	17.8	0.17	2.62	0.94	6.28	n.d.	8.08
M15	WB	28.1	19.0	31.0	31.4	0.15	0.43	0.65	1.82	n.d.	6.73
	WC	28.1	17.0	31.0	29.8	0.65	0.72	5.43	1.12	n.d.	6.48
	EC	29.7	17.0	31.2	31.2	0.23	0.13	0.78	1.05	n.d.	6.32
	EB	28.9	15.0	29.3	30.6	0.36	0.44	1.43	1.49	n.d.	6.93

Table 3. Sediment porewater nutrients in New River Estuary. ID indicates the location in the transect across the NRE at the designated site: the first letter denotes East ("E") or West ("W"), the second letter specifies Channel ("C") or Bank ("B") and the third letter ("A", "B", "C" or "D") specifies the bank location in the broader shallow portions of the estuary.

		Ferrous Iron (μM)		Hydrogen Sulfide (μM)		Nitrate + Nitrite (μM)	
Site	ID	Summer	Winter	Summer	Winter	Summer	Winter
AA1	EC	9.09	1.68	627.9	111.1	0.32	0.12
AA2	WC	1.92	5.49	11.7	16.4	0.37	0.21
JAX	WB	1.23	2.96	1.3	20.0	2.88	0.14
	C	9.84	1.71	973.4	800.7	0.58	2.37
	EBC	7.24	3.17	725.7	854.7	0.32	0.09
	EBB	3.05	3.83	396.7	470.5	0.23	n.d.
	EBA	1.58	13.12	481.8	10.6	0.15	0.01
M53	WB	1.38	2.71	1.4	62.8	1.25	1.11
	WC	6.99	1.20	544.1	1100.4	0.58	0.16
	EC	6.59	1.81	413.3	37.2	0.47	0.09
	EB	0.98	1.91	3.1	1023.7	6.57	n.d.
M50	WBA	1.43	2.26	4.6	7.7	0.63	8.64
	WBB	0.98	2.36	0.4	958.3	0.89	0.40
	WC	3.27	1.20	784.2	1159.9	0.28	1.14
	C	1.40	1.30	890.9	963.7	1.68	1.50
	EC	2.87	9.83	832.4	563.2	0.49	0.35
	EBD	4.87	1.46	894.7	290.7	0.26	0.39
	EBC	1.88	1.20	875.6	1121.8	0.37	0.52
	EBB	4.47	1.20	944.2	1072.7	0.16	0.30
	EBA	0.90	1.20	1.3	3.4	0.28	0.29
M47	WBA	0.00	n.d.	0.2	n.d.	0.67	0.81
	WBB	0.00	1.16	516.3	1038.7	0.12	0.31
	WC	0.67	1.36	773.9	398.6	0.16	0.07
	EC	0.20	1.21	360.2	1673.4	0.47	0.37
	EBB	1.07	1.85	668.6	1361.5	0.68	0.41
	EBA	0.12	1.32	0.9	1.6	0.29	0.92
M43	WB	0.57	1.36	0.0	1080.8	0.27	4.66
	WC	0.17	2.66	397.3	854.6	0.71	0.40
	EC	4.01	1.71	629.0	903.1	0.16	0.29
	EBC	1.32	0.77	817.3	1068.6	0.21	0.76
	EBB	0.50	1.56	264.9	488.1	0.18	0.07
	EBA	0.00	0.96	11.6	4.5	0.14	2.05

		Ferrous Iron (μM)		Hydrogen Sulfide (μM)		Nitrate + Nitrite (μM)	
Site	ID	Summer	Winter	Summer	Winter	Summer	Winter
M39	WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	WC	2.43	1.11	734.3	667.1	0.01	0.11
	EC	6.06	1.46	531.1	1242.2	0.09	0.59
	EBC	1.98	1.56	709.5	492.3	0.05	0.21
	EBB	0.13	0.87	0.2	935.7	0.07	0.83
	EBA	4.25	2.87	0.3	22.8	1.44	2.68
M36	WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	WC	1.40	1.30	1690.6	2.6	0.07	0.20
	EC	12.23	1.31	74.3	29.4	0.12	0.06
	EBC	3.30	1.41	4.1	204.7	0.24	0.02
	EBB	3.00	1.01	0.0	11.7	0.33	0.17
	EBA	0.42	1.21	0.0	30.9	1.85	1.48
M31	WBA	1.99	12.33	1.3	3.3	0.27	3.46
	WBB	2.72	1.20	0.0	14.5	0.22	0.08
	WBC	18.41	7.41	0.0	11.7	0.07	0.26
	WC	4.35	20.62	13.8	5.3	0.03	0.07
	EC	12.95	17.54	0.4	2.8	0.09	0.14
	EB	n.d.	4.68	n.d.	14.3	0.24	0.61
172	WB	59.56	22.49	n.d.	871.1	1.47	1.54
	WC	n.d.	1.54	n.d.	4.7	0.16	9.46
	EC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EB	2.99	36.46	92.1	50.3	0.24	0.27
M18	WBA	n.d.	67.51	n.d.	0.9	1.03	0.14
	WBB	1.72	2.07	0.0	8.5	0.21	0.37
	WC	2.04	1.76	0.0	0.9	1.32	0.14
	EC	59.34	49.40	0.0	5.3	0.20	0.13
	EBB	3.51	110.38	0.0	20.5	2.09	0.10
	EBA	3.13	25.18	n.d.	30.7	0.18	0.22
M15	WB	1.83	5.33	0.0	6.8	0.24	0.55
	WC	1.56	3.72	0.7	1.1	0.33	2.15
	EC	1.64	1.83	0.0	1.9	1.70	4.63
	EB	2.04	7.90	n.d.	0.9	0.03	0.20

Table 4. Sediment characteristics in the New River Estuary. ID indicates the location in the transect across the NRE at the designated site: the first letter denotes East ("E") or West ("W"), the second letter specifies Channel ("C") or Bank ("B") and the third letter ("A", "B", "C" or "D") specifies the bank location in the broader shallow portions of the estuary.

		Organics (%)		Ammonium (μmol g^{-1} wet sed)		Benthic Chl *a* (μg chl a $(g\ sed)^{-1}$)		Grain Size (μm)	
Site	ID	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
AA1	EC	21.6	25.4	0.34	0.33	0.00	6.39	32.4	43.7
AA2	WC	3.2	3.0	0.14	0.20	0.00	0.00	35.8	344.1
JAX	WB	2.2	1.5	0.14	0.11	12.73	2.30	246.6	169.5
	C	14.7	23.0	0.36	1.24	5.98	4.68	35.7	54.7
	EBC	20.7	19.4	0.42	1.00	7.89	9.62	33.2	31.4
	EBB	17.5	14.3	0.35	0.55	6.44	1.66	20.6	39.3
	EBA	32.5	13.6	0.67	0.58	3.94	2.54	34.2	36.7
M53	WB	0.7	0.8	0.06	0.33	9.75	17.71	232.4	191.8
	WC	17.2	18.5	0.42	1.22	5.17	14.54	24.2	30.3
	EC	16.4	17.8	0.33	0.54	6.61	9.87	37.2	28.6
	EB	0.5	12.3	0.10	0.72	14.80	12.10	250.4	31.4
M50	WBA	0.4	0.4	0.12	0.52	12.54	23.70	263.4	281.3
	WBB	5.4	n.d.	0.10	0.62	2.32	6.21	226.0	20.9
	WC	16.3	15.8	0.35	0.71	2.87	10.87	38.6	48.6
	C	19.6	16.6	1.21	1.11	2.58	11.72	27.7	28.4
	EC	17.9	18.2	0.55	0.69	2.53	11.47	29.6	40.7
	EBD	19.6	n.d.	0.60	0.82	11.82	10.96	27.0	19.5
	EBC	16.8	16.7	0.68	0.77	44.74	9.77	33.7	26.6
	EBB	19.1	17.8	0.56	0.74	21.11	9.01	33.8	31.8
	EBA	3.2	0.7	0.14	0.52	17.82	25.35	180.3	219.8
M47	WBA	2.8	0.6	0.17	0.54	11.11	17.53	175.6	179.3
	WBB	10.4	9.9	0.32	0.72	9.85	7.03	14.0	26.2
	WC	14.7	17.6	0.50	1.04	4.60	13.48	19.4	58.6
	EC	n.d.	18.1	n.d.	n.d.	0.00	6.04	15.9	31.8
	EBB	15.4	17.0	0.65	0.87	6.23	10.74	14.3	24.1
	EBA	0.9	0.4	0.27	0.29	13.53	17.98	203.0	331.7
M43	WB	1.3	0.5	0.16	0.17	7.06	9.10	227.0	311.3
	WC	5.1	9.5	0.16	0.58	11.59	4.75	119.0	26.0
	EC	10.2	15.3	0.35	0.91	8.81	8.37	18.8	30.4
	EBC	12.3	16.0	0.53	0.79	4.39	0.00	14.6	73.9
	EBB	11.6	12.0	0.14	0.33	5.03	3.17	21.8	30.0
	EBA	1.6	0.6	0.23	0.39	22.56	42.65	252.4	229.0

		Organics (%)		Ammonium ($\mu mol\ g^{-1}$ wet sed)		Benthic Chl *a* (μg chl a $(g\ sed)^{-1}$)		Grain Size (μm)	
Site	ID	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
M39	WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	WC	11.7	11.5	0.45	0.47	4.25	25.41	29.1	30.6
	EC	13.8	14.5	0.66	1.01	7.33	5.08	46.7	27.6
	EBC	7.8	11.0	0.58	n.d.	3.66	3.15	20.7	59.3
	EBB	0.4	13.0	0.18	0.06	12.56	3.67	313.2	27.0
	EBA	4.1	0.4	0.22	0.33	20.31	15.91	237.0	302.0
M36	WB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	WC	9.5	9.0	1.53	0.36	18.73	5.12	25.8	n.d.
	EC	5.9	n.d.	0.70	0.28	6.69	1.63	69.7	41.9
	EBC	6.1	10.6	0.29	0.28	10.90	2.96	28.2	47.6
	EBB	n.d.	1.0	n.d.	n.d.	7.91	2.21	51.4	209.8
	EBA	0.9	0.5	n.d.	0.28	15.31	36.83	215.5	198.9
M31	WBA	7.6	0.6	n.d.	0.17	3.53	0.00	21.0	442.8
	WBB	11.8	8.8	0.09	0.38	9.94	0.60	22.0	59.1
	WBC	11.1	11.7	1.41	0.43	18.67	0.00	60.9	28.1
	WC	9.2	9.5	0.19	1.21	4.92	17.35	28.3	33.9
	EC	8.1	11.3	0.26	0.42	10.87	0.00	27.6	40.3
	EB	1.9	0.8	0.25	0.09	29.27	0.00	226.0	233.3
172	WB	0.8	0.7	0.07	0.28	3.11	8.53	233.9	256.6
	WC	n.d.	n.d.	n.d.	0.07	n.d.	3.55	23.6	346.3
	EC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	EB	5.0	1.0	0.73	n.d.	27.12	5.69	32.9	190.7
M18	WBA	3.1	2.2	0.24	0.22	3.46	8.74	231.2	174.7
	WBB	2.1	0.5	n.d.	n.d.	7.37	3.53	106.8	268.1
	WC	2.0	1.9	0.32	n.d.	7.80	7.27	145.6	153.5
	EC	3.3	2.6	0.30	0.22	15.96	7.40	205.8	63.8
	EBB	3.7	6.0	0.22	0.37	10.85	5.03	110.3	104.7
	EBA	0.8	0.4	n.d.	0.33	2.55	7.72	222.1	229.7
M15	WB	1.0	0.2	0.44	0.09	7.93	5.80	226.2	282.4
	WC	0.3	0.1	0.12	0.13	4.03	7.84	181.8	251.4
	EC	0.3	0.4	0.08	0.07	4.84	3.96	226.7	278.7
	EB	2.8	1.3	0.16	0.55	n.d.	20.41	280.9	157.6

Table 5. Potential rate measurements of denitrification and anammox in the New River Estuary. Mean rates ± standard deviation are reported in nmol N g^{-1} wet sediment hr^{-1} for summer and winter. ID indicates the location in the transect across the NRE at the designated site: the first letter denotes East ("E") or West ("W"), the second letter specifies Channel ("C") or Bank ("B") and the third letter ("A", "B", "C" or "D") specifies the bank location in the broader shallow portions of the estuary. The rates in red color indicate identified hotspots.

		Denitrification (nmol N g^{-1} wet sed hr^{-1})		Anammox (nmol N g^{-1} wet sed hr^{-1})	
Site	ID	Summer	Winter	Summer	Winter
AA1	EC	6.29 ± 0.10	99.85 ± 7.50	0.42 ± 0.01	12.28 ± 1.23
AA2	WC	4.37 ± 0.32	4.45 ± 0.29	0.38 ± 0.01	0.56 ± 0.03
JAX	WB	24.37 ± 0.84	53.62 ± 15.63	1.62 ± 0.01	3.51 ± 1.15
	C	11.53 ± 1.37	25.01 ± 0.44	0.99 ± 0.18	1.85 ± 0.64
	EBC	21.77 ± 1.08	19.09 ± 0.82	2.08 ± 0.19	1.31 ± 0.10
	EBB	15.91 ± 2.17	14.87 ± 0.16	1.09 ± 0.23	1.22 ± 0.02
	EBA	6.35 ± 0.41	11.24 ± 1.43	0.47 ± 0.00	0.95 ± 0.15
M53	WB	2.36 ± 0.04	15.24 ± 0.43	0.13 ± 0.00	0.46 ± 0.01
	WC	7.27 ± 0.30	20.12 ± 1.43	0.48 ± 0.00	0.77 ± 0.07
	EC	8.05 ± 1.36	9.68 ± 0.77	0.65 ± 0.08	0.42 ± 0.01
	EB	6.66 ± 0.31	9.93 ± 0.97	0.61 ± 0.00	0.31 ± 0.02
M50	WBA	0.72 ± 0.13	10.67 ± 0.98	0.02 ± 0.00	0.28 ± 0.05
	WBB	16.70 ± 1.73	1.87 ± 0.61	0.91 ± 0.11	0.05 ± 0.02
	WC	9.58 ± 0.80	0.53 ± 0.03	0.64 ± 0.04	0.01 ± 0.00
	C	6.99 ± 0.62	1.74 ± 0.24	0.15 ± 0.02	0.04 ± 0.01
	EC	1.17 ± 0.10	2.40 ± 0.13	0.03 ± 0.00	0.05 ± 0.01
	EBD	4.75 ± 0.31	1.09 ± 0.06	0.14 ± 0.01	0.02 ± 0.00
	EBC	5.01 ± 0.14	6.00 ± 0.47	0.19 ± 0.00	0.20 ± 0.02
	EBB	13.30 ± 0.03	5.89 ± 1.18	1.02 ± 0.20	0.23 ± 0.04
	EBA	2.68 ± 0.19	4.31 ± 0.62	0.10 ± 0.01	0.12 ± 0.04
M47	WBA	21.10 ± 0.46	9.90 ± 0.68	1.68 ± 0.09	0.37 ± 0.04
	WBB	10.21 ± 2.39	0.67 ± 0.28	0.37 ± 0.03	0.01 ± 0.01
	WC	9.91 ± 3.00	0.89 ± 0.20	0.68 ± 0.16	0.02 ± 0.00
	EC	3.26 ± 0.97	1.38 ± 0.78	0.17 ± 0.05	0.02 ± 0.02
	EBB	4.14 ± 0.43	2.69 ± 0.16	0.15 ± 0.01	0.06 ± 0.00
	EBA	0.46 ± 0.17	5.23 ± 0.01	0.02 ± 0.01	0.17 ± 0.00
M43	WB	3.39 ± 0.43	5.45 ± 1.08	0.14 ± 0.02	0.23 ± 0.05
	WC	1.19 ± 0.16	6.80 ± 0.09	0.02 ± 0.00	0.17 ± 0.01
	EC	1.21 ± 0.00	1.49 ± 1.54	0.02 ± 0.00	0.03 ± 0.03
	EBC	2.19 ± 0.91	0.30 ± 0.00	0.04 ± 0.02	0.00 ± 0.00
	EBB	0.65 ± 0.13	1.90 ± 0.21	0.01 ± 0.00	0.04 ± 0.01
	EBA	0.10 ± 0.00	7.44 ± 0.76	0.00 ± 0.00	0.17 ± 0.04

		Denitrification (nmol N g^{-1} wet sed hr^{-1})		Anammox (nmol N g^{-1} wet sed hr^{-1})	
Site	ID	Summer	Winter	Summer	Winter
M39	WB	n.d. ± n.d.	n.d. ± n.d.	n.d. ± n.d.	n.d. ± n.d.
	WC	4.63 ± 1.35	19.87 ± 6.09	0.15 ± 0.05	0.55 ± 0.10
	EC	6.20 ± 0.23	1.06 ± 0.31	0.24 ± 0.03	0.02 ± 0.00
	EBC	8.77 ± 0.29	6.60 ± 0.78	0.27 ± 0.01	0.16 ± 0.02
	EBB	0.59 ± 0.04	6.76 ± 1.39	0.02 ± 0.00	0.19 ± 0.04
	EBA	0.15 ± 0.05	2.68 ± 0.06	0.01 ± 0.00	0.09 ± 0.00
M36	WB	n.d. ± n.d.	n.d. ± n.d.	n.d. ± n.d.	n.d. ± n.d.
	WC	2.90 ± 0.53	5.43 ± 0.59	0.09 ± 0.02	0.10 ± 0.02
	EC	4.92 ± 0.72	4.76 ± 0.67	0.14 ± 0.02	0.08 ± 0.01
	EBC	7.94 ± 1.60	6.76 ± 0.95	0.24 ± 0.04	0.13 ± 0.02
	EBB	9.20 ± 0.38	5.58 ± 0.38	0.26 ± 0.06	0.20 ± 0.05
	EBA	0.02 ± 0.00	4.02 ± 1.06	0.00 ± 0.00	0.16 ± 0.00
M31	WBA	2.92 ± 0.20	10.73 ± 1.17	0.09 ± 0.01	0.63 ± 0.18
	WBB	5.21 ± 0.00	2.33 ± 0.12	0.14 ± 0.01	0.05 ± 0.00
	WBC	6.64 ± 0.38	12.91 ± 0.75	0.20 ± 0.01	0.41 ± 0.09
	WC	3.10 ± 0.01	14.47 ± 0.57	0.08 ± 0.00	0.56 ± 0.09
	EC	6.52 ± 0.58	2.37 ± 0.52	0.18 ± 0.01	0.08 ± 0.02
	EB	0.16 ± 0.02	7.90 ± 1.01	0.01 ± 0.00	0.14 ± 0.02
172	WB	0.19 ± 0.07	7.52 ± 0.19	0.01 ± 0.00	0.20 ± 0.02
	WC	n.d. ± n.d.	n.d. ± n.d.	n.d. ± n.d.	n.d. ± n.d.
	EC	3.49 ± 0.20	1.33 ± 0.50	0.13 ± 0.04	0.07 ± 0.03
	EB	2.43 ± 0.79	4.68 ± 0.10	0.06 ± 0.02	0.23 ± 0.06
M18	WBA	0.40 ± 0.02	1.85 ± 0.06	0.01 ± 0.00	0.05 ± 0.00
	WBB	6.19 ± 0.31	2.29 ± 0.13	0.38 ± 0.02	0.07 ± 0.00
	WC	3.73 ± 0.78	5.72 ± 0.12	0.16 ± 0.03	0.20 ± 0.02
	EC	9.11 ± 0.25	7.48 ± 1.28	0.48 ± 0.03	0.23 ± 0.03
	EBB	5.74 ± 0.39	4.31 ± 0.23	0.24 ± 0.02	0.06 ± 0.01
	EBA	0.14 ± 0.01	0.10 ± 0.03	0.01 ± 0.00	0.01 ± 0.00
M15	WB	0.46 ± 0.04	1.55 ± 0.15	0.03 ± 0.00	0.14 ± 0.02
	WC	0.45 ± 0.05	1.84 ± 0.19	0.03 ± 0.00	0.11 ± 0.02
	EC	0.71 ± 0.01	2.52 ± 0.16	0.05 ± 0.00	0.07 ± 0.00
	EB	0.34 ± 0.05	2.55 ± 0.35	0.01 ± 0.00	0.06 ± 0.00

Table 6. Abundance of *nosZ* and *hzo* genes in the New River Estuary. Mean copy number (gene copies g^{-1} wet sediment ± standard deviation) are reported for *nosZ* Clade I, Clade II and hzo. Sites not examined in the molecular study are indicated by "n.a." Total *nosZ* was calculated by summing Clade I and Clade II of *nosZ* gene abundance. ID indicates the location in the transect across the NRE at the designated site: the first letter denotes East ("E") or West ("W"), the second letter specifies Channel ("C") or Bank ("B") and the third letter ("A", "B", "C" or "D") specifies the bank location in the broader shallow portions of the estuary.

Site	ID	*nosZ* Clade I		*nosZ* Clade II		Total *nosZ*		*hzo*	
		Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
AA1	EC	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
AA2	WC	6.70E+07 ± 4.80E+06	2.02E+07 ± 3.42E+05	6.37E+05 ± 1.06E+05	5.26E+04 ± 2.46E+03	6.77E+07	2.02E+07	1.74E+05 ± 4.76E+03	1.87E+04 ± 4.40E+03
JAX	WB	2.74E+07 ± 2.55E+06	1.74E+08 ± 7.51E+06	4.56E+05 ± 1.63E+04	2.32E+06 ± 3.06E+05	2.78E+07	1.76E+08	3.22E+04 ± 4.99E+03	1.26E+05 ± 3.79E+03
	C	7.22E+07 ± 5.37E+05	1.65E+08 ± 8.95E+05	7.37E+05 ± 1.74E+05	1.86E+06 ± 1.20E+05	7.30E+07	1.67E+08	1.04E+05 ± 1.33E+04	1.80E+05 ± 1.50E+04
	EBC	8.24E+07 ± 5.61E+06	9.59E+07 ± 4.00E+06	1.30E+06 ± 2.98E+05	9.38E+05 ± 6.85E+04	8.37E+07	9.69E+07	9.90E+04 ± 7.91E+03	9.13E+04 ± 8.81E+03
	EBB	6.13E+07 ± 3.20E+06	1.33E+08 ± 8.14E+06	7.50E+05 ± 1.69E+05	1.12E+06 ± 3.77E+04	6.21E+07	1.34E+08	5.42E+04 ± 1.19E+04	1.57E+05 ± 1.37E+04
	EBA	3.95E+07 ± 1.71E+06	1.56E+08 ± 1.14E+07	1.59E+05 ± 1.46E+04	1.01E+06 ± 3.86E+04	3.97E+07	1.57E+08	1.81E+05 ± 1.90E+04	1.23E+05 ± 1.46E+04
M53	WB	3.84E+07 ± 2.67E+06	2.13E+08 ± 2.65E+07	1.17E+06 ± 1.78E+05	3.32E+06 ± 3.28E+05	3.96E+07	2.17E+08	4.24E+04 ± 9.89E+03	4.47E+05 ± 2.30E+04
	WC	9.89E+06 ± 5.68E+05	2.04E+08 ± 1.53E+07	2.10E+06 ± 3.25E+05	2.68E+06 ± 2.75E+05	1.20E+07	2.07E+08	1.32E+05 ± 9.43E+03	4.65E+05 ± 1.35E+04
	EC	2.75E+06 ± 9.93E+04	9.86E+07 ± 4.45E+05	5.82E+05 ± 4.68E+04	6.94E+05 ± 3.70E+04	3.33E+06	9.93E+07	3.98E+04 ± 4.05E+03	2.88E+05 ± 4.06E+04
	EB	6.82E+06 ± 2.59E+05	2.03E+08 ± 3.05E+07	1.88E+06 ± 2.52E+05	2.84E+06 ± 4.18E+05	8.70E+06	2.06E+08	6.20E+04 ± 3.37E+03	4.71E+05 ± 1.12E+04
M50		n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
M47	WBA	1.75E+08 ± 1.27E+07	8.05E+07 ± 9.06E+06	4.58E+06 ± 6.36E+05	1.18E+06 ± 5.89E+04	1.80E+08	8.17E+07	6.61E+04 ± 7.59E+03	8.78E+04 ± 1.46E+04
	WBB	1.59E+08 ± 8.56E+06	1.05E+08 ± 8.23E+06	4.83E+06 ± 3.06E+05	9.34E+05 ± 5.52E+04	1.64E+08	1.06E+08	7.95E+05 ± 1.06E+05	1.44E+05 ± 1.61E+04
	WC	6.18E+07 ± 3.57E+06	1.45E+08 ± 1.37E+07	6.29E+05 ± 3.34E+04	2.50E+06 ± 1.10E+05	6.25E+07	1.48E+08	4.99E+04 ± 1.29E+03	2.37E+05 ± 3.32E+04
	EC	8.53E+07 ± 2.81E+06	1.62E+08 ± 2.70E+07	8.79E+05 ± 8.44E+04	1.36E+06 ± 5.47E+04	8.61E+07	1.63E+08	9.93E+04 ± 4.67E+03	2.04E+05 ± 3.03E+04
	EBB	7.83E+07 ± 3.19E+06	1.04E+08 ± 1.67E+07	7.63E+05 ± 9.04E+04	5.14E+05 ± 3.06E+04	7.91E+07	1.04E+08	1.48E+05 ± 7.03E+03	1.54E+05 ± 1.42E+04
	EBA	2.73E+07 ± 3.07E+06	7.50E+07 ± 1.03E+07	4.56E+05 ± 8.91E+04	1.16E+06 ± 1.18E+05	2.77E+07	7.62E+07	1.89E+04 ± 2.32E+03	9.09E+04 ± 1.17E+04
M43		n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
M39	WB	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
	WC	8.59E+07 ± 2.62E+06	2.48E+08 ± 1.24E+07	3.82E+06 ± 1.27E+05	3.52E+06 ± 1.54E+05	8.97E+07	2.51E+08	1.43E+05 ± 1.17E+04	4.54E+05 ± 6.76E+04
	EC	2.82E+08 ± 1.56E+07	4.86E+07 ± 6.75E+06	7.10E+06 ± 5.10E+05	2.33E+05 ± 2.19E+04	2.89E+08	4.88E+07	2.17E+05 ± 2.53E+04	5.88E+04 ± 1.18E+03
	EBC	5.93E+07 ± 8.61E+05	8.90E+07 ± 7.53E+06	1.19E+06 ± 2.95E+04	9.19E+05 ± 1.22E+05	6.05E+07	8.99E+07	9.99E+04 ± 2.23E+04	2.84E+05 ± 5.33E+04
	EBB	4.09E+07 ± 9.80E+05	1.85E+07 ± 1.38E+06	1.72E+06 ± 1.14E+05	7.29E+04 ± 2.16E+04	4.26E+07	1.86E+07	4.60E+04 ± 1.79E+03	4.67E+04 ± 6.55E+03
	EBA	5.54E+07 ± 5.82E+06	5.48E+07 ± 2.37E+06	1.20E+06 ± 1.16E+05	3.83E+05 ± 4.17E+04	5.66E+07	5.52E+07	5.10E+04 ± 6.94E+03	1.65E+05 ± 2.91E+04
M36		n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
M31	WBA	7.11E+07 ± 6.90E+06	1.01E+07 ± 6.35E+05	1.58E+06 ± 5.59E+04	1.08E+05 ± 1.50E+04	7.26E+07	1.03E+07	1.76E+05 ± 1.55E+04	3.34E+04 ± 4.78E+03
	WBB	1.01E+08 ± 6.28E+06	1.17E+08 ± 1.14E+07	4.26E+06 ± 6.07E+05	1.35E+06 ± 1.66E+05	1.05E+08	1.19E+08	2.66E+05 ± 5.92E+04	4.01E+05 ± 5.92E+04
	WBC	4.29E+07 ± 2.01E+06	1.00E+08 ± 5.10E+05	1.60E+06 ± 4.93E+04	1.03E+06 ± 1.22E+05	4.45E+07	1.01E+08	1.32E+05 ± 1.35E+04	2.85E+05 ± 4.05E+04
	WC	8.86E+07 ± 2.38E+06	2.62E+08 ± 1.41E+07	8.36E+05 ± 1.05E+05	3.58E+06 ± 2.67E+05	8.94E+07	2.65E+08	5.42E+04 ± 3.51E+03	1.30E+06 ± 9.81E+04
	EC	8.25E+07 ± 3.30E+06	9.68E+07 ± 5.16E+06	2.47E+06 ± 3.01E+05	1.29E+06 ± 7.39E+04	8.49E+07	9.81E+07	1.53E+05 ± 2.58E+04	4.04E+05 ± 5.17E+04
	EB	7.19E+07 ± 2.36E+06	1.36E+07 ± 3.31E+06	8.82E+05 ± 1.65E+05	8.40E+04 ± 1.17E+04	7.28E+07	1.37E+07	1.69E+04 ± 3.78E+03	3.28E+04 ± 3.28E+03
172		n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
M18		n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.
M15	WB	1.62E+07 ± 2.17E+05	2.99E+07 ± 7.96E+05	1.10E+04 ± 1.81E+03	2.47E+05 ± 1.64E+04	1.62E+07	3.01E+07	3.74E+02 ± 1.08E+02	4.54E+04 ± 2.54E+03
	WC	1.46E+07 ± 9.87E+05	1.50E+07 ± 2.11E+05	8.33E+03 ± 1.00E+03	2.51E+05 ± 1.52E+04	1.46E+07	1.52E+07	5.79E+02 ± 1.46E+02	2.11E+04 ± 8.67E+02
	EC	1.86E+07 ± 1.24E+06	8.79E+06 ± 3.74E+05	1.58E+04 ± 2.73E+03	1.00E+05 ± 6.88E+02	1.86E+07	8.89E+06	1.78E+03 ± 4.91E+02	9.72E+03 ± 1.32E+03
	EB	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a. ± n.a.	n.a.	n.a.	n.a. ± n.a.	n.a. ± n.a.

Table 7. Descriptive statistics for total, hotspot, and modspot sites sampled in the New River Estuary. Activities are presented in nmoles N g^{-1} wet sediment hr^{-1}.

	Total			Hotspot			Modspot		
	Mean	Median	SD	Mean	Median	SD	Mean	Median	SD
Denitrification	7.02	4.72	11.00	33.87	21.77	26.98	4.88	4.31	4.01
Anammox	0.41	0.15	1.19	2.35	1.31	3.07	0.18	0.14	0.18

Table 8. Contribution of denitrification and anammox hotspots to N_2 production. Contribution of denitrification and anammox hotspots to percent N_2 production was calculated separately for the individual pathways.

		Percent (%)		
	Total	Sites	Denitrification	Anammox
Denitrification	9	7.3	35.6	x
Anammox	13	10.6	x	60.9

Table 9. Results from regression analyses of potential N_2 activities with respective functional gene abundance. Analyses for hotspots and modspots were conducted separately. Analyses for unrelated parameters were not run and indicated by an "x".

		nosZ Clade I		*nosZ* Clade II		Total *nosZ*		*hzo*	
		R^2	p-value	R^2	p-value	R^2	p-value	R^2	p-value
Denitrification	modspots	**0.13**	**0.007**	0.06	0.079	**0.13**	**0.007**	x	x
	hotspots	0.04	0.840	0.08	0.453	0.01	0.855	x	x
Anammox	modspots	x	x	x	x	x	x	**0.10**	**0.021**
	hotspots	x	x	x	x	x	x	0.04	0.566

Table 10. BIOENV model results. Subsets of environmental variables were analyzed to find the best correlation to biological data for denitrification and anammox hotspots and modspots. The best models are reported.

		Correlation	p-value	Environmental Parameters
Denitrification	modspots	0.250	0.008	S %organics
	hotspots	0.412	0.146	BW Salinity, BW NO_3^-, PW H_2S
Anammox	modspots	0.274	0.001	BW Salinity, BW NH_4^+
	hotspots	0.476	0.019	BW Salinity, BW NH_4^+

Figure 1. Sampling sites and contour plots of anammox and denitrification rates in the New River Estuary. Sixty-two sampling sites were established along 12 transects in the estuary (A). Denitrification (B-C) and anammox (D-E) activities for summer (B&D) and winter (C&E) are presented in nmol N g^{-1} wet sediment hr^{-1}. Hotspots are shown in red.

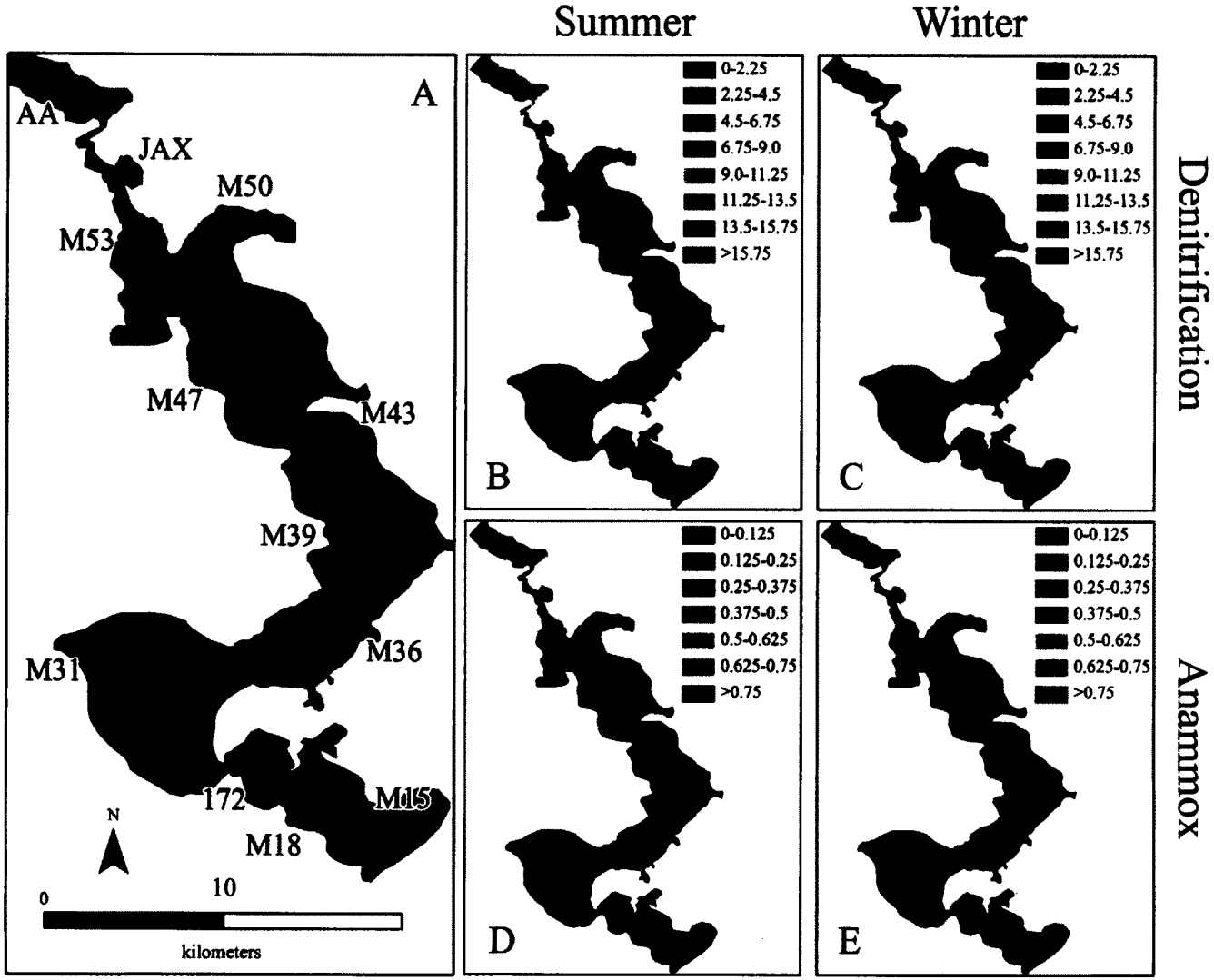

Summer
Winter
Denitrification
Anammox
A
AA
JAX
M50
M53
M47
M43
M39
M36
M31
172
M15
M18
N
0
10
kilometers
B
C
D
E
0-2.25
2.25-4.5
4.5-6.75
6.75-9.0
9.0-11.25
11.25-13.5
13.5-15.75
>15.75
0-0.125
0.125-0.25
0.25-0.375
0.375-0.5
0.5-0.625
0.625-0.75
>0.75

Figure 2. Identification of biogeochemical hotspot using Tukey's method for outlier detection. Outliers were identified as points beyond the whiskers (Q3 + 1.5* IQR) and designated hotspots (*).

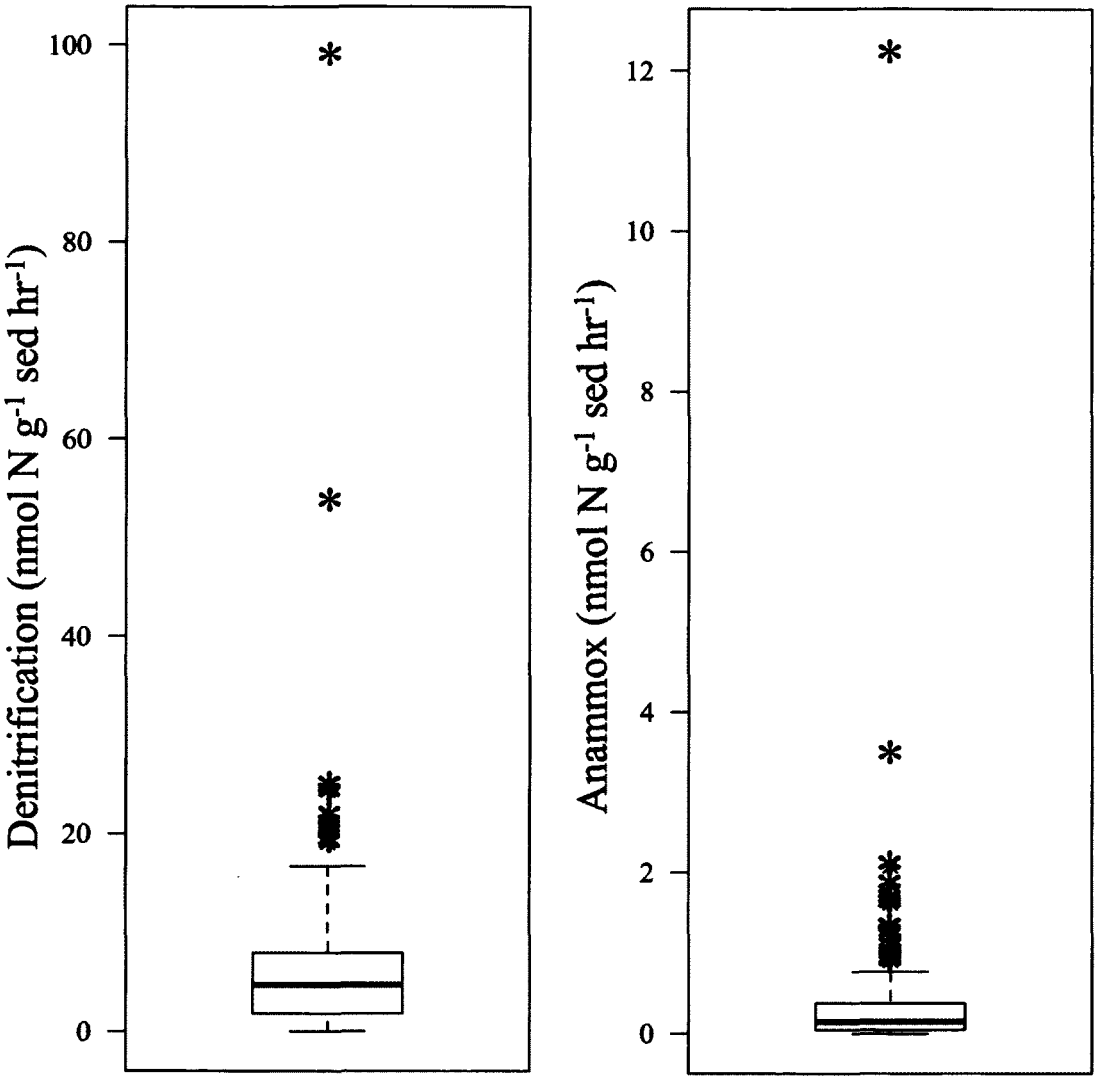
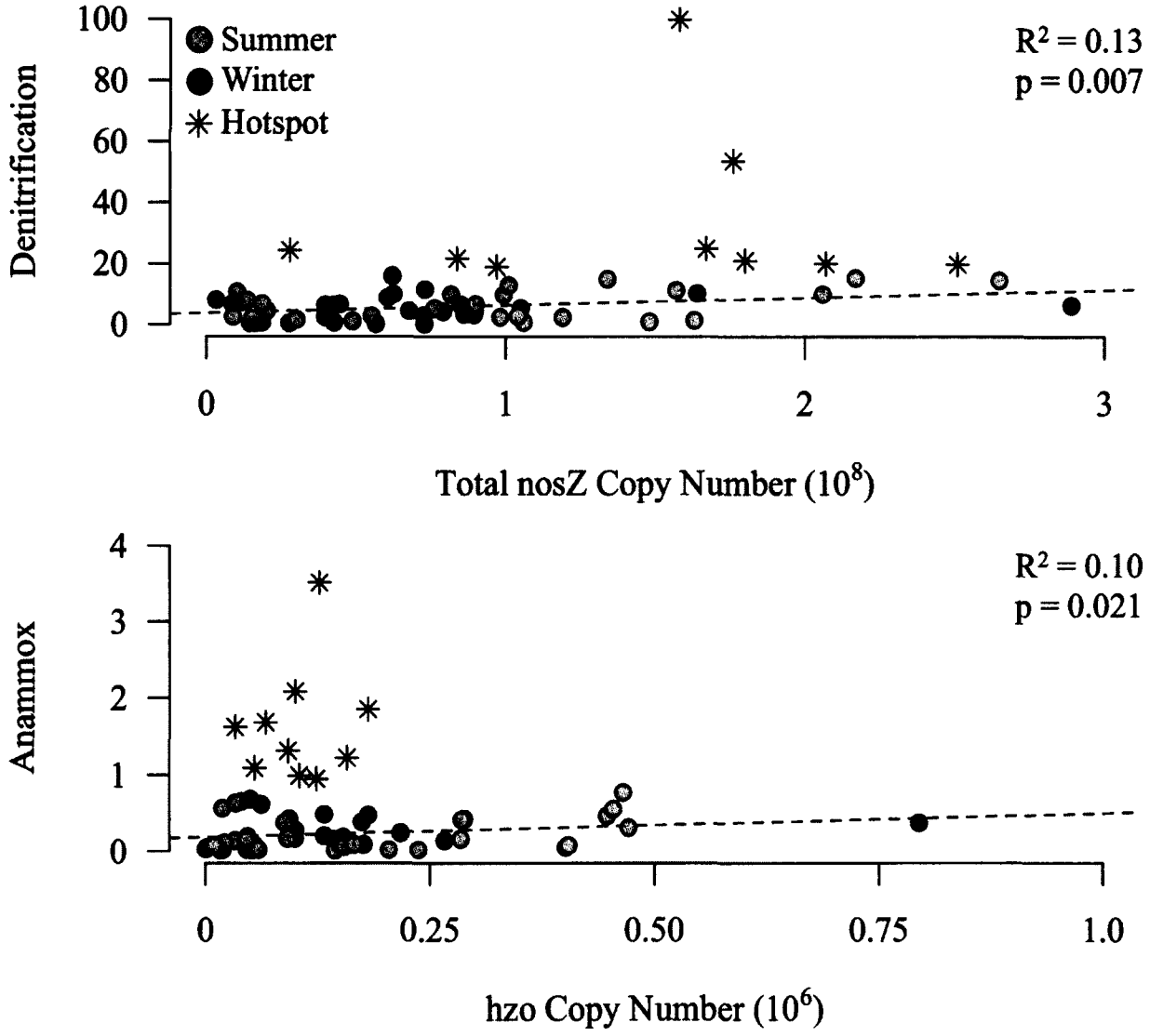

Denitrification (nmol N g-1 sed hr-1)
0
20
40
60
80
100
Anammox (nmol N g-1 sed hr-1)
0
2
4
6
8
10
12

Figure 3. Biological factors of hotspots and modspots. Regressions of potential activities and gene abundance for denitrification and anammox are shown for modspots. Hotspots (*) lie above the regression line and show no correlation with gene abundance. R^2 and p values are shown for regressions of modspots with hotspots removed. Rates for denitrification and anammox are presented in nmol N g^{-1} wet sediment hr^{-1}.


Denitrification
100
80
60
40
20
0
Summer
Winter
Hotspot
R² = 0.13
p = 0.007
0
1
2
3
Total nosZ Copy Number (10⁸)
Anammox
4
3
2
1
0
R² = 0.10
p = 0.021
0
0.25
0.50
0.75
1.0
hzo Copy Number (10⁶)

CHAPTER 4

Distribution of denitrifying communities in response to environmental factors as revealed through *nirS* gene microarray analysis

ABSTRACT

Knowledge of community structural response to environmental variation is essential for understanding the microbial regulation of nitrogen removal processes in aquatic systems. In particular, it is important to elucidate the environmental factors that support higher abundance of different denitrifying populations as they significantly contribute to overall denitrification activities. A customized microarray containing 165 *nirS* gene probes (archetypes) was designed from all available *nirS* sequences detected in environments worldwide to represent sequences separated by >15% divergence. We conducted *nir*S gene microarray analysis with sediment samples collected in two seasons (summer and winter) at four sites in the New River Estuary, NC, USA to identify the abundant members of the denitrifying community and examine their responses to changing environmental variables over spatial and temporal scales. In addition, the contribution of specific archetypes to overall denitrifying activities was evaluated. The abundance of specific archetypes associated with marine and estuarine environments and *Thauera linaloolentis* positively correlated with increased denitrification activities. The co-variation of archetypes formed two different denitrifier assemblages in the NRE, each associated with different environmental conditions. Together these findings highlight the importance of habitat type in the composition of denitrifying communities and the essential role of individual members within the community function.

INTRODUCTION

Denitrification is the major pathway for removal of fixed nitrogen (N) in estuarine sediments and plays a mitigating role in eutrophication of coastal systems (Seitzinger et al. 2006). Knowledge of denitrifier community structural response to environmental variation is essential for understanding the microbial regulation of nitrogen removal processes in aquatic systems (Braker et al. 2000). Denitrifying community structure in sediments is continually changing in response to habitat specific environmental factors (Zumft 1997, Braker et al. 2000, Jones & Hallin 2010, Wei et al. 2015). Individual members within a denitrifying community respond differently to the same environmental conditions as result of differences in cellular metabolism and regulation (Tiedje 1988, Cavigelli & Robertson 2000, Liu, Mao, et al. 2013). Therefore, the denitrifying community composition is an important factor influencing overall denitrification activities. In order to assess community composition in relation to potential activities under different environmental conditions, it is necessary to characterize the diversity of this functional guild as well as changes in relative abundance of its members (Braker et al. 2000, Taroncher-Oldenburg et al. 2003). It is also important to elucidate the environmental factors that support higher abundance of different denitrifying populations as often contribute steadily to overall nitrogen removal activities (Bulow et al. 2008).

Denitrification is a respiratory process in which nitrogenous oxides serve as terminal electron acceptors in the oxidation of organic carbon (Tiedje 1988). It is a stepwise reduction pathway of nitrate (NO_3^-) and nitrite (NO_2^-) to gaseous N species (nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2), mediated by a series of enzymes; nitrate reductase (Nar/Nap), nitrite reductase (Nir), nitric oxide reductase (Nor)

and nitrous oxide reductase (Nos). The key step is nitrite reduction converting NO_2^- to NO. The production of NO distinguishes denitrifying bacteria from other NO_3^--respiring organisms. Two distinct Nir enzymes have been described but never found in the same cell: one containing c and d_1-type hemes (cd_1-Nir) and the other a copper (Cu-Nir) (Brittain et al. 1992). NirS is the more widely distributed enzyme in the aquatic environment (Jones & Hallin 2010) and present in over 70% of known denitrifiers (Braker et al. 2000), although there is evidence to suggest that the diversity of both *nir*S and *nir*K containing denitrifiers is currently underestimated (Wei et al. 2015).

Molecular analyses targeting the cd_1 type nitrite reductase gene (*nir*S) have been employed in previous studies to successfully detect denitrifying bacteria from environmental samples and to gain better understanding of the diversity and composition of community structure (Braker et al. 1998, 2000, Hallin & Lindgren 1999, Priemé et al. 2002, Yan et al. 2003). In particular, functional gene microarray is a widely used, high-throughput, automated technique that allows the quantification and identification of particular guilds (Jayakumar et al. 2004, Bulow et al. 2008). These arrays can be used to analyze genes encoding for enzymes involved in biogeochemical cycles (Torsvik & Øvreås 2002). Comparative analysis in relation to environmental factors can be used to obtain information on the linkage between the phylogeny and ecology of functional groups of bacteria responsible for denitrification.

A transect study was conducted to identify denitrification hotspots across 13 sites in the New River Estuary (NRE), NC, USA (Chapter 3). A small number of sites demonstrated anomalously high activity that accounted for 35.6% of denitrification activity. An increase in denitrifier gene abundance could not account for the increase in

activities and it was hypothesized that different groups of denitrifiers might be present at these hotspots and responsible for the elevated N removal. The diversity and composition of the denitrifying microbial community was investigated using *nir*S microarray in a subsample of the transect sites; this included four sites along an estuarine salinity gradient over two seasons in the NRE. The objectives of the study were to: 1) Link the diversity of the denitrifying community to potential denitrification activities; 2) Identify the archetypes responsible for overall denitrification activities of a community; and 3) Elucidate the environmental factors influencing the composition and function of the denitrifying communities in the NRE.

MATERIALS AND METHODS

Seasonal Sampling of the New River Estuary

Transect sampling was conducted in the New River Estuary (NRE), Onslow Bay, North Carolina, USA during the summer and winter, 2009, along salinity and nutrient gradients as described in detail in the previous chapter (Chapter 3). In this study, four sites during summer and winter were further examined for denitrifier community structure and included the upper-estuary site Jax (34.73N, 77.43W), two mid-estuary sites M47 (34.68N, 77.39W) and M31 (34.59N, 77.40W), and a lower-estuary site M15 (34.55N, 77.35W) (Figure 1). All samples and measurements were collected in the channel west of the indicated channel markers.

Environmental Parameter and Potential Denitrification Measurements

Procedures for collection and processing of environmental parameters were also described in detail in the previous chapter (Chapter 3). Environmental parameters pertaining to this study included water column depth, temperature, salinity and dissolved oxygen (DO), sediment porewater ferrous iron (Fe^{2+}), hydrogen sulfide (H_2S), nitrate plus nitrite ($NO_3^- + NO_2^-$ or NO_x^-), ammonium (NH_4^+), as well as sediment percent organic content (%organics), free plus extractable NH_4^+, benthic chlorophyll a, and mean sediment grain size. Potential denitrification was measured using ^{15}N stable isotope analysis. Sediments from each site were stored at -80°C for molecular analysis.

Microarray Oligonucleotide Probe Design

Microarray BC013 (for BioComplexity 13) contained a comprehensive collection of probes specific for a range of genes involved in nitrogen transformations including 165 nirS (nitrite reductase; cytochrome cd1 containing) genes derived from isolates, various environmental clones as well as cultured organisms. The BCO13 array applied the internal standard ratio method, previously outlined for field samples (Ward et al. 2007, Bulow et al. 2008, Ward 2008, Peng et al. 2013). The array contains an internal standard to filter and quantify signal intensity of the probes and allow for comparison across arrays. Each 90-mer oligonucleotide probe contains a 70-mer nirS archetype sequence and a 20-mer oligo internal standard sequence. Four arrays, each containing six blocks were printed on each slide.

Target Preparation

Hybridization targets were prepared with 50 ng of genomic DNAs from 8 sediment samples, which were used for quantitative PCA assays in Chapter 3. Genomic DNA was digested with HinF1 following standard protocol. Products were precipitated with a series of 100% and 70% ethanol washes and dried under vacuum. Cleaned products were labeled by incorporating amino-allyl-dUTP during a random priming labeling reaction using the Klenow enzyme and random primers supplied in the BioPrime labeling kit (Invitrogen). The standard dNTP mixture was replaced with 1.2 mM dACG and a 1:12 mixture of dNTP to dUaa. Products were again precipitated in ethanol, dried under vacuum and eluted to a final volume of 50 μL.

A volume equivalent to 1000 ng of dUaa labeled DNA was dried under vacuum for conjugation to Cy3 in duplicates. The DNA labeled with dUaa was hydrated in 4.5 μL of 100 mM $NaCO_3$ buffer (pH 9) and incubated in the dark at room temperature for 15 minutes. Next, 4.5 μL of Cy3 dissolved in DMSO (889-pmol μL^{-1}) were added; incubation in the dark proceeded for an additional 3 hours to overnight. After adding 4.5 μL of 4 M hydroxylamine, the reaction mixtures were incubated for 15 minutes to quench the fluorescent reaction. The labeled products from parallel reactions were pooled and cleaned using Qiaquick kit (Qiagen, Valencia, CA, USA) with some modification. Qiaquick PE buffer was replaced with a mixture of 0.5 mL of 1 M KPO_4 (pH 8.5), 7.625 mL of H_2O, and 42.25 mL of ethanol. Qiaquick EB buffer was replaced with 10 μL of KPO_4 (pH 8.5) and 2.49 mL of H_2O. The concentration of the target was measured on a Perkin Elmer LS 55 Luminescence Spectrometer using a PicoGreen assay (Molecular Probes). The remaining product was dried down and stored in -80°C or -20°C freezer.

Prehybridization, hybridization, and post-hybridization washes were done as previously described (Taroncher-Oldenburg et al. 2003, Ward et al. 2007). Briefly, hybridization mixtures, containing 1 μg of Cy3-labelled target and 1 μL of the Cy5-labeled reverse complimentary 20mer reference (0.5 pmol) in a total volume of 200 μL, were made for each target and transported to the ozone-free Array Facility (Princeton University, NJ, USA). Hybridization mixtures were heated to 95°C for 5 minutes in a wet block covered with foil, and cooled on ice for 2 minutes. The hybridization mixture (100 μL) was added to each gasketed well in a hybridization chamber, which was secured in an oven rack and rotated at 65°C overnight. A series of post hybridization washes in 45 mL of low-stringency (1SSC and 0.1% SDS), medium-stringency (0.1SSC and 0.1% SDS), or high-stringency (0.1SSC) buffer and MilliQ water were conducted in the Array Facility on a shaker for 20 minutes at 100 rpm (Taroncher-Oldenburg et al. 2003, Ward et al. 2007). Microarrays were dried by centrifugation (1,700 g for 5 min) and were immediately scanned on a GenePix 4000A scanner (Axon Instruments,Inc., Foster City, Calif.) using the GenePix Pro software provided with the scanner.

Quantification of Hybridization Signals

Hybridization values were obtained from the median fluorescence or background fluorescence data reported on the GenePix Pro software-derived spreadsheet. Scanned arrays were normalized and filtered following the method of Bouskill et al. (2011) with some modifications. Signal intensities for all features were calculated by subtracting the background fluorescence for each channel. The median background fluorescence across both channels and for all features was calculated and used to identify significant signals

(signal intensity greater than two standard deviations above the median background fluorescence). The ratio of Cy3:Cy5 fluorescence was calculated for each feature and features with no Cy3 signal were not included in further analyses. The ratio of Cy3:Cy5 fluorescence was normalized to the average fluorescence of the mixall features for respective blocks in the array to account for uneven hybridization across the slide. The raw microarray image was also checked to ensure that all anomalous signals were identified and removed. Data were normalized as relative fluorescence ratio (RFR) by converting the signal intensity of each probe to a percentage of the total signal intensity (sum of all significant signals) to allow for comparison between arrays. RFRs with >1% were considered robust signals and used for subsequent analyses. RFRs with <1% were not included in further analyses.

Ecological Analysis

All statistical analyses were conducted in R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing); packages used in each analysis are indicated below. Spearman's rank correlation was used to examine relationships between potential denitrification activities, community structure and environmental variables. RFR data were used to calculate the Shannon diversity index (*H*) seasonally at each site (Package 'vegan') to provide information on the species diversity of a community. The relationship between spatial and temporal patterns in denitrifier community composition and environmental parameters was explored through detrended correspondence analysis on transformed data (DCA; Package 'vegan'). A heat map generated from Spearman's Correlation coefficients was used to investigate relationships among archetypes and to

assess the influence of environmental parameters on the abundance of particular archetypes and potential denitrification activities (Package 'pheatmap').

RESULTS

Environmental Characteristics in the New River Estuary

Bottom water temperature was generally uniform throughout the estuary during each season with means ranging from 27.7 ± 0.3°C in the summer to 14.4 ± 3.0°C in the winter (Table 1). Salinity increased gradually with movement seaward (Table 1). Winter sampling was coincident with large freshwater inputs from the watershed that completely freshened the uppermost stations Jax and M47. Elevated levels of bottom water NO_3^- and NH_4^+ were observed throughout the estuary during winter sampling (Table 1). Highest bottom water NO_3^- and NH_4^+ concentrations were also observed at Jax and M47 with similar concentrations around 13.3 μM and 14.9 μM for NO_3^- and NH_4^+, respectively. Bottom water hypoxia (DO levels < 5 mg/L) was observed throughout the estuary during summer, but was not evident in the winter (Table 1).

Iron (Fe^{2+}) concentration in porewater varied between sites and seasons with highest observed concentration of Fe^{2+} at 20.6 μM at M31 during the winter (Table 2). Hydrogen sulfide (H_2S) concentrations ranged from 0.7 to >900 μM, with elevated concentrations at Jax and M47 during both seasons (Table 2). Porewater NO_x^- was generally low (<1 μM) with the exception of higher concentrations (>2 μM) at Jax and M15 during winter (Table 2).

Sediment %organics were highest at the 3 upper most sites (Jax, M47, M31), coincident with the elevated porewater H_2S concentrations in these sites during both

seasons (Table 3). Extractable sediment NH_4^+ concentrations in the winter exceeded 1μmol NH_4^+ g^{-1} wet sediment in the three upper most sites (Jax, M47 and M31) (Table 3). Benthic chlorophyll *a* was highest in mid-estuary during winter at levels >13 μg chlorophyll *a* g^{-1} sediment (Table 3). Sediment grain size increased from fine at the upper three sites to fine-medium grained sands towards the mouth of the estuary (Table 3).

Denitrification Activities in the New River Estuary

The potential for denitrification in the NRE was present at every site but varied greatly in magnitude. Potential denitrification rates were described in great detail in Chapter 3. Briefly, at the sites that were examined in this study, rates ranged from 0.45 nmol N g^{-1} h^{-1} at the mouth of the estuary and increased to 11.53 nmol N g^{-1} h^{-1} at the upstream sites during summer (Figure 2). Rates were slightly higher and more spatially variable during winter, ranging from 0.89 to 25.01 nmol N g^{-1} h^{-1}.

Denitrifier Community Composition

When considering RFR values >1%, the number of *nirS* archetypes was reduced from 165 to 79 of the most abundant archetypes in the NRE. Regressions of duplicate arrays demonstrate strong reproducibility with R^2 values >0.606 (Table 4).

Examination of the overall community composition showed slightly higher number of archetypes contributing to a greater diversity during the winter at each of the respective sites (Table 5). Greatest overall diversity was observed at the mid-estuary sties (M47 and M31) during both seasons. Shannon Evenness index was very similar between sites and seasons indicating an equal distribution of abundant denitrifiers within sites. Jax

and M15 sites demonstrated higher evenness in summer when the number of abundant archetypes was lowest. The lowest value for evenness was observed at M15 in winter and concurrent with one of the highest fluorescent signals from an archetype observed in this study. The relative contribution to the overall fluorescent signal was calculated for each site and the same mid-estuary sites (M47 and M31) that contained the highest number of abundant archetypes during both seasons also had the highest percent contribution to total signal. Potential denitrification activities did not correlate with Shannon diversity index nor did it correlate with total abundant archetype and their contribution to overall RFR; however, it should be noted that the highest potential activities occurred seasonally at Jax where some of the lowest diversity was observed.

The Influence of Environmental Factors on The Distribution and Composition of Denitrifier Communities

DCA showed a clear separation of sites M31 and M15 in winter (Cluster I) from M47 and Jax in winter as well as all sites in summer (Cluster II; Figure 3). This separation was attributed to the 23 archetypes abundant only at sites M31 and M15 in winter and 38 archetypes specific to Cluster II. Environmental factors associated with the clustering of different archetypes varied. Porewater Fe^{2+}, sediment Chl *a* positively associated with Cluster I while bottom water NO_3^- and NH_4^+, porewater H_2S, sediment NH_4^+ and %organics were more influential in Cluster II. Several environmental parameters significantly or marginally influenced overall sedimentary denitrifier community composition in the NRE (Figure 3b).

While the influence of environmental parameters can be generalized to the overall denitrifying communities in the NRE, it is also useful to identify environmental parameters affecting the distribution of individual archetypes. This was quantitatively examined through a heat map analysis showing Spearman rank correlations across the combined data set (Figure 4). The most abundant archetypes were Nir149 and Nir153, contributing to 3.91% of the overall RFR at M31 in summer and M15 in winter, respectively. Both archetypes were positively associated with porewater Fe^{2+}, sediment Chl *a,* and negatively correlated with temperature. Nir149 and Nir153 represent *nir*S sequences obtained from Choptank River sediments in the Chesapeake Bay.

Nir149 was also one of the most widespread archetypes in the NRE, with significant detection at 6 of the 8 examined samples. Other prevalent archetypes (Nir -4, -5, -8, -40, -44, -47, -81) detected seasonally throughout the upper- and mid-estuary were positively correlated with porewater H_2S and sediment %organics and negatively correlated with salinity. The *nir*S sequences that the aforementioned probes include are also widespread in their origins: Nir5 and Nir47 were designed from Chesapeake Bay sediment isolates, Nir8 was originally derived from the Arabian Sea water column, and South China Sea sediment isolates made up Nir -81. Several widespread denitrifiers in culture were detected and included: *Pseudomonas grimontii* (Nir4), the rhizobacterium *Azospirillum brasilense* (Nir40), and the "very common and easily culturable denitrifier" *Pseudomonas stutzeri* (Nir44; Lalucat et al. 2006).

Heat map analysis also enabled the investigation of relationships among archetypes and revealed archetypes specific to particular sites and seasons (Figure 3). Of particular interest were archetypes representing known denitrifying aromatic carbon

degraders (represented by shaded boxes) and co-varying archetypes (open boxes color coded by covariance; Figure 5). Co-occurring archetypes Nir -20, -34, -54, -67, -96, -101, -151, -152, -153, and -154 were observed only at M31 and M15 in winter. These archetypes were positively correlated with porewater Fe^{2+} and negatively correlated with porewater H_2S and sediment %organics. The probes were designed from *nir*S sequences that largely originated from estuarine sediments and marine water column with the exception of Nir67 representing *Azoarcus tolulyticus* $Tol4^T$, a toluene degrading denitrifier (Song & Ward 2003). Interestingly, Nir154, an archetype that co-varies with Nir67, closely clusters with another known aromatic compound degrading denitrifier, *Aromatoleum aromaticum*, represented by Nir93.

Similarly, Jax contained several archetypes (Nir -14, -16, -50, -80, -119, and -121) that were below detection at other sites and even varied in abundance seasonally within the site. While most of these archetypes are commonly found in estuarine and marine systems, it is interesting to note that Nir50 and Nir80 represent the denitrifiers *Thauera linaloolentis* and *A. tolulyticus* 2FB2, respectively. *T. linaloolentis* is a dominant member of wastewater treatment plant microbial communities and capable of degrading aliphatic monoterpenes (Foss & Harder 1998, Liu, Frostegård, et al. 2013). *A. tolulyticus* 2FB2 was isolated from estuarine sediments of the highly contaminated Arthur Kill, New Jersey (Song & Ward 2003), commonly referred to as the "Chemical Coast". Covariance of Nir -14, -16, -119 occurred with *T. linaloolentis* with Nir119 clustering with Nir50. Porewater H_2S, sediment %organics, and NH_4^+ were positively correlated with these archetypes.

The archetype Nir93, representing *Aromatoleum aromaticum*, also capable of aromatic carbon degradation (Rabus 2005), was abundant at M15 in summer and then again further upstream at Jax in winter. Nir93 co-occurred with three archetypes originally described in the Choptank River in Chesapeake Bay (Nir2 and Nir 132) as well as an offshore Washington Coast clone (Nir15). All archetypes co-occuring with Nir93 negatively correlated with sediment NH_4^+ and Chl *a*.

DISCUSSION

The relationship between diversity and denitrification activities was explored to identify linkages between structure and function of denitrifier communities in the NRE. The highest diversity of denitrifiers, comprising 32-41% of the overall denitrifier community at M47 and M31, was not reflected in higher potential denitrification activities. In fact, the lowest denitrification activity measurement in this study (M47 in winter) was associated with some of the highest diversity of archetypes in the study. Alternatively, the highest denitrification activities were seasonally observed at Jax where we found some of the lowest diversity. This suggests that this site could provide conditions that do not contribute to a greater diversity of denitrifiers but offer a stable environment that promotes greater denitrification activities. Additionally, the overall diversity of the denitrifying community may not be as important as the individual members and their make up of the community. Environmental conditions do not always generate the same response by denitrifiers due to the physiological differences within this diverse functional group (Cavigelli & Robertson 2000). Affinity for substrate and uptake constants/capacity as well as enzyme sensitivity to oxygen differ among denitrifiers

(Philippot & Hallin 2005, Bowles et al. 2012). Thus, denitrifiers better adapted to the specific conditions of their habitat are selected for and reflected in the community structure (Schloter et al. 2000). This is corroborated by the findings of a previous study (Chapter 3) reporting anomalously high activities or "hotspots" at transects across this site. At hotspots, elevated denitrifier (*nosZ*) gene abundance was not contributing to the higher activities. Instead it was suggested that the presence of microbes with higher substrate affinity or metabolic capability could be contributing to the greater potential for denitrification. Similarly, Jayakumar et al. (2004) described higher intensity of denitrification associated with specific *nir*S genes in the Arabian Sea Oxygen Minimum Zone in samples with high NO_3^- and low DO. In this study, we describe several archetypes abundant at Jax (Nir14, -16, -50, and -119) that positively correlated with potential denitrification as well as bottom water NO_3^- and NH_4^+, porewater H_2S, sediment NH_4^+ and %organics. Perhaps these archetypes represent denitrifying bacteria that are either more efficient in substrate use, have a higher affinity for substrate, and/or are better adapted to the site's environmental conditions and are thus capable of contributing to higher denitrification activity.

A majority of archetypes detected in the NRE were commonly found in estuarine and marine systems. Archetypes representing soil and wastewater treatment denitrifiers were also found. While the biogeographic distribution of microorganisms is still an area of contention among microbial ecologists (Martiny et al. 2006), geographic distance and local environmental conditions clearly affect denitrifier community structure. Habitat specific factors, or the abiotic and biotic features associated with particular habitats, may have a stronger effect on the abundance and diversity of denitrifiers in different

environments (Smith et al. 2007, Jones & Hallin 2010, Wei et al. 2015), and this appears to be the case in the NRE as well given the abundance and widespread distribution of marine and estuarine denitrifiers.

The detection of marine and estuarine denitrifiers in the NRE was an expected outcome; however, the presence of denitrifiers typically dominating wastewater treatment plant (WWTP) microbial communities was a bit more surprising but not unexplainable. Prior to 1998, the City of Jacksonville and the U.S. Marine Corps base at Camp Lejeune WWTPs were inefficient and frequently discharged partially treated waste into the NRE (Mallin et al. 2005). The effluent discharge pipe from the city was located in Wilson Bay, the location of the Jax site, and M31 is located near the Marine Corps WWTP pipe discharge into French Creek. Once the facilities were upgraded, point source pollution was significantly reduced and the effluent pipes were removed. Environmental conditions in the upper and mid-estuary are rich in organic carbon and NO_3^- and could possibly sustain the WWTP-associated denitrifiers in Wilson and Stone Bays.

The presence of denitrifiers capable of aromatic carbon degradation throughout the NRE might be indicative of the amount of contamination entering the estuary from the watershed, particularly explosives coming from The United States Marine Corps Base at Camp Lejeune. 2, 4, 6, Trinitrotoluene (TNT) and other explosives are major contaminants in areas surrounding military installations (Ullah et al. 2010). Traces of these explosives can persist in the environment for decades (Darrach et al. 1998), providing a steady supply of substrate for microorganisms capable of aromatic carbon degradation (Bradley et al. 1994, Ullah et al. 2010, Smith et al. 2013). Recent studies on the microbial communities in the Gulf of Mexico following the deep-water horizon oil

spill showed remarkably different community structure in the deep-sea sediments relative to the surface. The relative abundance of toluene degrading denitrifiers and their detection throughout the estuary may indicate that the NRE is contaminated with man made aromatic hydrocarbons.

Spatial and temporal variation of *nir*S communities was also observed corresponding to different environmental conditions of NRE. Denitrifying communities formed two distinct clusters based on the environmental factors that constrained particular archetypes. Cluster I had a strong correlation with porewater Fe^{2+} and sediment Chl *a*. This may indicate that denitrification is coupled to enhanced nitrification in the presence of benthic microalgae (BMA) at M31 and M15. BMA can regulate microbial processes through direct competition (Joye & Anderson 2008) or by the release of O_2 and organic C (Smith & Underwood 1998, Underwood & Smith 1998). Anderson et al. (2013) observed net autotrophy accompanied by BMA uptake of NH_4^+ in the sediments of shallow water sites in close proximity to M31 and M15. The study also noted a decline in BMA as freshwater discharge increased. Freshwater discharge was highest in winter 2009, as mentioned in Chapter 2, which could have reduced the BMA community to levels that allowed for a mutualistic relationship for resources rather than a competition for substrate. Higher denitrification activities were found during daytime fueled by a coupling of BMA and nitrification in shallow estuarine system where NH_4^+ was not limited (Rysgaard et al. 1994, An & Joye 2001). Higher levels of NH_4^+ than NO_3^- were measured at the sites closest to the mouth of the estuary. Together, the observed correlations and the environmental conditions offer additional support for the possibility of a benthic microalgae-nitrification-denitrification coupling in Cluster I.

Cluster II correlated positively with sediment % organics, porewater H_2S, and NH_4^+ but negatively with salinity. In general, the distribution of *nir*S communities are often closely related to salinity as the major environmental determinant (Santoro 2009, Jones & Hallin 2010). The correlation with sediment NH_4^+ in Cluster II is likely not indicative of coupled nitrification-denitrification because high concentration of H_2S inhibits nitrification (Joye & Hollibaugh 1995). Alternatively, the denitrifiers in the NRE may be tolerant to the high H_2S levels throughout the estuary and/or capable of using H_2S as an electron donor (Cardoso et al. 2006). This indicates a linkage between nitrogen and sulfur cycle in the NRE. Similar correlations between NH_4^+, H_2S and anammox were observed in our earlier study of N_2 production in the NRE (Lisa et al. 2014). Thus, the clustering of communities based on the detection of specific *nirS* archetypes correlated with different environmental factors emphasizes the importance of habitat type in the structure and function of denitrifying communities.

CONCLUSIONS

We successfully applied *nir*S microarray to investigate the link among denitrifying community structures, denitrification activities and environmental conditions. The greatest diversity of *nir*S archetypes was detected at the mid-estuary mesohaline sites, but diversity did not correspond to elevated denitrifying activities. Alternatively, the abundance of specific archetypes were associated with increased potential for denitrification. Finally, co-variation of archetypes allowed for the formation of two different denitrifier assemblages associated with different environmental conditions. Together these findings highlight the importance of habitat type in the distribution of denitrifying communities and the link between structure and function of denitrifying community.

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Table 1. Bottom water parameters in the New River Estuary. Missing data points not determined at the time of sampling are indicated by "n.d."

Site	Temperature (°C)		Salinity		Nitrate (μM)		Ammonium (μM)		Dissolved Oxygen (mg/L)	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Jax	27.7	n.d.	12.3	5.7	0.64	13.30	2.75	14.89	3.0	7.0
M47	27.3	11.8	18.8	5.2	0.70	13.78	1.27	14.76	4.4	10.2
M31	27.8	n.d.	23.3	12.3	0.17	6.44	0.51	9.41	n.d.	7.4
M15	28.1	17.0	31.0	29.8	0.65	0.72	5.43	1.12	n.d.	6.5

Table 2. Sediment porewater characteristics in the New River Estuary.

Site	Ferrous Iron (μM)		Hydrogen Sulfide (μM)		Nitrate + Nitrite (μM)	
	Summer	Winter	Summer	Winter	Summer	Winter
Jax	9.84	1.71	973.4	800.7	0.58	2.37
M47	0.67	1.36	773.9	398.6	0.16	0.07
M31	4.35	20.62	13.8	5.3	0.03	0.07
M15	1.56	3.72	0.7	1.1	0.33	2.15

Table 3. Sediment characteristics in the New River Estuary.

Site	Sediment Organics (%)		Ammonium (μmol/g wet sed)		Benthic Chl *a* (μg chl a (g sed)$^{-1}$)		Grain Size (μm)	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Jax	14.71	22.96	0.36	1.24	5.98	4.68	35.7	54.7
M47	14.66	17.62	0.50	1.04	4.60	13.48	19.4	58.6
M31	9.21	9.51	0.19	1.21	4.92	17.35	28.3	33.9
M15	0.34	0.13	0.12	0.13	4.03	7.84	181.8	251.4

Table 4. Reproducibility of duplicate *nirS* microarrays. RFR values >1% were considered valid; regressions between duplicate arrays were run to examine the reproducibility of the arrays.

Site	R^2	
	Summer	Winter
Jax	0.816	0.890
M47	0.796	0.733
M31	0.867	0.606
M15	0.840	0.722

Table 5. Denitrifying community characteristics in the New River Estuary. Abundant archetypes were defined as archetypes contributing to >1% of the relative fluorescence ratio in each array. Diversity and evenness measures were based on microarray signal hybridization data of abundant archetypes.

Site	Abundant Archetypes				Shannon Diversity		Shannon Evenness	
	number		(% RFR)		(H)		H/log(S)	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Jax	19	21	26.4	28.6	2.9233	3.0121	0.9928	0.9894
M47	24	27	32.7	41.3	3.1408	3.2581	0.9883	0.9886
M31	26	28	39.8	39.2	3.1966	3.3038	0.9811	0.9915
M15	15	23	20.8	36.8	2.6810	3.0640	0.9900	0.9772

Figure 1. Sampling sites in the New River Estuary, NC, USA. Four sites were examined along a salinity and nutrient gradient and included Jax, M47, M31, and M15. Sampling was conducted during the summer and winter.

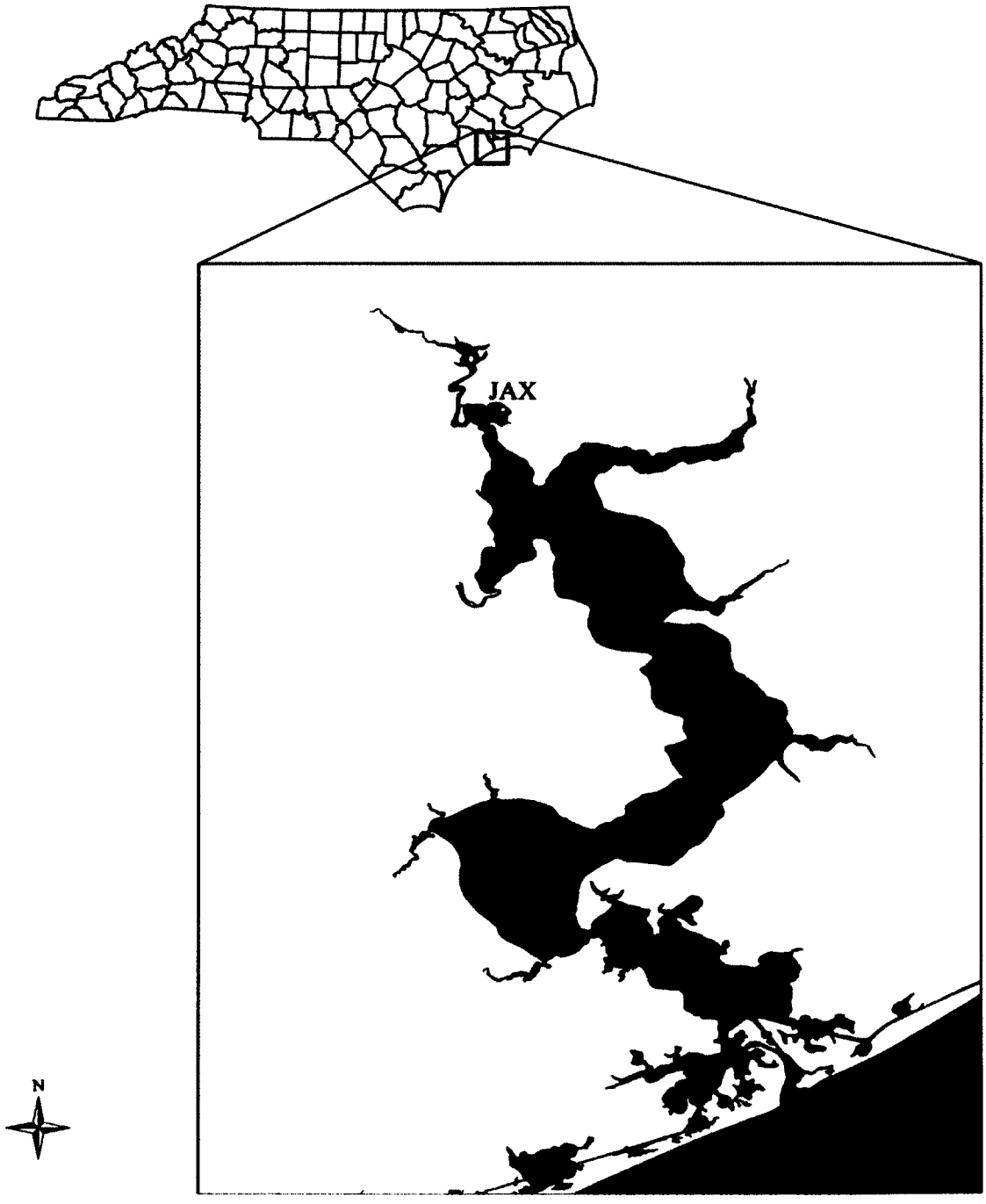

JAX
N

Figure 2. Sedimentary denitrification activities. Denitrification activities for the examined sites in this study were previously described in Chapter 3. Activities are presented in nmol N g^{-1} sediment hr^{-1} for summer and winter. Error bars represent standard deviation.

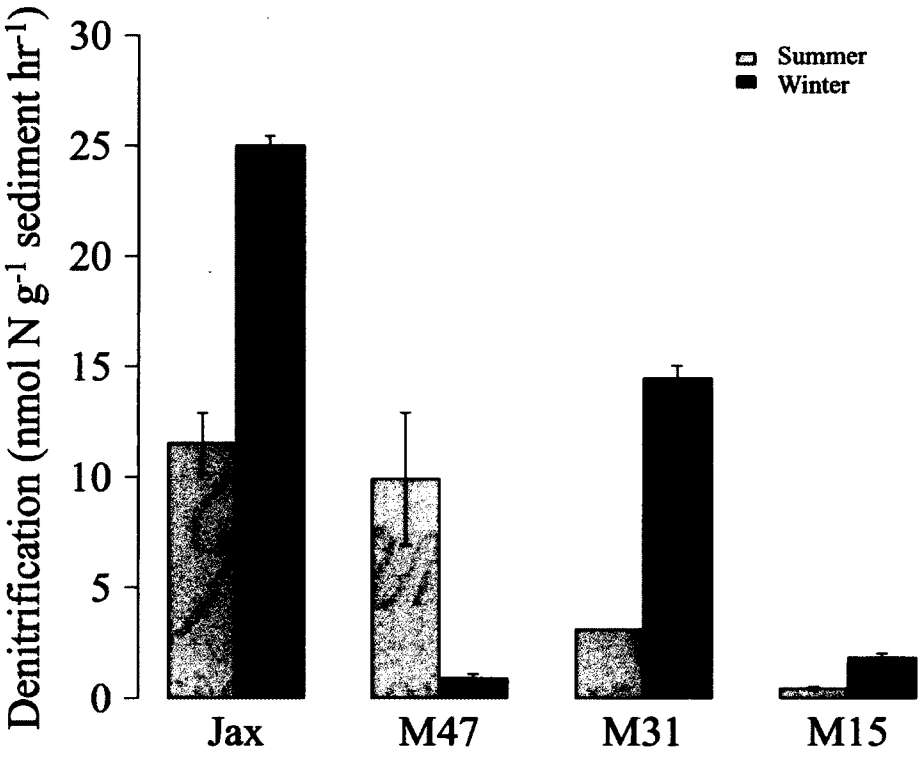

Denitrification (nmol N g-1 sediment hr-1)
30
25
20
15
10
5
0
Summer
Winter
Jax
M47
M31
M15

Figure 3. Detrended correspondence analysis of abundant *nirS* archetypes and environmental parameters. Sites formed two clusters (Cluster 1 & 2) based on microarray signal hybridization data of abundant archetypes. Seasons corresponding to the sites are S (summer) and W (winter). Environmental parameters are from the bottom water (BW), porewater (PW), and sediments (S).

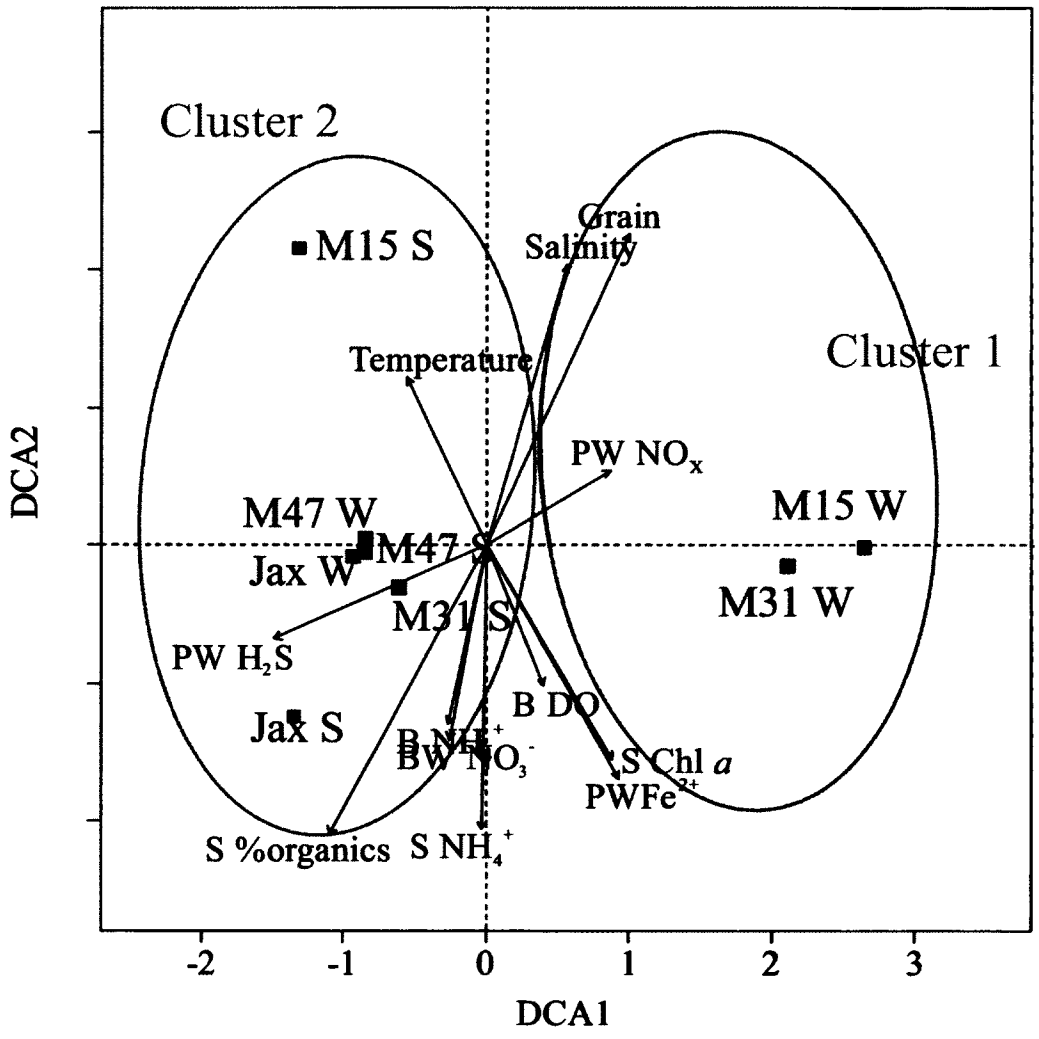

Cluster 2
Cluster 1
M15 S
Grain
Salinity
Temperature
PW NO_x
M47 W
M47 S
Jax W
M31 S
M15 W
M31 W
PW H_2S
Jax S
B DO
B NH_4^+
BW NO_3^-
S Chl *a*
PWFe^{2+}
S %organics
S NH_4^+
DCA2
DCA1
-2
-1
0
1
2
3

Figure 4. Heat map analysis showing Spearman's correlations for biological and environmental parameters. Colors depict r-values returned by correlation analysis between the 79 abundant *nir*S archetypes, environmental parameters, and denitrification activities. Eculidean distance measures were used to cluster columns based on similarities of r-values with dark red indicating strong positive correlations and dark blue indicating strong negative correlations. *nir*S archetypes (Nir) are followed by an identification number, environmental parameters include bottom water (BW), porewater (PW), and sediment (S) parameters, and denitrification activities.

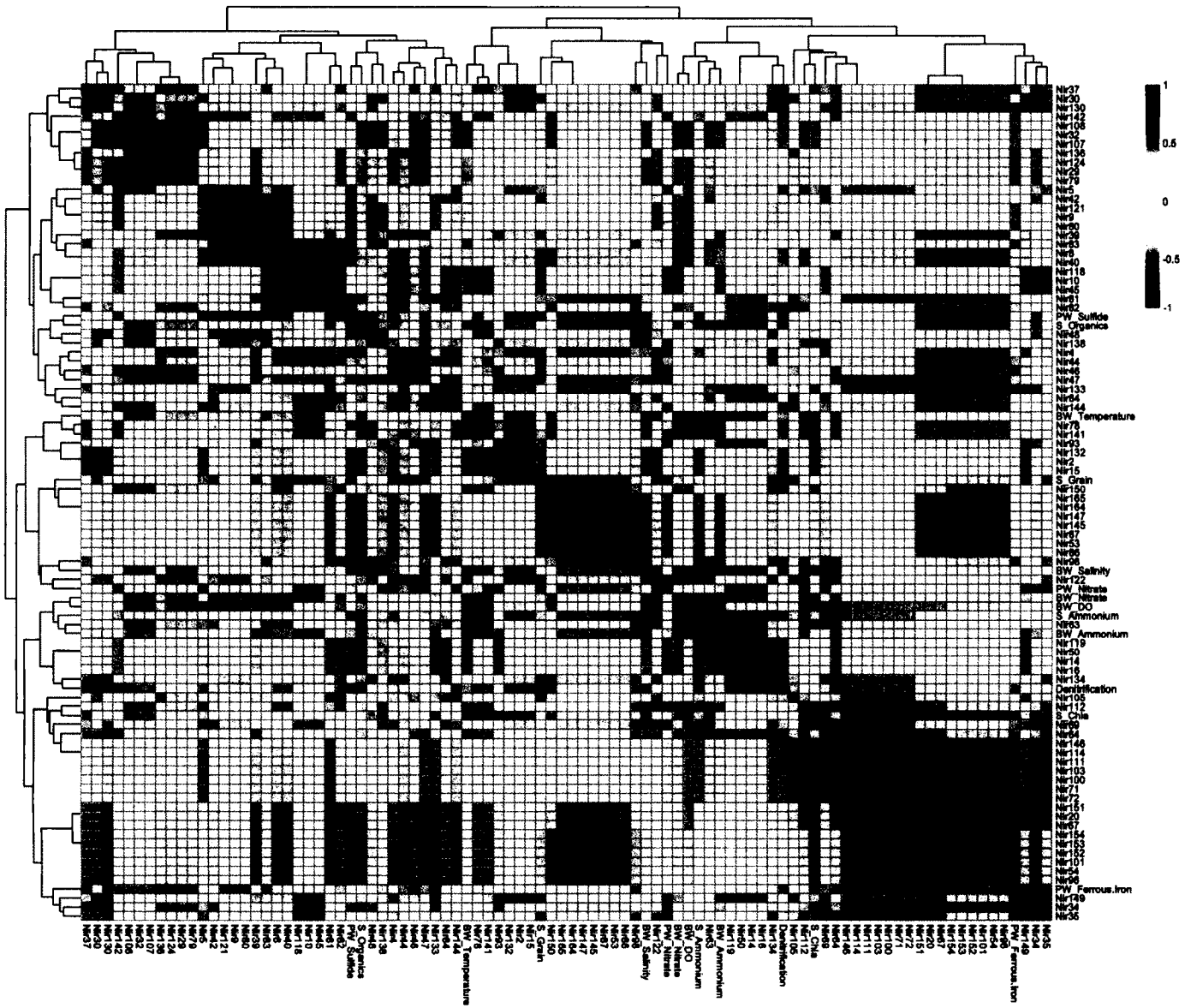
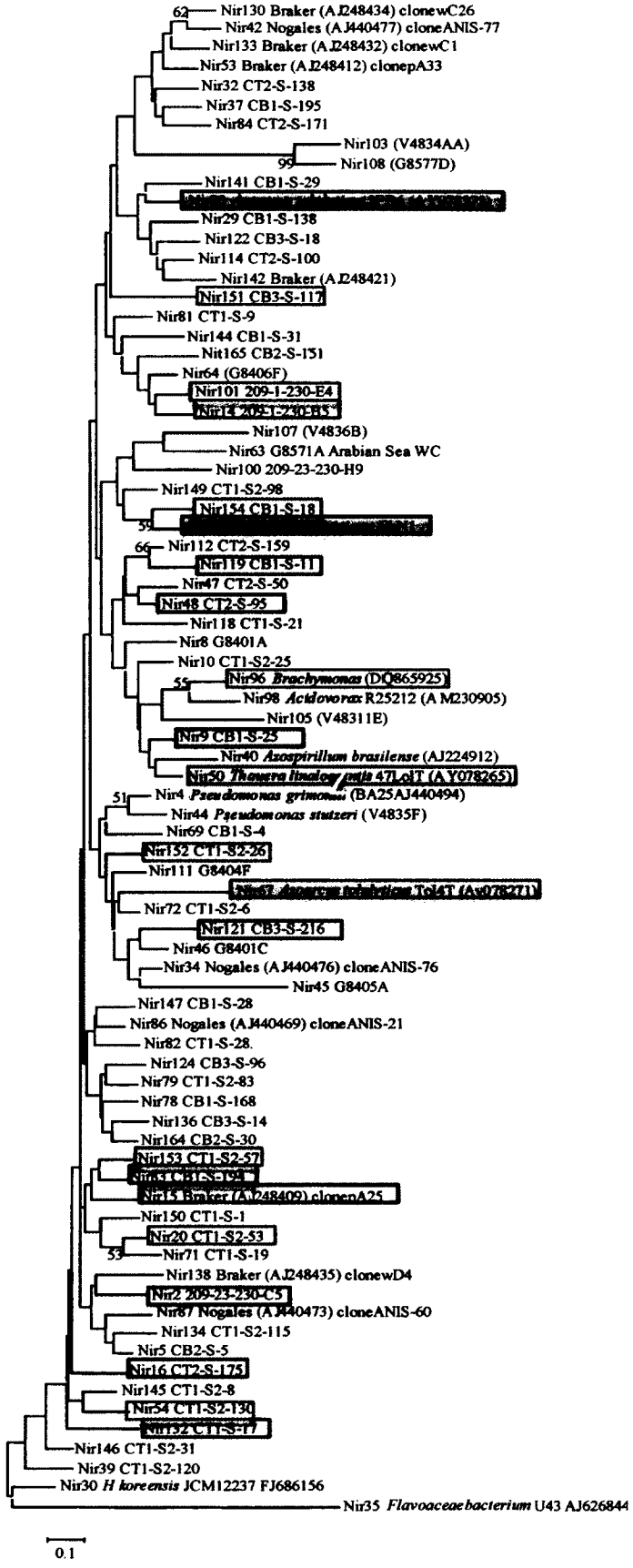

1
0.5
0
-0.5
-1
PW_Sulfide
S_Organics
BW_Temperature
S_Grain
BW_Salinity
PW_Nitrate
BW_Nitrate
BW_DO
S_Ammonium
BW_Ammonium
Denitrification
S_Chla
PW_Ferrous.Iron

Figure 5. Phylogenetic Tree of *nirS* sequences in microarray BCO13. Neighbor-joining trees were constructed and Bootstrap analysis with 1000 replicates was used to estimate confidence. Shaded boxes around archetypes depict known aromatic carbon degraders. Open boxes correspond to the co-occurrence of the archetypes with the known aromatic carbon degraders.


62
Nir130 Braker (AJ248434) cloneWC26
Nir42 Nogales (AJ440477) cloneANIS-77
Nir133 Braker (AJ248432) cloneWC1
Nir53 Braker (AJ248412) clonepA33
Nir32 CT2-S-138
Nir37 CB1-S-195
Nir84 CT2-S-171
Nir103 (V4834AA)
99
Nir108 (G8577D)
Nir141 CB1-S-29
Nir29 CB1-S-138
Nir122 CB3-S-18
Nir114 CT2-S-100
Nir142 Braker (AJ248421)
Nir151 CB3-S-117
Nir81 CT1-S-9
Nir144 CB1-S-31
Nit165 CB2-S-151
Nir64 (G8406F)
Nir101 209-1-230-E4
Nir107 (V4836B)
Nir63 G8571A Arabian Sea WC
Nir100 209-23-230-H9
Nir149 CT1-S2-98
Nir154 CB1-S-18
59
66
Nir112 CT2-S-159
Nir119 CB1-S-11
Nir47 CT2-S-50
Nir48 CT2-S-95
Nir118 CT1-S-21
Nir8 G8401A
Nir10 CT1-S2-25
55
Nir96 Brachymonas (DQ865925)
Nir98 Acidovorax R25212 (AM230905)
Nir105 (V48311E)
Nir9 CB1-S-25
Nir40 Azospirillum brasilense (AJ224912)
Nir50 Thauera linaloolentis 47LolT (AY078265)
51
Nir4 Pseudomonas grimontii (BA25AJ440494)
Nir44 Pseudomonas stutzeri (V4835F)
Nir69 CB1-S-4
Nir152 CT1-S2-26
Nir111 G8404F
Nir72 CT1-S2-6
Nir121 CB3-S-216
Nir46 G8401C
Nir34 Nogales (AJ440476) cloneANIS-76
Nir45 G8405A
Nir147 CB1-S-28
Nir86 Nogales (AJ440469) cloneANIS-21
Nir82 CT1-S-28.
Nir124 CB3-S-96
Nir79 CT1-S2-83
Nir78 CB1-S-168
Nir136 CB3-S-14
Nir164 CB2-S-30
Nir153 CT1-S2-57
Nir15 Braker (AJ248409) clonepA25
Nir150 CT1-S-1
Nir20 CT1-S2-53
53
Nir71 CT1-S-19
Nir138 Braker (AJ248435) clonewD4
Nir2 209-23-230-C5
Nir87 Nogales (AJ440473) cloneANIS-60
Nir134 CT1-S2-115
Nir5 CB2-S-5
Nir16 CT2-S-175
Nir145 CT1-S2-8
Nir54 CT1-S2-130
Nir146 CT1-S2-31
Nir39 CT1-S2-120
Nir30 H koreensis JCM12237 FJ686156
Nir35 Flavoaceaebacterium U43 AJ626844
0.1

CHAPTER 5

Tidal and seasonal dynamics of microbial communities responsible for sedimentary nitrogen cycling in the Cape Fear River Estuary

ABSTRACT

Tidal and seasonal fluctuations in the oligohaline reaches of estuaries may alter geochemical features that influence structure and function of microbial communities involved in sedimentary nitrogen (N) cycling. In order to evaluate sediment community responses to small-scale (tidal) and large-scale (seasonal) changes in different tidal regimes, nitrogen cycling rates and genes were quantified in three sites that span a range of tidal influence in the upper portion of The Cape Fear River Estuary. Environmental parameters were monitored during low and high tides in winter and spring. ^{15}N tracer incubation experiments were conducted to measure nitrification, denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonia (DNRA). Abundances of functional genes including bacterial and archaeal ammonia monooxygenases (*amo*A), nitrite reductases (*nir*S and *nrf*A), nitrous oxide reductase (*nos*Z), and hydrazine oxidoreductase (*hzo*) were measured using quantitative PCR assays. Denitrification rates were highest among the measured N cycling activities while bacteria carrying *nrf*A genes were most abundant. A discernable pattern in the short-term variation of N cycling activities and gene abundance was not apparent under the different tidal regimes. Significant seasonal variation in nitrification, denitrification, and anammox activities as well as bacterial *amo*A, *nir*S and *nos*Z gene abundance were observed, largely explained by increases in substrate availability during winter, with sediment ammonium playing a central role. These results suggest the coupling of nitrification to N removal pathways is primarily driven by organic carbon mineralization and independent of tidal or salinity changes. The linkage between structure and function of microbial communities was also evident as changes in denitrification and nitrification activities strongly reflected variations in the abundance of the respective functional genes.

INTRODUCTION

Estuaries are highly dynamic systems that form the transition between freshwater and marine environments. The interface between these two environments supports a tight coupling of biogeochemical activities that are important in regulating the amount of fixed nitrogen (N) in coastal systems. Microbially mediated N recycling processes such as nitrification and dissimilatory nitrate reduction to ammonium (DNRA) occur simultaneously with the removal processes of denitrification and anaerobic ammonium oxidation (anammox) in estuarine sediments (Capone et al. 2008). Each pathway can be influenced by a wide range of interacting factors including: temperature, salinity, dissolved oxygen (DO), substrate availability (nitrate + nitrite (NO_3^- + NO_2^-), ammonium (NH_4^+), dissolved organic carbon (DOC), and hydrogen sulfide (H_2S) (Cornwell et al. 1999, Dalsgaard et al. 2005, Seitzinger et al. 2006). These environmental factors change over small and large temporal scales in estuarine ecosystems and thus, differentially influence the activities and structure of N cycling microbial communities on these time scales.

Tidal fluctuations in the oligohaline reaches of aquatic systems result in small-scale changes in sediment and porewater chemistry, primarily as a result of movement of the water masses during the ebb and flood of the tide (Rocha & Cabral 1998, Usi et al. 1998, Mortimer et al. 1999). Such changes in the supply of oxygen and salinity influence the availability of organic material as well as the species and distribution of N, creating a bottom up control on biogeochemical cycling. Expansion of the oxic zone as a result of tidal forcing may support higher rates of remineralization and nitrification in intertidal estuarine sediments (Rocha & Cabral 1998, Mortimer et al. 1999). Enhanced nitrification

can provide substrate for denitrification when NO_3^- diffuses to anoxic zones; however, inhibitory effects on denitrification in intertidal sediments can also be sustained through prolonged oxygen exposure during ebb tide or flushing of nutrients during flood tide (Thompson et al. 1995, Usi et al. 1998). In addition, distinct differences in microbial community structure have been observed between high and low tides, linked to variability in environmental conditions (Chauhan et al. 2009, Kara & Shade 2009, Olapade 2012). Temperature, light and nutrient availability contributed to greater bacterial diversity and abundance observed during high and outgoing tide (Olapade 2012) while significant shifts in phylotype abundance occurred in response to elevated supply of dissolved organics during low tide (Chauhan et al. 2009). These studies primarily examine the effects of tidal fluctuation on planktonic microbial communities. However, little is known about the effects of tidal exchange on benthic communities in the oligohaline reaches of estuaries, where a major portion of N cycling takes place. Variations in N cycling activities and microbial community structure are likely to occur as a result of changes in sediment chemistry and the movement of different water masses with changing tides, greatly influencing estuarine N cycling.

Larger scale temporal shifts corresponding to seasonality influence sedimentary N cycling processes in various ecosystems (Jorgensen & Sorensen 1985, Berounsky & Nixon 1990, Rysgaard et al. 1995, Eriksson et al. 2003, Dunn et al. 2013, Lisa et al. 2014). Literature reports of peak N cycling activity vary both for season and for the reaction described; peaks often coincide with temperature as well as oxygen dynamics (Rysgaard et al. 1995, Dunn et al. 2013) or weather events that provide additional sources of substrate to support elevated processes (Eriksson et al. 2003, Lisa et al. 2014).

Seasonal changes in benthic microbial communities are also apparent, with significant shifts in composition and metabolism in response to changes in temperature observed in heterotrophic microbial communities (Kristensen 1993, Desnues et al. 2007). This trend is also evident in autotrophic microbial communities where particular ammonia oxidizing archetypes are more prevalent during specific seasons and correlated with temperature, DON and NH_4^+ concentrations (Bouskill et al. 2011). The seasonal reoccurrence of ammonia oxidizing organisms suggests the return of indigenous communities following large perturbations in more stable systems.

The greatest changes in geochemical conditions occur in oligohaline reaches of estuaries where fluctuations in salinity and nutrients occur tidally and seasonally, yet little is known about the response of sedimentary N cycling activities and sediment microbial communities to small or large-scale changes under these environmental conditions. The extent to which temporal variability in the environment influences both activities and structure of microbial communities together has largely escaped attention and can have implications for coupling of sedimentary N cycling processes and the overall ability to remove N from the ecosystem. It is therefore important to consider the effects of dynamic environmental conditions on microbial community structure and function when examining the temporal effects on estuarine biogeochemical cycling over short and long-term scales. The objective of this study was to investigate the linkage between the abundance and activities of microbial communities responsible for N cycling processes under short and long term changes in a tidal estuarine ecosystem. We examined the effects of temporal changes on nitrification, denitrification, anammox, and DNRA

activities and respective functional gene abundance in subtidal estuarine sediments at three tidal sites in the oligohaline reaches of the Cape Fear River Estuary, NC, USA.

MATERIALS AND METHODS

Study System

The Cape Fear River Estuary (CFRE), located in southeastern North Carolina (Figure 1), makes up a 72 km long portion of the lower Cape Fear River proper and empties directly into Long Bay (Mallin et al. 1999, Dafner et al. 2007). The Cape Fear River proper is a sixth-order brown water stream originating in the Piedmont (Mallin et al. 1999). Two fifth-order blackwater tributaries originating in the coastal plains, The Black and Northeast Cape Fear Rivers, also empty into the lower portion of the Cape Fear River proper.

The CFRE watershed encompasses 23,310 km^2, the largest in North Carolina, and supports one fifth of the population of the state. One half of the land within the basin is forested while the remaining fifty percent is dedicated to cropland and pastureland or is urbanized (Lin et al. 2006). Within the Cape Fear River basin, the most industrialized river basin in the state, are 641 licensed point discharges, a harbor, and state port in Wilmington (Mallin et al. 1999, 2000). High levels of inorganic nutrients enter the system through these point discharges and additional nonpoint sources such as runoff from urban, suburban, and livestock facilities.

Seasonal Sampling of the CFRE

Sampling was conducted during two seasons (winter and spring) in the upper portion of the estuary over the course of a tidal cycle in 2012. Sampling sites were selected based upon tidal salinity variation. Salinity, a conservative environmental parameter often used a proxy for changes in other geochemical conditions, was used to monitor the magnitude of tidal influence on our study sites. Sites included, Indian Creek (IC; 34.2842N, 77.9981W), where salinity is <0.1 and invariant with tidal stage. Two other sites, Navassa (NAV; 34.2589N, 77.9846W) and Horseshoe Bend (HB; 34.2422N, 77.9681W), had larger changes in salinity throughout the tidal cycle, ranging from 0 to 15. At each site, all sampling and measurements were conducted in the channel and at a subtidal portion of the west bank during both low and high tides. This sampling scheme allowed for seasonal comparison between winter and spring at each site under the two extreme tidal conditions. The design enabled us to deconvolute the effects of small and large-scale temporal variation in geochemical conditions on N cycling activities and community structure, seasonally and tidally.

Environmental Parameter Measurements

Environmental parameters including water column depth, temperature, salinity and dissolved oxygen (DO), were measured within 1 meter of the bottom at the time of sampling using a 6820 multi parameter YSI data sonde (YSI Incorporated, Yellow Springs, OH). Surface and bottom waters were 0.7μm (GF/F) filtered and stored on ice prior to nutrient analysis. Ammonium and NO_3^- concentrations were measured spectrophotometrically on a Bran Luebbe segmented flow nutrient auto analyzer using

phenol hypochlorite and Cd-reduction/azo dye methods, respectively following modified standard EPA methods (Long & Martin 1997). Samples designated for DOC and TDN analysis were stored in glass vials, preserved with H_3PO_4 and refrigerated until analysis. The samples were analyzed within one week on a Shimadzu 5050A analyzer following standard operating procedures.

Sediment samples were collected as previously described in Chapter 2 using a petite ponar grab (Wildco, Buffalo NY). The top 2 cm of sediment were collected and divided into aliquots to be characterized for solid phase sediment properties, N cycling rate determinations, and microbial molecular analyses. Sediment percent organic content was determined by loss on ignition (LOI) of dried sediments (500°C for 4 hours). Sediment NH_4^+ (free plus extractable) was measured by phenol hypochlorite following KCl extraction using a 1:1 ratio of 2M KCl to sediments (Mackin & Aller 1984). Sediments from each site were stored at -80°C for molecular analysis.

N cycling rates

Four N cycling reaction rates were measured: nitrification, denitrification, anammox, and DNRA. Anaerobic sediment slurry incubations using $^{15}NO_3^-$ (99 atom% 15N enrichment) were conducted to measure potential rates of, denitrification, anammox and DNRA in estuarine sediments.

Nitrification rates were determined under aerobic conditions using an isotope pool dilution method modified from Wessel & Tietema (1992). Incubations were conducted using 20g of sediment incubated in 80 mL filtered site water amended with 70μM NO_3^- at 0.7 15N atom% (Carini et al. 2010). Initial and final samples were taken at time (t) t_i and

t_f from the same incubation container and conducted for 24 hours in triplicate. $^{15}NO_3^-$ was measured on an Elemental Analyzer Isotope Ratio Mass Spectrometer (EA-IRMS; Delta V, Thermo Fisher Scientific) following $^{15}NO_3^-$ isolation using the Devarda's reduction and alkaline acid trap diffusion technique (Sigman et al. 1997, Holmes et al. 1998). Dilution of the isotope pool from t_i to t_f was used to calculate the rates of nitrification (Tobias et al. 2003).

Sediment slurry incubation experiments with ^{15}N tracer were conducted to measure potential rates of denitrification and anammox following the method of Lisa et al. (2014). Production of $^{29}N_2$, and $^{30}N_2$ was measured on a Gas Bench - Isotopic Ratio Mass Spectrometer (GB-IRMS; Delta V Plus, Thermo Fisher Scientific, Waltham, MA) and used to calculate the rate of anammox and denitrification following the method of Thamdrup & Dalsgaard (2002) as modified by Song and Tobias (2011). Percent anammox (%anammox) was estimated based on the rates of anammox and total N_2 production in each sample.

The accumulation of $^{15}NH_4^+$ in the same incubations was measured to calculate potential rates of DNRA (Tobias et al. 2001) according to the modifications of Song et al. (2014). Briefly, NH_4^+ was isolated from the slurry by alkaline acid trap diffusion following the addition of 7 mL of 40 ppt NaCl solution, 0.15 g MgO, and 3 μmoles of unlabeled NH_4^+ carrier. DNRA rate calculations were based on the concentration and ^{15}N mole fraction excess of extractable NH_4^+ as well as the $^{15}NO_3^-$ mole fraction (Tobias et al. 2001). Percent DNRA (%DNRA) was estimated based on the contribution of DNRA to total NO_3^- reduction (denitrification + anammox + DNRA).

Quantitative PCR Assays of functional genes

Sediment DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Griffiths et al. 2000, DeAngelis et al. 2010) with two additional modifications, 1.) the amount of wet sediment was increased from 0.5 to 0.75 g and 2.) Thermo Savant Fast Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad, CA) was used for cell disruption. DNA extractions were carried out on IC and HB sites from winter and spring sampling events only.

Real time PCR assays were used to quantify abundance of functional genes in order to obtain a quantitative measurement of the genetic potential a site has to carry out a particular biogeochemical reaction. Primers targeting genes encoding the catalytic subunits of relevant enzymes were used to quantify the abundances of microorganisms capable of N cycling processes. Thermal cycling, fluorescent data collection, and data analysis were carried out using the ABI 7500 Fast Real Time PCR System (Version 1.4). Assays were carried out in a volume of 20 μL containing 0.5 to 1.0 ng of template DNA and SYBR green using Go-Taq qPCR Master Mix (Promega Corporation, Madison, WI). PCR specificity was monitored by analysis of dissociation curves. The R^2 values for the standard curves were >0.96 for all runs.

Abundances of ammonia oxidizing bacteria and archaea were quantified by targeting ammonia monooxygenase (*amo*A) genes under the following PCR conditions: 95°C for 10 m; 50 cycles consisting of 95°C for 15 s, 53°C for 45 s, 72°C for 30 s, and 80°C for 30s (data acquisition); followed by dissociation step consisting of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s. Bacterial *amo*A gene fragments were amplified using the PCR primers amoA-1F and amoA-2R (Rotthauwe et al. 1997, Gao et

al. 2014) and archaeal *amo*A gene fragments were detected with Arch-amoAF and Arch-amoAR (Francis et al. 2005, He et al. 2007).

Denitrifying bacterial abundance was assessed by quantifying nitrite reductase (*nir*S) and nitrous oxide reductase (*nos*Z) genes. Primers used to quantify *nir*S genes were

nirS1F (Braker et al. 1998) and nirS-q-R (Mosier & Francis 2010). The thermal cycling conditions were modified as follows: 95°C for 15 m; 38 cycles of 95°C for 15 s, 62.5°C for 30 s, 72°C for 30 s, and 84°C for 35 s (data acquisition), with a dissociation step of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s. qPCR of *nos*Z genes were carried out using the primers nosZ2F and nosZ2R (Henry et al. 2006) under the following modified conditions: 95°C for 10 m; 50 cycles of 95°C for 15 s, 55°C for 45 s, 72°C for 35 s, and 75°C for 35 s (data acquisition), and a dissociation step of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s.

Abundance of anammox bacteria was quantified using qPCR primers HZOQPCR1F and HZOQPCR1R following the methods of Long et al. (2013). DNRA bacterial abundance was determined by quantify *nrfA* gene abundance following the method of Song et al. (2014).

Statistical Analysis

Data were examined for normality using the Shapiro-Wilk Test and log transformed to meet the assumptions of statistical inference for parametric tests as well as reduce the range of the data. Paired-T Tests were used to identify differences among activities and gene abundance between tides and seasonally between paired samples.

Pearson's product moment correlation analysis was used to investigate correlations between environmental conditions, potential N cycling activities and functional gene abundance. N transformation rates were also analyzed in conjunction with bacterial functional gene abundances as well as environmental parameters to determine if correlations between the variables exist. Due to the robust nature of the analyses, $\alpha=<0.05$ was retained to delineate significant relationships between all response and explanatory variables. All statistical analyses were conducted in R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing).

RESULTS

Environmental Parameters

Changes in salinity were negligible at the IC site ranging from 0.01 to 0.10 during both seasons (Table 1). Changes in salinity during the winter sampling event were greater at the two downstream sites, with salinity varying from 0.1 to 6.4 and up to 8.0 within the 6 hour tidal exchange at NAV and HB, respectively. The overall salinity at the downstream sites was higher during the spring low tide sampling, accompanied by lower amplitude of change between tidal stages. Bottom water temperature was constant throughout the sites and reflected typical seasonal conditions. DO in bottom water remained >6 mg L^{-1} during all sampling events.

Water column dissolved nutrients remained spatially uniform at the three sites within a tide and season, but were quite variable between tidal stage and season (Table 2). Bottom water NO_3^- levels were elevated in the winter but remained relatively constant between tidal stages. Seasonal and tidal trends for bottom water NH_4^+ were not observed.

DOC and TDN levels were both highest in winter. DOC was highly variable with the incoming tide while TDN consistently decreased with the incoming tide. The ratio of bottom water DOC:NO_x was also higher during the winter and elevated at high tide at all sites.

Sediment %organics were variable across tidal stage and season and increased with downstream movement towards NAV and HB (Table 3). Sediment extractable NH_4^+ was consistently low for both seasons. The greatest increase in sediment extractable NH_4^+ was also observed downstream at NAV and HB, with the incoming tide where the largest changes in salinity occurred.

N Cycling Rates & Their Correlation to Environmental Parameters

Tidal variations in all process rates - nitrification, denitrification, anammox, and DNRA - between low and high tide were not significantly different from one another ($p > 0.05$) (Figure 2). Seasonal variations were specific to particular N cycling processes. Several environmental parameters, including temperature, DO, and dissolved nutrients, showed strong seasonal variation and correlated with particular N cycling pathways, with sediment extractable NH_4^+ taking on a strong central role in connecting the N cycling activities.

Nitrification rates ranged from 0.02 to 5.72 nmols N g^{-1} wet sediment $hour^{-1}$ and were significantly higher in the winter ($p < 0.001$; Table 4a). Nitrification was negatively correlated with temperature, and positively correlated with DO levels, DOC, TDN, bottom water NH_4^+, as well as sediment extractable NH_4^+ (Table 5).

Denitrification was the highest of all N cycling processes, ranging from 2.50 to 44.21 nmols N g^{-1} wet sediment $hour^{-1}$, with significantly higher activities also occurring in the winter ($p=0.008$; Table 4b). Temperature negatively correlated with denitrification rates (Table 5). Interestingly, increases in DO and sediment extractable NH_4^+ were accompanied by increases in denitrification activities (Table 5).

Anammox activities were on the same order of magnitude as nitrification, ranging from 0.17 to 4.77 nmols N g^{-1} wet sediment $hour^{-1}$ (Table 4c). Highest anammox activities were also observed during winter ($p < 0.05$). Anammox activities were inversely correlated with temperature, but positively responded to increasing DO levels, DOC, TDN and bottom NH_4^+ (Table 5). Once again, sediment extractable NH_4^+ correlated with anammox activities (Table 5).

DNRA was the lowest of all N cycling activities throughout the study, ranging from 0.00 to 1.89 nmols N g^{-1} wet sediment $hour^{-1}$ (Table 4d). Differences in activities both tidally and seasonally were not significant for DNRA ($p > 0.05$). DNRA was positively correlated with salinity and negatively correlated with bottom water nitrate (Table 5). Sediment extractable NH_4^+ was also positively correlated with DNRA activities (Table 5).

Mean %anammox was 11.58 and overall %anammox spanned the broad range of 1.9 to 44.6% contribution to N_2 production. %DNRA also varied, ranging from 0 to 24.7% with a mean of 2.56% contribution to NO_3^- reduction. Seasonal, spatial, and tidal variations and correlations with environmental parameters were not significant for either %anammox or %DNRA (Table 5).

Functional Gene Abundance & Its Correlation to Environmental Parameters

Variations in functional gene copy numbers showed no discernable pattern with respect to the different tidal stages and were not significantly different from one another between low and high tides (Figure 3). However, seasonal trends in gene abundance were evident and correlated with particular environmental parameters.

Bacterial and archaeal *amo*A genes showed very similar distribution patterns in abundance. Bacterial *amo*A gene abundance ranged from 1.70×10^4 to 5.28×10^5, while archaeal *amo*A ranged from 1.12×10^4 to 2.15×10^5 gene copies g^{-1} wet sediment (Tables 7a and 7b). Bacterial *amo*A gene copy number was significantly higher in the winter ($p=0.040$), while archaeal *amo*A gene abundance was not seasonally influenced ($p>0.05$). As a result, bacterial *amoA* genes comprised a higher proportion of total *amo*A gene abundance during the winter, but this trend did not hold during the spring. Abundance of AOB and AOA also correlated with differing environmental parameters. Bacterial *amoA* gene abundance was negatively correlated with temperature and positively correlated with DO (Table 5). Alternatively, archaeal amoA gene abundance was correlated with DOC (Table 5). Positive correlations with sediment extractable NH_4^+ were common in both AOB and AOA communities (Table 5).

Abundance of *nir*S genes ranged from 6.37×10^5 to 6.40×10^7 and *nos*Z genes ranged from 1.61×10^6 to 5.99×10^7 copies g^{-1} wet sediment (Tables 7c and 7d). Both *nir*S and *nos*Z genes were also most abundant during winter ($p=0.031$ and $p=0.045$ respectively), but did not differ tidally. Several environmental parameters were commonly correlated with both *nir*S and *nos*Z gene abundance; these parameters

included temperature, DO, and sediment extractable NH_4^+ (Table 5). TDN was the only parameter that correlated with *nir*S gene abundance.

Abundance of *hzo* genes ranged from 1.38×10^4 to 5.80×10^5 gene copies g^{-1} wet sediment (Table 7e). Seasonal variations in *hzo* gene abundance were not present ($p>0.05$). Environmental parameters examined in this study showed no correlations with *hzo* gene abundance (Table 5). Bacteria possessing *nrf*A were the most abundant out of all groups in the examined communities, ranging from 1.10×10^7 to 1.54×10^9 gene copies g^{-1} wet sediment (Table 7f). Abundance of *nrf*A was not significantly different between two seasons ($p>0.05$). Sediment organics and sediment extractable NH_4^+ positively correlated with *nrf*A gene copy number (Table 5).

Correlations Among Rates and Functional Gene Abundance

Changes in N cycling activities strongly reflected changes in their respective functional genes for denitrification and nitrification but not for anammox and DNRA (Figure 4). Nitrification rates were positively correlated with both bacterial and archaeal *amo*A gene abundances (Table 5). Similarly, increases in denitrification rates reflected increases in both *nir*S and *nos*Z gene abundances. However, anammox and DNRA activities were not correlated with abundance of their respective genes.

DISCUSSION

Overall, denitrification was dominant among the four N cycling processes measured in this study with respect to rates and gene copy numbers that were consistent with other estuaries of similar trophic status (Dalsgaard et al. 2005, Henry et al. 2006, Seitzinger et al. 2006, Smith et al. 2007, Dong et al. 2009, Henderson et al. 2010). The strong positive relationship between denitrifier community abundance and potential denitrification rates suggest that denitrifiers are well adapted and poised to respond to increasing nutrients, despite the continually changing conditions in the tidal oligohaline reaches of the CFRE.

Nitrification rates were within the lower quartile of published values (Caffrey et al. 2007) but gene abundance (AOA and AOB) were not remarkably different from other systems (AOB (Stehr et al. 1995, Bernhard et al. 2007) and AOA (Mosier & Francis 2008, Moin et al. 2009)). Despite low nitrifying activities, the reflection of changes in nitrification rates with changes in AOB and AOA gene abundance, indicate that a large portion of the nitrifying community is active. Significant correlations between nitrification and denitrification rates with respective functional gene abundance have been observed in other coastal systems and suggest that the microorganisms present in these dynamic environments, including the CFRE, are metabolically active and tolerant to the constant changing conditions (Bernhard et al. 2007, Caffrey et al. 2007, Dong et al. 2009, Petersen et al. 2012).

Anammox, anammox % and *hzo* gene copy number were consistent with the range of activities (Dalsgaard et al. 2005, Nicholls & Trimmer 2009) and gene abundance (Dang et al. 2010, Lisa et al. 2014) reported in other estuarine systems. No significant

correlation between gene abundance and activities of anammox communities was observed in this study. This may be a result of both the physiological nature of the organisms involved in anammox as well as the dynamic environmental conditions of the sampling sites. Anammox bacteria prefer stable environmental conditions with a continual and simultaneous low supply of substrate and oxygen. Additionally, due to their slow growth and specific metabolic requirements, anammox bacteria are typically out-competed by heterotrophic denitrifiers in most dynamic environments such as estuaries (Dalsgaard et al. 2005).

DNRA activities were substantially lower than those measured in other estuaries (Tobias et al. 2001, Gardner et al. 2006, McCarthy et al. 2008) despite similar values of *nrf*A gene abundance (Dong et al. 2009, Song et al. 2014). Decoupling between abundance and activity of DNRA bacteria can be explained by the metabolic versatility of their anaerobic energetic pathways including fermentation, denitrification, anammox and sulfate reduction (Simon 2002, Kartal et al. 2007). Given the diversity of organisms possessing DNRA metabolism, it is also not particularly surprising that a correlation between activities and *nrf*A gene abundance was not observed (Woods 1938).

Short-term temporal variation in N cycling processes and functional gene abundance was observed in the upper CFRE during tidal exchange; however a significant, clear pattern was not found. These results are contrary to several studies that observed significant changes in activities and structure of microbial communities in intertidal sediments exposed to the atmosphere during low tide. The difference in findings could primarily be a result of a reduced capacity for solute exchange due to slower diffusion in continually inundated sediments of CFRE. Under these

circumstances of continual inundation, advection or seepage is less likely to accelerate solute exchange as it would in intertidal systems thereby not affecting N cycling communities or resulting activities simply with the changing tide.

Nitrification, denitrification, and anammox rates and respective gene abundance (with the exception of hzo); however, did show significant long-term variation, with elevated values observed during winter. Temperature was shown to be a major driver of nitrification, denitrification, and anammox activities, as well as functional gene abundance in ammonia oxidizing and denitrifying communities in the CFRE; however, co-variation between temperature and nutrients supply is likely contributing to higher winter activities. Generally, metabolic processes and microbial community composition positively respond to increases in temperature, as is sometimes the case with nitrification activities and AOB abundance (Berounsky & Nixon 1990, Cébron et al. 2003), denitrification and denitrifier abundance (Nowicki 1994, Szukics et al. 2010). However, the inverse relationships between nitrification and denitrification and temperature in this study, as well as the studies conducted by Kemp et al. (1990) and Bernhard et al. (2007), suggests seasonally variable environmental factors other than temperature have a stronger control on activities and structure of microbial communities in the oligohaline reaches of CFRE. The inverse relationships between N cycling activities and community structure with temperature in this study can be explained by a higher supply of nutrients, particularly DOC, TDN, and sediment extractable NH_4^+, to the upper reaches of the CFRE during the winter. Taking the relationship of the three variables into consideration, it is likely that elevated N supply during the winter and lack of competition for DIN is driving the availability of dissolved N and sediment extractable NH_4^+ ($r=0.82$, $p<0.001$;

r=0.47, p=0.024, respectively). This elevated supply of NH_4^+ during the winter may be responsible for higher winter nitrification activities and AOB abundance that can in turn support elevated denitrification and anammox activities in the CFRE. Although not directly measured in this study, our data suggest that a greater abundance of ammonia oxidizers and higher nitrification activities are, at least in part, coupled to denitrification and anammox activities and support a higher abundance of denitrifying communities. This conclusion is corroborated by an earlier study in the upper CFRE, which showed 43% of denitrification activities were coupled to nitrification (Hines et al. 2012). These findings are also supported by the occurrence of substrate induced stimulation of coupled nitrification-denitrification and nitrification-anammox in other marine and estuarine systems (Seitzinger 1994, Rysgaard et al. 1995, Cornwell et al. 1999, Lam et al. 2007, Caffrey et al. 2007, Crowe et al. 2012). Caffrey et al (2007) also noted that sediment NH_4^+ concentrations, as a result of organic matter remineralization, were highly central to nutrient cycling; results from this study suggest this is also the case in the CFRE.

Another important finding in this study is the correlations between AOB and AOA with different environmental parameters. Abundance of both AOB and AOA were significantly positively correlated with nitrification activities; all three factors were correlated with sediment extractable NH_4^+. However, DOC concentrations only correlated with changes in abundance of AOA. The response of AOB and AOA to different environmental conditions introduces the concept of niche differentiation and provides a clue to understand the role of AOA and AOB in estuarine environments (Bernhard & Bollmann 2010). The ecological concept of niche differentiation involves the partitioning of resources to allow organisms to occupy a habitat. The physiological

capabilities of AOA include tolerance to varying environmental conditions and ability to incorporate organic carbon in growth (Kelly et al. 2011). Evidence that AOA are capable of mixotrophic metabolisms with capabilities to assimilate organic acids has been demonstrated in soils and marine systems (Ouverney et al. 2000, Hallam et al. 2006, Stahl & de la Torre 2012b). The correlation between DOC and abundance of AOA can be explained by the potential for mixotrophic metabolism in AOA, supporting niche differentiation between AOB and AOA in the CFRE. The occupation of this particular niche by AOA allows the organisms to successfully compete with AOB for reduced N species, effectively contributing to estuarine sedimentary nitrification in the CFRE.

Increases in DNRA activities corresponded with increases in salinity in the CFRE, despite the lack of a significant difference between low and high tides. Large increases in salinity have been found to favor DNRA over denitrification (Gardner et al. 2006, Giblin et al. 2010); however, DNRA never exceeded denitrification and was consistently an order of magnitude lower than denitrification. Low activities in this study, consistent with findings in oligohaline systems (Giblin et al. 2013) suggest that the conditions necessary to allow DNRA to proceed were not met. Metabolic requirements of DNRA include a strong redox potential, higher C:N ratio, and an ample supply of labile organic matter (Tobias et al. 2001, Koop-Jakobsen & Giblin 2010). Although C lability and redox potential were not measured in this study, we can conclude that conditions in the upper reaches of the CFRE were not favorable for DNRA, despite the elevated salinity at particular tides

CONCLUSIONS

Denitrification activities were highest among the measured N cycling processes while bacteria capable of DNRA were most abundant. The strong centrality of sediment NH_4^+ levels and the potential for coupled nitrification-denitrification and nitrification-anammox supports the role of nutrients and substrate availability as drivers of these processes rather than temperature and salinity. Alternatively, salinity was found to positively influence DNRA activities. We observed significant correlations between nitrification activities and *amo*A gene abundances in both AOB and AOA, suggesting that both bacteria and archaea are equally important to NH_4^+ oxidation in the CFRE. Similar trends occurred with denitrification activities and abundance of denitrifying bacterial communities possessing both *nir*S and *nos*Z genes. The strong correlation between nitrifier and denitrifier microbial communities and rate measurements implies that abundances of microbial members is important in determining the magnitude of nitrification and denitrification activities.

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Table 1. Water column physical parameters in the Cape Fear River Estuary. Measurements of bottom water physical parameters were taken at the west bank and west channel of Indian Creek (IC), Navassa (NAV), and Horseshoe Bend (HB). Measurements were taken at high and low tides during the winter and spring in 2012.

		Salinity				Temperature (°C)				Dissolved Oxygen (mg L^{-1})			
		Winter		Spring		Winter		Spring		Winter		Spring	
Site	Location	low	high	low	high	low	high	low	high	low	high	low	high
IC	Bank	0.09	0.10	0.10	0.10	11.9	12.1	24.0	24.0	9.86	9.91	6.59	6.50
	Channel	0.09	0.10	0.10	0.11	11.9	12.1	24.0	23.9	9.90	9.87	6.61	6.38
NAV	Bank	0.10	2.30	2.84	4.70	12.1	12.7	24.0	23.9	9.87	9.62	5.92	6.03
	Channel	0.10	6.35	2.95	6.63	11.9	12.9	24.0	23.8	9.84	9.16	6.02	5.95
HB	Bank	0.19	7.44	6.02	11.10	12.2	13.2	23.9	23.6	9.86	9.17	6.01	6.14
	Channel	1.74	8.01	6.21	15.73	12.2	13.1	23.9	23.9	9.65	9.13	6.11	6.04

Table 2. Water column dissolved nutrients in the Cape Fear River Estuary. Measurements of bottom water nutrients were taken at the west bank and west channel of Indian Creek (IC), Navassa (NAV), and Horseshoe Bend (HB). Measurements were taken at high and low tides during the winter and spring in 2012.

		Nitrate (μM)				Ammonium (μM)				Dissolved Organic Carbon (μM)				Total Dissolved Nitrogen (μM)				Carbon:Nitrogen			
		Winter		Spring		Winter		Spring		Winter		Spring		Winter		Spring		Winter		Spring	
Site	Location	low	high	low	high	low	high	low	high	low	high	low	high	low	high	low	high	low	high	low	high
IC	Bank	71.5	72.6	55.5	55.3	12.4	13.6	4.0	5.4	1109	1255	490	884	94.1	93.7	46.9	38.7	15.5	17.3	8.8	16.0
	Channel	72.2	74.8	62.5	54.3	17.4	12.7	4.3	5.8	1207	1241	447	457	96.3	92.4	46.8	42.7	16.7	16.6	7.2	8.4
NAV	Bank	67.2	16.2	47.8	43.6	13.4	6.9	8.5	7.4	1213	1298	521	487	89.8	78.1	45.0	39.8	18.1	80.2	10.9	11.2
	Channel	66.1	38.3	37.5	35.1	12.6	15.6	7.3	8.3	1135	1266	478	465	91.7	69.2	41.1	37.7	17.2	33.0	12.8	13.3
HB	Bank	61.1	31.5	32.8	32.6	13.3	13.9	7.1	10.5	1491	1322	546	909	91.4	62.9	41.2	34.1	24.4	41.9	16.7	27.9
	Channel	35.2	8.1	15.8	28.4	59.6	7.0	5.9	9.3	1232	1084	499	428	79.9	54.7	40.0	31.3	35.0	133.7	31.5	15.1

Table 3. Sediment characteristics in the Cape Fear River Estuary. Sediments from the west bank and west channel of Indian Creek (IC), Navassa (NAV), and Horseshoe Bend (HB) were characterized at high and low tides during the winter and spring in 2012.

		Organics (%)				Ammonium (μmol g wet sed^{-1})			
		Winter		Spring		Winter		Spring	
Site	Location	low	high	low	high	low	high	low	high
IC	Bank	5.6	8.2	13.0	13.3	0.33	0.33	0.01	0.22
	Channel	0.2	0.2	0.5	0.2	0.02	0.02	0.00	NA
NAV	Bank	NA	6.8	49.4	14.5	0.08	0.77	0.01	0.01
	Channel	0.2	2.5	0.6	0.9	0.01	0.08	0.00	0.00
HB	Bank	13.2	11.3	19.8	19.1	0.05	0.29	0.01	0.00
	Channel	10.9	5.9	25.3	13.3	0.51	1.04	0.15	0.00

Table 4. Tidal and seasonal fluctuations in sedimentary N cycling activities in the Cape Fear River Estuary. Sediments from the west bank and west channel of Indian Creek (IC), Navassa (NAV), and Horseshoe Bend (HB) were collected at high and low tides during the winter and spring in 2012. Average N cycling activities ± standard deviation are presented as follows: A) nitrification, B) denitrification, C) anammox, and D) dissimilatory nitrate reduction to ammonium (DNRA). Lost samples are indicated by NA.

A

		Nitrification (nmol N g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	3.31 ± 0.00	1.88 ± 0.15	0.11 ± 0.02	0.05 ± 0.00
	Channel	1.22 ± 0.26	1.04 ± 0.00	0.12 ± 0.03	0.03 ± 0.02
NAV	Bank	4.64 ± NA	3.54 ± 0.10	0.36 ± NA	0.13 ± NA
	Channel	0.52 ± 0.13	0.65 ± 0.08	0.02 ± 0.02	0.07 ± NA
HB	Bank	2.26 ± NA	3.52 ± 0.11	0.14 ± 0.08	0.55 ± 0.38
	Channel	3.71 ± NA	5.72 ± 0.11	NA ± NA	0.17 ± NA

B

		Denitrification (nmol N g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	42.83 ± 3.30	18.40 ± 1.95	7.03 ± 0.72	6.41 ± 1.48
	Channel	5.69 ± 0.60	9.59 ± 1.26	5.62 ± 1.22	5.30 ± 2.60
NAV	Bank	27.00 ± 0.11	44.21 ± 1.84	7.10 ± 1.10	9.25 ± 0.99
	Channel	2.50 ± 1.22	18.84 ± 1.68	6.98 ± 0.35	11.72 ± 0.24
HB	Bank	14.69 ± 0.76	11.42 ± 0.66	3.84 ± 0.28	7.24 ± 1.70
	Channel	30.43 ± 1.16	30.55 ± 1.11	19.36 ± 0.33	23.22 ± 0.76

C

		Anammox (nmol N g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	4.77 ± 0.16	2.37 ± 0.22	0.24 ± 0.01	0.28 ± 0.01
	Channel	0.17 ± 0.10	1.15 ± 0.13	1.15 ± 0.99	1.78 ± 0.12
NAV	Bank	1.90 ± 0.73	3.86 ± 0.44	2.51 ± 0.79	1.30 ± 0.21
	Channel	2.01 ± 0.08	1.84 ± 0.25	0.52 ± 0.03	1.35 ± 0.22
HB	Bank	2.63 ± 0.20	1.62 ± 0.16	0.27 ± 0.01	1.03 ± 0.13
	Channel	2.51 ± 0.27	4.14 ± 0.01	0.38 ± 0.03	0.65 ± 0.00

D

		DNRA (nmol N g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	0.60 ± 0.03	0.12 ± 0.09	0.11 ± 0.10	0.24 ± 0.24
	Channel	0.14 ± 0.03	NA ± NA	0.00 ± 0.00	0.12 ± 0.17
NAV	Bank	0.16 ± NA	1.66 ± 1.09	NA ± NA	0.22 ± 0.02
	Channel	0.06 ± 0.00	0.33 ± 0.15	0.02 ± 0.02	0.36 ± 0.28
HB	Bank	0.21 ± NA	0.52 ± 0.38	0.04 ± 0.02	0.24 ± 0.13
	Channel	NA ± NA	NA ± NA	1.89 ± 1.21	1.69 ± 0.06

Table 5. Correlation analysis of N cycling activities and gene abundance with environmental parameters. Pearson Product Moment Correlation Analysis was conducted to identify correlations among different environmental parameters, activities, and gene abundance for pooled samples. Bold values indicate significant correlations.

	Bottom Water														Sediment			
	Salinity		Temperature		DO		DOC		TDN		Nitrate		Ammonium		Organics		Ammonium	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Nitrification	-0.07	0.7469	**-0.82**	**0.0000**	**0.80**	**0.0000**	**0.78**	**0.0000**	**0.68**	**0.0004**	-0.27	0.2048	**0.52**	**0.0103**	0.14	0.5255	**0.75**	**0.0001**
Denitrification	0.19	0.3835	**-0.45**	**0.0273**	**0.43**	**0.0371**	0.38	0.0662	0.30	0.1485	-0.40	0.0516	0.32	0.1221	0.27	0.2151	**0.67**	**0.0004**
Anammox	-0.08	0.7220	**-0.57**	**0.0034**	**0.55**	**0.0054**	**0.49**	**0.0161**	**0.49**	**0.0149**	-0.14	0.5245	0.32	0.1279	0.01	0.9502	**0.61**	**0.0019**
DNRA	**0.48**	**0.0343**	0.00	0.9964	-0.03	0.8984	-0.03	0.9115	-0.14	0.5596	**-0.74**	**0.0002**	-0.05	0.8305	0.41	0.0793	**0.49**	**0.0338**
Bacterial *amo*A	0.18	0.5495	**-0.68**	**0.0077**	**0.66**	**0.0102**	0.47	0.0901	0.51	0.0625	-0.32	0.2711	0.36	0.2122	0.23	0.4277	**0.65**	**0.0153**
Archaeal *amo*A	0.00	0.9941	-0.52	0.0556	0.51	0.0612	**0.57**	**0.0339**	0.44	0.1142	-0.19	0.5083	0.24	0.4186	0.51	0.0628	**0.61**	**0.0268**
*nir*S	-0.24	0.3807	**-0.64**	**0.0098**	**0.67**	**0.0063**	0.37	0.1742	**0.58**	**0.0239**	-0.15	0.5851	0.28	0.3139	-0.18	0.5222	**0.71**	**0.0048**
*nos*Z	0.24	0.3974	**-0.58**	**0.0235**	**0.55**	**0.0340**	0.40	0.1381	0.43	0.1110	-0.40	0.1440	0.33	0.2310	0.31	0.2578	**0.68**	**0.0069**
hzo	-0.13	0.6424	-0.47	0.0804	0.49	0.0607	0.41	0.1250	0.45	0.0944	-0.16	0.5770	0.04	0.8923	0.20	0.4686	0.47	0.0918
*nrf*A	0.36	0.1885	-0.10	0.7169	0.08	0.7797	0.21	0.4580	-0.04	0.8796	-0.47	0.0807	0.06	0.8312	**0.79**	**0.0004**	**0.60**	**0.0227**

Table 6. Abundance of relevant N cycling genes in the Cape Fear River Estuary during low and high tides. Sediments from the west bank and west channel of Indian Creek (IC), Navassa (NAV), and Horseshoe Bend (HB) were collected at high and low tides during the winter and spring in 2012. Gene copy numbers are presented for: A) bacterial ammonia monooxygenase (Bacterial *amo*A), B) archaeal ammonia monooxygenase (Archaeal *amo*A), C) nitrite reductase (*nir*S), D) nitrous oxide reductase (*nos*Z), E) hydrazine oxidoreductase (*hzo*), and F) cytochrome C nitrite reductase (*nrf*A). Average values are presented as gene copy number ± standard deviation. NA indicates lost samples.

A		Bacterial *amo*A (copy number g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	1.59E+05 ± 5.67E+04	3.52E+05 ± 3.77E+04	NA ± NA	5.09E+03 ± 1.07E+03
	Channel	1.40E+05 ± 2.26E+04	5.28E+05 ± 7.69E+04	NA ± NA	4.51E+04 ± 7.41E+03
HB	Bank	4.22E+05 ± 8.03E+04	2.59E+05 ± 1.48E+04	1.70E+04 ± 8.78E+03	4.66E+03 ± 2.00E+03
	Channel	6.30E+03 ± 2.38E+03	4.84E+04 ± 1.28E+03	2.75E+04 ± 5.86E+02	8.22E+03 ± 6.28E+02

B		Archaeal *amo*A (copy number g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	2.07E+05 ± 8.27E+04	1.34E+05 ± 4.70E+04	1.93E+05 ± 8.73E+04	1.91E+04 ± 5.54E+03
	Channel	1.20E+05 ± 6.85E+04	2.15E+05 ± 7.15E+04	NA ± NA	2.30E+04 ± 1.49E+04
HB	Bank	2.00E+05 ± 7.34E+04	1.83E+05 ± 5.34E+04	3.82E+04 ± 1.48E+04	1.30E+05 ± 7.05E+03
	Channel	1.80E+04 ± 5.04E+03	NA ± NA	3.11E+04 ± 1.78E+04	1.12E+04 ± 6.09E+02

C		*nir*S (copy number g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	1.41E+07 ± 6.77E+05	1.04E+07 ± 1.97E+05	2.99E+06 ± 2.40E+05	6.37E+05 ± 5.68E+04
	Channel	3.20E+07 ± 8.38E+05	6.40E+07 ± 2.03E+06	NA ± NA	5.45E+06 ± 3.67E+05
HB	Bank	3.15E+07 ± 3.02E+06	1.84E+07 ± 1.74E+06	5.70E+06 ± 4.58E+05	4.84E+06 ± 4.32E+04
	Channel	4.60E+06 ± 1.07E+05	1.32E+07 ± 1.23E+06	1.60E+07 ± 2.01E+06	4.50E+06 ± 2.45E+05

D		*nos*Z (copy number g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	1.53E+07 ± 1.19E+06	2.41E+07 ± 1.92E+06	1.76E+07 ± 1.89E+06	3.08E+06 ± 8.27E+05
	Channel	2.54E+07 ± 1.29E+06	5.99E+07 ± 1.46E+06	NA ± NA	1.10E+07 ± 1.93E+06
HB	Bank	2.32E+07 ± 2.05E+06	3.35E+07 ± 2.18E+06	9.46E+06 ± 2.09E+06	2.99E+06 ± 1.12E+06
	Channel	4.14E+06 ± 5.99E+05	1.15E+07 ± 1.06E+06	8.97E+06 ± 1.82E+05	1.61E+06 ± 3.72E+05

E		*hzo* (copy number g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	4.24E+05 ± 4.58E+04	1.46E+05 ± 8.89E+03	1.69E+05 ± 2.48E+04	3.74E+04 ± 1.36E+03
	Channel	1.44E+05 ± 1.86E+04	5.80E+05 ± 7.01E+04	NA ± NA	5.09E+04 ± 7.74E+03
HB	Bank	1.40E+05 ± 5.90E+04	2.72E+05 ± 4.88E+04	2.40E+05 ± 2.91E+04	1.12E+05 ± 6.35E+03
	Channel	7.92E+04 ± 3.31E+03	2.00E+05 ± 1.15E+05	2.22E+05 ± 6.40E+04	1.38E+04 ± 2.95E+03

F		*nrf*A (copy number g^{-1} wet sed)			
		Winter		Spring	
Site	Location	Low	High	Low	High
IC	Bank	3.10E+08 ± 2.74E+07	6.49E+08 ± 3.86E+07	9.20E+08 ± 3.32E+07	2.23E+08 ± 9.43E+06
	Channel	6.04E+08 ± 1.51E+07	1.54E+09 ± 9.08E+07	NA ± NA	2.09E+08 ± 1.67E+06
HB	Bank	3.97E+08 ± 7.42E+07	1.04E+09 ± 7.15E+07	6.27E+08 ± 5.37E+07	4.91E+08 ± 3.21E+07
	Channel	3.42E+07 ± 2.25E+06	3.32E+07 ± 2.43E+07	1.36E+08 ± 5.58E+07	1.10E+07 ± 6.33E+06

Table 7. Correlation analysis of N cycling activities with respective functional gene abundance. Correlations between nitrification and bacterial ammonia monooxygenase (Bacterial *amo*A), nitrification and archaeal ammonia monooxygenase (Archaeal *amo*A), denitrification and nitrite reductase (*nir*S), denitrification and nitrous oxide reductase (*nos*Z), anammox and hydrazine oxidoreductase (*hzo*), and dissimilatory nitrate reductase to ammonium (DNRA) and cytochrome C nitrite reductase (*nrf*A). Bold values indicate significant correlations. Correlation analyses were not run for unrelated parameters, as indicated by "x".

	Microbial Gene Abundance											
	Bacterial *amo*A		Archaeal *amo*A		*nir*S		*nos*Z		*hzo*		*nrf*A	
Process	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Nitrification	**0.8309**	**0.0002**	**0.6061**	**0.0216**	x	x	x	x	x	x	x	x
Denitrification	x	x	x	x	**0.6989**	**0.0037**	**0.6803**	**0.0053**	x	x	x	x
Anammox	x	x	x	x	x	x	x	x	0.2901	0.2943	x	x
DNRA	x	x	x	x	x	x	x	x	x	x	-0.0639	0.8436

Figure 1. Sampling sites in the Cape Fear River Estuary, NC, USA. Sampling sites in the upper, oligohaline portion of the CFRE include Indian Creek (IC), Navassa (NAV) and Horseshoe bend (HB).

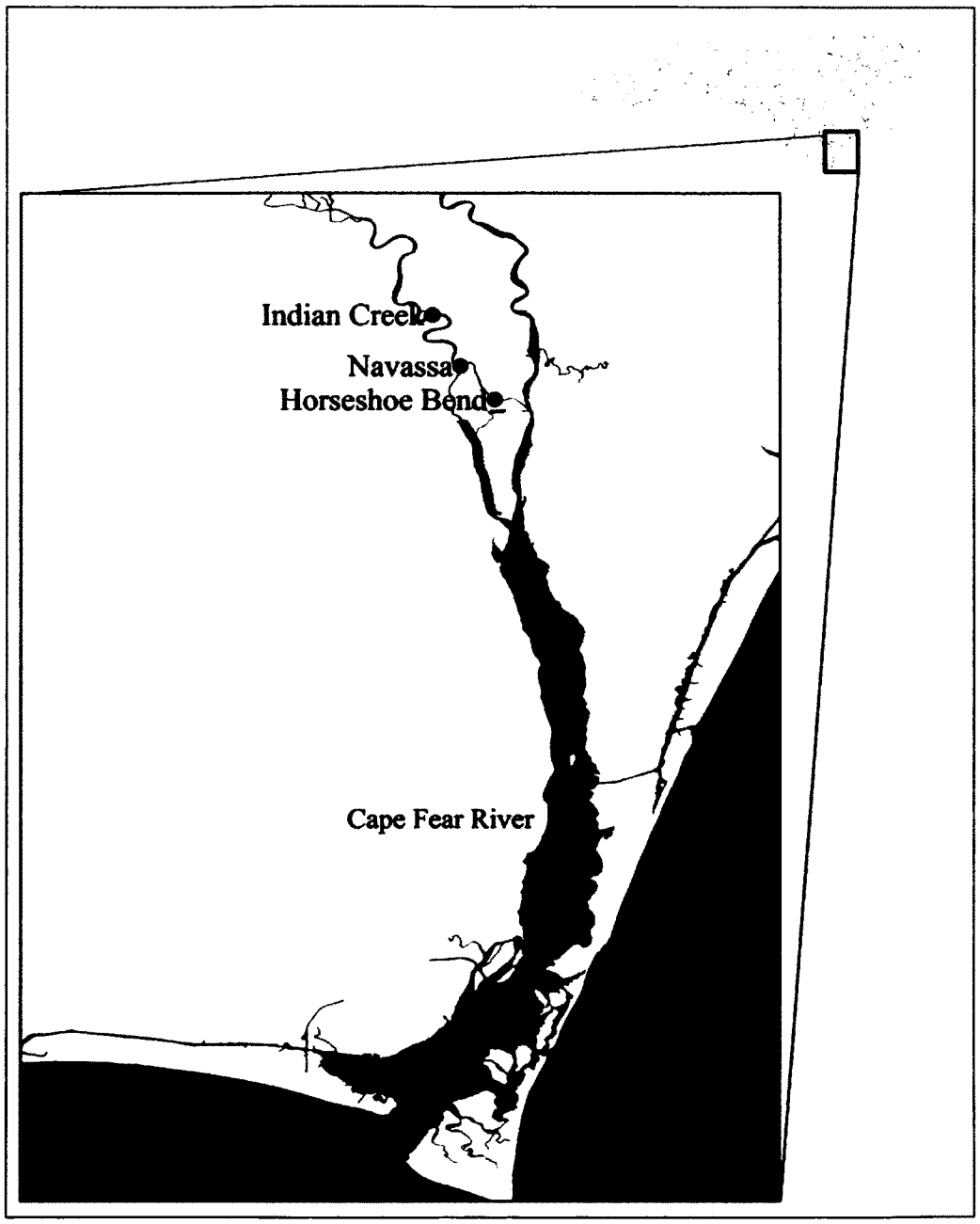

Indian Creek
Navassa
Horseshoe Bend
Cape Fear River

Figure 2. Fluctuation of sedimentary nitrogen cycling activities at high and low tides. N cycling values are presented in nmol N g^{-1} sediment hr^{-1} for two of six total sites Indian Creek (IC) and Horseshoe bend (HB) during winter only. Activities are presented as follows: A) nitrification, B) denitrification, C) anammox, and D) dissimilatory nitrate reduction to ammonium (DNRA). Error bars represent standard deviation.

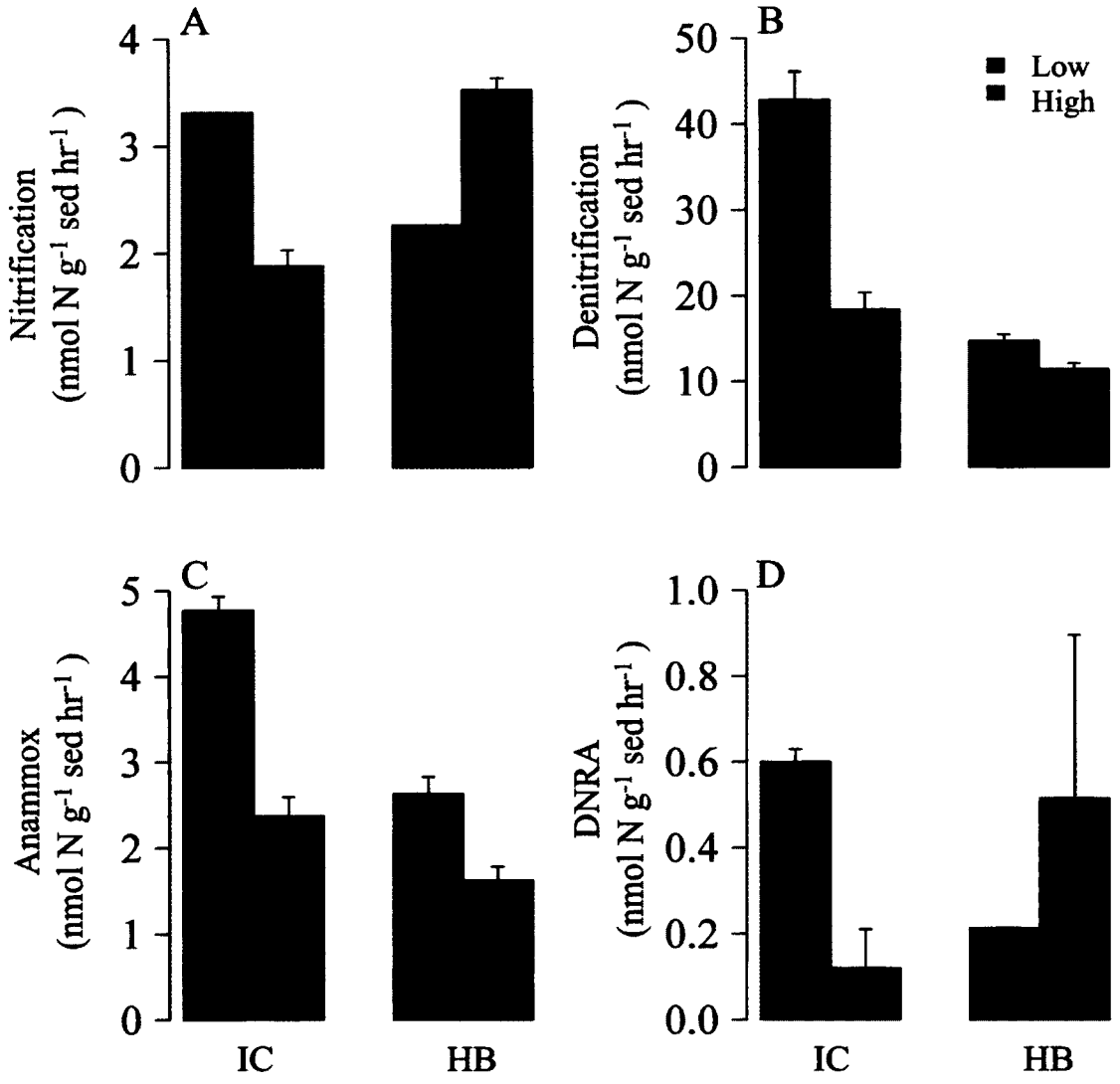

A
B
C
D
Nitrification (nmol N g-1 sed hr-1)
Denitrification (nmol N g-1 sed hr-1)
Anammox (nmol N g-1 sed hr-1)
DNRA (nmol N g-1 sed hr-1)
Low
High
IC
HB
IC
HB

Figure 3. Sedimentary nitrogen cycling functional gene abundance during low and high tides. Gene copy numbers are presented for two of six total sites Indian Creek (IC) and Horseshoe bend (HB) during winter only. Genes include: A) bacterial ammonia monooxygenase (Bacterial *amo*A), B) archaeal ammonia monooxygenase (Archaeal *amo*A), C) nitrite reductase (*nir*S), D) nitrous oxide reductase (*nos*Z), E) hydrazine oxidoreductase (*hzo*), and F) cytochrome C nitrite reductase (*nrf*A). Error bars represent standard deviation.

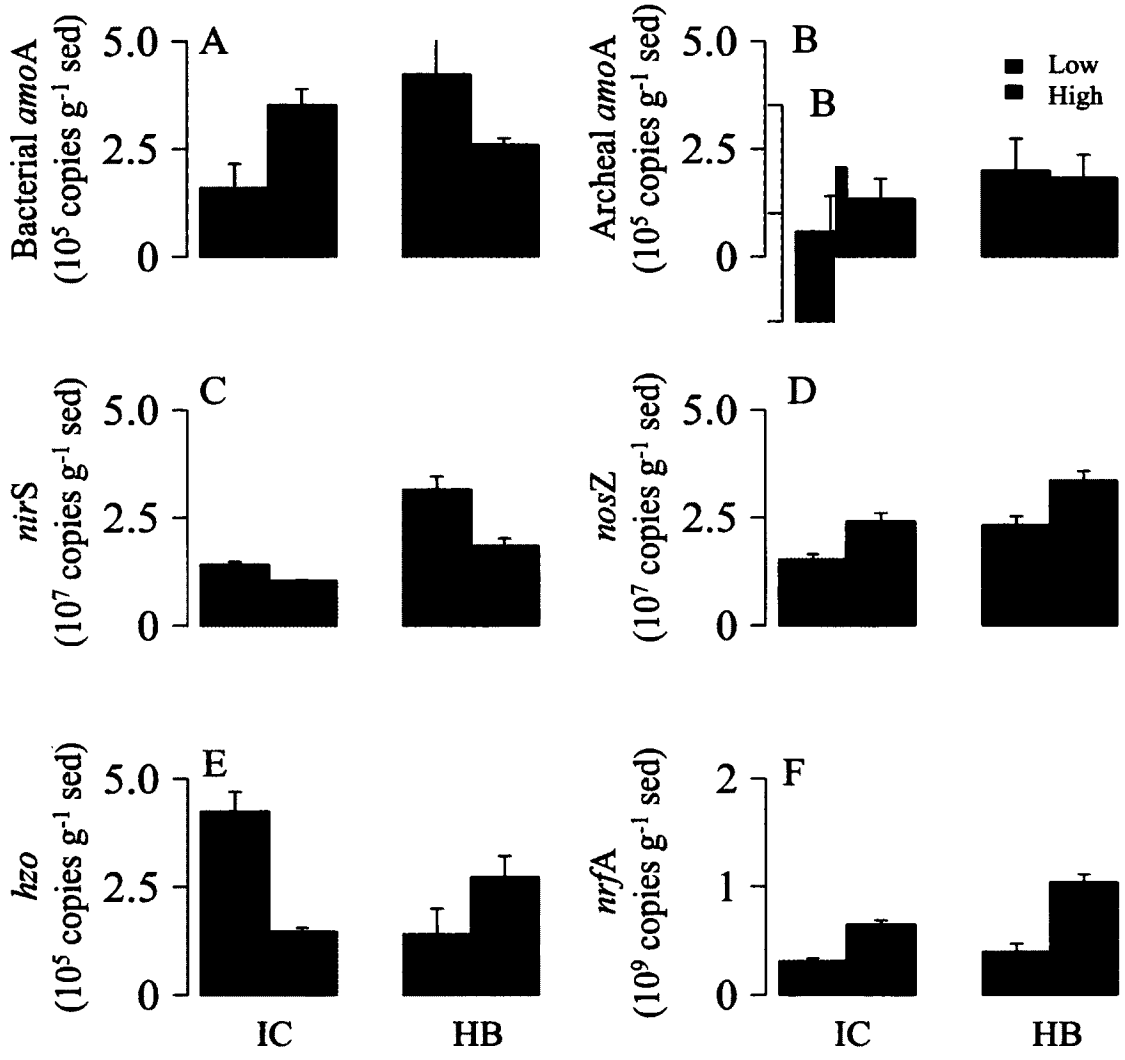
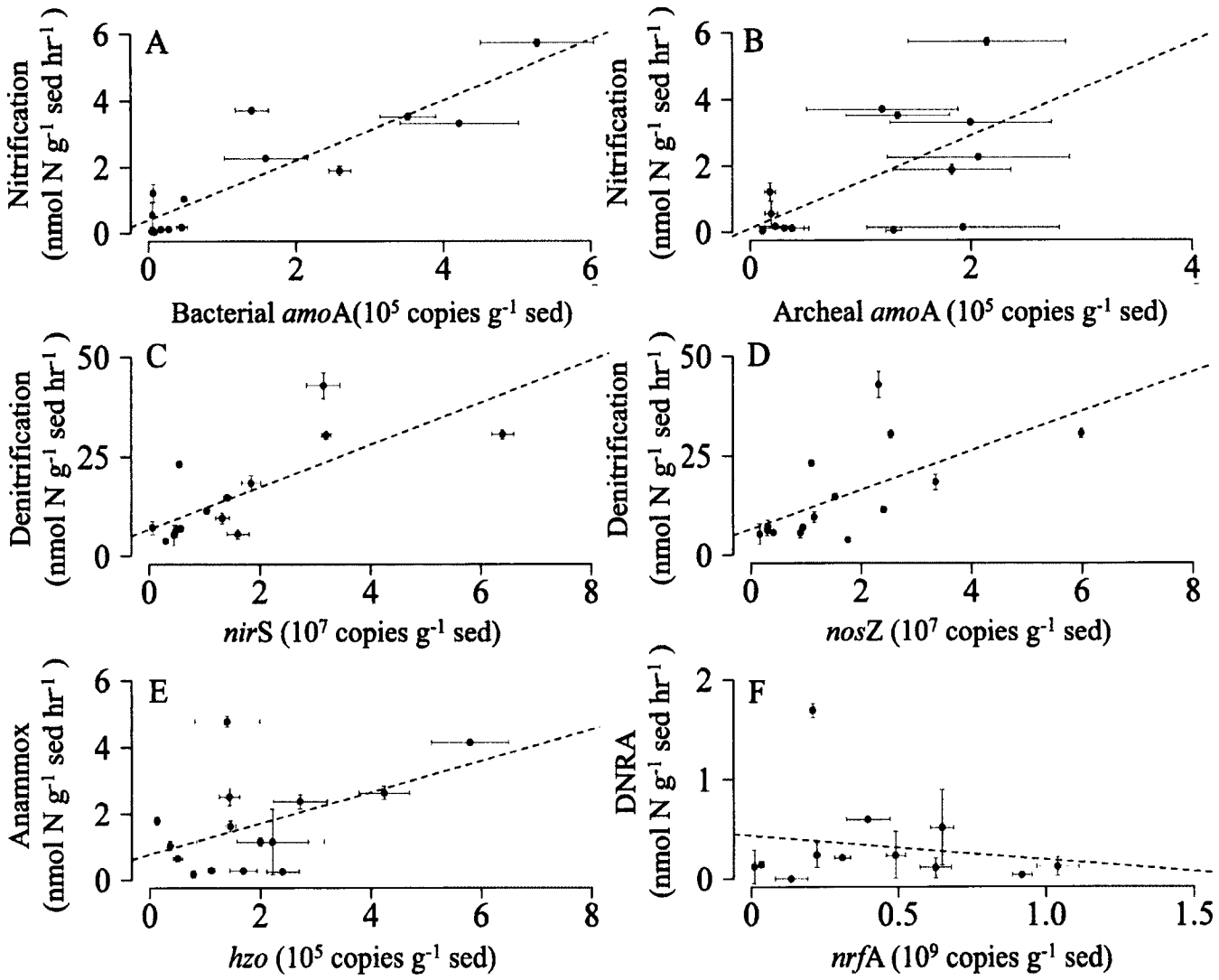

Bacterial amoA
(10^5 copies g^-1 sed)
5.0
2.5
0
A
Archeal amoA
(10^5 copies g^-1 sed)
B
B
Low
High
nirS
(10^7 copies g^-1 sed)
C
nosZ
(10^7 copies g^-1 sed)
D
hzo
(10^5 copies g^-1 sed)
E
nrfA
(10^9 copies g^-1 sed)
2
1
F
IC
HB
IC
HB

Figure 4. Correlation analyses of nitrogen cycling activities and respective functional gene abundance in the Cape Fear River Estuary. Correlations are presented for: A) nitrification and bacterial ammonia monooxygenase (Bacterial *amo*A), B) nitrification and archaeal ammonia monooxygenase (Archaeal *amo*A), C) denitrification and nitrite reductase (*nir*S), D) denitrification and nitrous oxide reductase (*nos*Z), E) anammox and hydrazine oxidoreductase (*hzo*), and F) dissimilatory nitrate reductase to ammonium (DNRA) and cytochrome C nitrite reductase (*nrf*A). Error bars represent standard deviation.


A
Nitrification (nmol N g-1 sed hr-1)
Bacterial amoA(10^5 copies g-1 sed)
B
Nitrification (nmol N g-1 sed hr-1)
Archeal amoA (10^5 copies g-1 sed)
C
Denitrification (nmol N g-1 sed hr-1)
nirS (10^7 copies g-1 sed)
D
Denitrification (nmol N g-1 sed hr-1)
nosZ (10^7 copies g-1 sed)
E
Anammox (nmol N g-1 sed hr-1)
hzo (10^5 copies g-1 sed)
F
DNRA (nmol N g-1 sed hr-1)
nrfA (10^9 copies g-1 sed)

CHAPTER 6

Distribution and niche differentiation of nitrifying prokaryotes in the Cape Fear River Estuary

ABSTRACT

Within the nitrifying prokaryotes exist the ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB). Patterns of the distribution and role of the AOB have been extensively studied, while the role of AOA and NOB in estuarine nitrification and interactions among nitrifying prokaryotes remain unclear. We conducted next generation sequencing of 16S rRNA gene (rDNA) and rRNA of archaea and bacteria to examine relative abundance of present and active nitrifying communities in association with environmental parameters to elucidate distribution and niche differences among the nitrifying prokaryotes. We found higher abundance of AOA at the rDNA level, but AOB and NOB were more abundant in the active nitrifying communities. The detection of either "*Ca.* Nitrosoarchaeum spp." or "*Ca.* Nitrosopumilus spp." varied based on salinity and ammonium concentrations. Negative correlations between *Nitrosococcus* spp. and *Nitrosomonas* spp. were observed in the AOB and a decrease of *Nitrospira* spp. coincided with the prevalence of *Nitrospina* spp when salinity increased. The formation of two nitrifying consortia were also proposed based on co-variations between "*Ca.* Nitrosopumilus spp." and *Nitrospina spp.* as well as *Nitrosomonas spp.* and "*Ca.* Nitrotoga spp.". Overall, salinity and NH_4^+ concentration were found to be important environmental variables in the development of niche differentiation of AOA, AOB and NOB communities in the Cape Fear River Estuary.

INTRODUCTION

Microbial communities are often primarily composed of rare taxa, with few abundant species that drive most ecosystem functions (Pedrós-Alió 2006). The rare species are proposed to be either dormant or slow growing. Rare taxa are usually described as the "seed bank" that contains the genetic diversity that could initiate shifts in community composition in response to environmental changes (Finlay et al. 1997, Pedrós-Alió 2006). These shifts can be manifested through increased growth rates of rare taxa when conditions are favorable (Campbell et al. 2011) and may occur continually to maintain the levels of microbial diversity observed in most ecosystems (Jones & Lennon 2010, Lennon & Jones 2011). Increases in ribosomal RNA content in a cell is considered to be a proxy of cell growth which can be used to assess the *in situ* growth rate of rare and abundant microorganisms (Campbell et al. 2011, Wilhelm et al. 2014). Further, the ratio of 16S rRNA:rDNA might be used as an index of microbial activity and growth to determine microbial community responses to environmental changes (Campbell et al. 2011, Campbell & Kirchman 2013).

Bacterial nitrifiers constitute a small portion (<1%) of the total prokaryotic community in natural environments (Ward & Carlucci 1985, Kirchman 2008). Archaea, on the other hand, are ubiquitous and abundant in the environment but the biogeochemical role of AOA in remains unclear (Francis et al. 2005). Nitrifying prokaryotes have an important role in the distribution of oxidized nitrogen (N) species in the environment through the oxidation of ammonia (NH_3) to nitrate (NO_3^-; Ward 1996). In the first step, NH_3 oxidation to nitrite (NO_2^-) is mediated by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA). NO_2^- is further oxidized to NO_3^-,

using oxygen derived from water (H_2O) by the enzyme nitrite oxidoreductase (Nrx) in nitrite oxidizing bacteria (NOB) (Hooper et al. 1997). AOA and AOB typically co-vary in the environment and the co-existence of AOA and AOB raises a question of niche differentiation between both groups. Niche differentiation involves the partitioning of resources to allow organisms to occupy a habitat. Niche separation of AOA and AOB, often related to salinity and NH_4^+ concentrations, may determine which organisms live and thrive, and where they are most abundant (de Bie et al. 2001, Bouskill et al. 2012, Bollmann et al. 2014). AOA sometimes outnumber AOB in marine and some estuarine systems (Francis et al. 2005, Wuchter et al. 2006, Caffrey et al. 2007, Abell et al. 2010) and often outnumber AOB in the oligotrophic environments due to their high affinity for NH_4^+ (Bollmann et al. 2014), further evoking the mystery surrounding their contribution to the global N cycle and role in nitrification.

Nitrite oxidizing bacteria convert NO_2^- to NO_3^- and belong to two phyla, *Proteobacteria* and *Nitrospirae.* The *Proteobacteria* phylum has four genera of NOB (*Nitrobacter*, *Nitrotoga*, *Nitrococcus*, and *Nitrospina*) while the *Nitrospirae* phylum contains the genus *Nitrospira* (Abeliovich 2006). Detecting and identifying *Nitrospira* spp. as an important NOB in many environmental samples led to a debunking of the consensus that *Nitrobacter* was the dominant NOB in the environment (Abeliovich 2006). However, *Nitrospina*-like bacteria were recently found to play an important role in N cycling in the Eastern Tropical Pacific. These findings further expand the knowledge pertaining to the role of NOB in the N cycling, but also reveal gaps in understanding the diversity and ecological features of this functional guild (Levipan et al. 2014).

The tidal study conducted in the Cape Fear River Estuary (CFRE; Chapter 5) provided an ideal environment to assess the role of AOA and AOB in sedimentary nitrification in response to the fluctuating environmental conditions We observed significant correlations between nitrification activities and *amo*A gene abundance in AOB and AOA, suggesting that both bacteria and archaea are equally important to NH_4^+ oxidation in the CFRE. The response of AOA and AOB to different environmental conditions provided some evidence to support niche differentiation and provided some clues to understand the role of AOA and AOB in estuarine environments. The composition of NOB was not examined previously; however, there might also be niche differentiation among NOB populations. Furthermore, by examining the ratio of rRNA:rDNA of nitrifying communities we might be able to identify more active nitrifiers corresponding to tidal and seasonal changes. Therefore, the objectives of this chapter were 1) To examine relative abundance of present and active nitrifying populations in sediments under high and low tide conditions; 2) To examine niche differences among the nitrifying populations; and 3) To identify environmental parameters affecting different nitrifying prokaryotes. In order to achieve the proposed objectives, next generation sequencing of 16S rRNA gene (rDNA) and rRNA of archaea and bacteria was conducted using the Ion Torrent Personal Genome Machine (PGM). We first examined the relative abundance of present and active prokaryotes by comparing 16S rDNA sequences with 16S rRNA sequences. We then identified known nitrifiers at the genus level to estimate the abundance of present and active AOA, AOB and NOB in the sediment communities. Finally, the environmental factors influencing the distribution of AOA, AOB and NOB were determined to address the concept of niche differentiation.

MATERIALS AND METHODS

Seasonal Sampling of the CFRE

Sampling was conducted during two seasons (winter and spring) in the upper portion of the estuary over the course of a tidal cycle in 2012 (described in detail in Chapter 5). The two sites selected for further analysis in this study were at the west banks of Indian Creek (IC; 34.2842N, 77.9981W), where salinity is <0.1 and invariant with tidal stage and Horseshoe Bend (HB; 34.2422N, 77.9681W), which had larger changes in salinity throughout the changing tides, ranging from 0 to15.

Environmental Parameter Measurements

Procedures for collection and processing of environmental parameters were described in detail in Chapter 5. Environmental parameters pertaining to this study included water column depth, temperature, salinity, dissolved oxygen (DO), dissolved organic carbon (DOC), and total dissolved nitrogen (TDN). Sediment porewater was examined for nitrate plus nitrite (NO_3^- + NO_2^- or NO_x^-) and ammonium (NH_4^+). Finally, sediment percent organic content (%organics) and free plus extractable NH_4^+ were quantified from sediments.

Sediment DNA and RNA extraction

Sediments from each site were stored at -80°C for molecular analysis. Sediment DNA and RNA were extracted in the same samples using the cetyltrimethylammonium bromide (CTAB) method (Griffiths et al. 2000, DeAngelis et al. 2010) modified as described in Chapter 5. Duplicate extractions were conducted and pooled. Samples for

DNA (30 μL volume) were treated with 1 μL RNase following and incubated at 37°C for 50 min. Samples designated for RNA were treated with DNAse using the TURBO DNA Free Kit (Life technologies) following manufacturer's protocol. RNA was quantified on a NanoDrop ND2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to 3ng μL^{-1} prior to the synthesis of cDNA. cDNA was synthesized using the iScript cDNA Synthesis Kit (Invitrogen, Burlington, Ontario, Canada). PCR was conducted in triplicates of each sample using Taq DNA Polymerase Kit (Invitrogen, Burlington, Ontario, Canada) with the primers 515 F and 915R. Both primers target the hyper-variable V4 and V5 regions of 16S rRNA genes in both bacteria and archaea. The primers were modified to include adaptor sequences used for the Ion Torrent PGM sequencer. The 915R primer also has an 8-bp barcode tag. The PCR conditions involved an initial denaturation step at 95°C for 3 min followed by 25 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min and a final extension step at 72°C for 5 min. Samples were pooled and amplicons were purified using UltraClean GelSpin DNA Purification Kit (Mo-Bio, Carlsbad, CA). The concentration of purified products was measured on a 2200 TapeStation instrument using D1K reagents (Agilent Technologies, Santa Clara, CA). The purified amplicons were sequenced using the Ion Torrent PGM with the Ion Torrent 400 bp sequencing kit protocol (Life Technologies, Grand Island, NY).

Bioinformatic and Ecological Analyses of 16S rDNA and rRNA sequences

FastQ file was downloaded from the Torrent Server using Torrent Suite v3.0 software after the completion of base calling (Life Technologies). Sequences were binned into 16 libraries according to the barcode sequence and initial quality filtering (>350 bp

and >25 of quality score) was conducted using the RDP Pipeline Initial Process (https://pyro. cme.msu.edu/init/form.spr). Acacia (Bragg et al. 2012) was used to de-noise the trimmed sequences. A total of 268,767 sequences were obtained after trimming and denoising (Table 1). In order to minimize the biases of sequence number differences in samples, 8900 sequences were randomly selected for downstream processing using OBIsample (http://www.cecill.info/licences/Licence_CeCILL_V2.1-en.html). The selected sequences were run through the Mothur Pipeline in the downstream analysis. Unique sequences were identified and aligned using the SILVA database. Sequences were screened for badly aligned sequences and chimeras that were subsequently discarded. Sequences were classified using the SILVA reference template and OTUs were grouped based on 97% similarity.

Differences between 16S rDNA and rRNA sequences were examined at the domain, phylum, and family levels. A further investigation of known nitrifiers was conducted at the genus level. Relative percent of nitrifying microorganisms were log (x+1) transformed to compare the ratio RNA to DNA. A heat map generated from Spearman's Correlation coefficients was used to investigate relationships among known nitrifiers and to assess the influence of environmental parameters on the abundance of particular genera and potential nitrification activities (Package 'pheatmap' R version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing).

RESULTS

Distribution of Archaea and Bacteria within 16S rDNA and 16S rRNA Sequences

Bacterial sequences comprised 53.5 to 86.5% of the overall 16S rDNA communities (Table 1). The number of archaea sequences represented anywhere from 13.5 to 46.5% of the total 16S rDNA sequences. The lowest seasonal archaeal abundance was observed at low tide during winter for both sites. Slightly higher abundances of archaea were found at HB. The relative abundance of bacteria comprising the 16S rRNA sequences increased at all sites while the relative abundance of archaea in the 16S rRNA samples was reduced to a range of 1.0 to 12.8%. Low tide winter sample was also the lowest in relative abundance of active archaea. IC spring high tide and both HB winter samples had the highest abundance of active archaea.

The pattern of decreasing relative abundance of archaea from present to active communities can be seen at the phyla level (Figure 2) and subsequently at each taxonomical level. The relative abundance of bacteria increases in the active communities at the phyla (Figure 3) and each taxonomical level. For the purpose of this chapter, known members of the nitrifying community were examined at the genus level with respect to their presence in the environment and their activity. It is important to note that a majority of the OTUs obtained from this study were unclassified on this level, we therefore only looked at known nitrifying genera in this study.

Present Members of CFRE Nitrifying Microbial Communities

Thaumarchaeota composed of 13.46% of total archaeal communities. The sequences associated with three *Candidatus* AOA genera, "*Ca.* Nitrosoarchaeum", "*Ca.*

Nitrosopumilus", and "*Ca.* Nitrosotalea", made up to 0.3% of the overall communities based on 16S rDNA sequences (Table 2). The relative abundance of AOA compared to AOB was greater on the DNA level. Shifts in the AOA populations between low and high tide were observed, and all three genera showed different patterns related to tide and site (Figure 4). "*Ca.* Nitrosoarchaeum spp." tended to decrease in relative abundance from low to high tide with one exception, an increase in abundance during winter at IC (Figure 4). The relative abundance of "*Ca.* Nitrosopumilus spp." was unchanging at HB between tides but varied at IC with no clear pattern pertaining to tidal stage. "*Ca.* Nitrosotalea spp." were consistently detected in the 16S rDNA communities at low tide but absent in high tide conditions.

The sequences assigned to the orders of Chromatiales (containing *Nitrosococcus*), Nirosomonadales (containing *Nitrosomonas* and "*Ca.* Nitrotoga" Desulfobacterales containing *Nitrospina*), and Nitrospirales (containing *Nitrospira*) contributed 0.02%, 0.43%, 0.13%, and 0.78% of total bacterial 16S rDNA sequences respectively. The relative abundance of NOB exceeded that of AOB and AOA at each site; AOB comprised the lowest relative abundance of the known nitrifying microorganisms on the DNA level (Table 2). The presence of AOB belonging to the genera of *Nitrosococcus* and *Nitrosomonas* varied between sites; *Nitrosococcus* spp. appeared to be restricted to low tide while *Nitrosomonas* spp. appeared at high tides and low tide IC spring (Figure 4). The nitrite oxidizing *Nitrospina* spp. and *Nitrospira* spp. were the most abundant nitrifiers in the bacterial communities, while "*Ca.* Nitrotoga spp." were only detected at IC high tide during winter.

Active Members of CFRE Nitrifying Microbial Communities

Active AOA made up 0.1% of the overall communities based on 16S rRNA sequences. Active members of the Thaumarchaeota comprised 6.03% of the overall active microbial communities and the relative abundance of AOA compared to AOB was lower on the RNA level. Shifts in the relative abundance of AOA populations between low and high tide were observed in the active communities (Table 2). "*Ca.* Nitrosoarchaeum spp." generally decreased at high tide, with one exception at IC during high tide in the winter. Active "*Ca.* Nitrosopumilus spp." were only detected during the winter at high tide at IC, but were always present at HB during both seasons during both tidal stages. "*Ca.* Nitrostolea spp." were undetectable in the RNA communities.

The pattern for nitrifying bacteria was opposite to that of the archaea as active nitrifying bacterial communities contributed to a greater relative abundance compared to their respective 16S rDNA counterparts (Table 2). Chromatiales (containing *Nitrosococcus*), Nirosomonadales (containing *Nitrosomonas* and "*Ca.* Nitrotoga") Desulfobacterales containing *Nitrospina*), and Nitrospirales (containing *Nitrospira*) contributed 0.08%, 0.98%, 2.34%, and 1.27% of the total RNA communities respectively. Active AOB belonging to the genus *Nitrosococcus* increased with the incoming tide during both seasons at IC and followed the opposite pattern at HB (Figure 4). *Nitrosomonas* 16S rRNA sequences increased at HB from low to high tides but were relatively stable at IC over the tidal cycle. The nitrite oxidizing "*Ca.* Nitrotoga spp." were only detected at IC low tide during spring and at HB high tide during winter. *Nitrospira* spp. were the most abundant of the active NOB at IC and outnumbered AOA and AOB. Though less abundant at HB, *Nitrospira* spp. made up a large percent of the active

nitrifying communities in the CFRE (Table 2). 16S rRNA sequences associated with *Nitrospina* were also elevated at HB during winter, with similar relative abundances to *Nitrospira*. Active *Nitrospira* spp. were lower at IC.

Comparison of 16S rDNA and 16S rRNA Sequences Associated with CFRE Nitrifying Microorganisms

A trend of decreases in active archaea relative to present archaea and subsequently increases in the relative abundance of active bacteria was observed from the domain to the genus level. AOA followed the same pattern of decreasing relative abundance when we examined their presence and activity within the microbial community (Figure 5B). The dotted line represents a 1:1 ratio of RNA:DNA. In most cases for the AOA (top) and again for the total AOA sequences (bottom), the points fell below this line indicating a decrease in 16S rRNA relative to 16S rDNA. There were three cases where an increase in relative abundance of a AOA from present to active was observed (Figure 5A); the relative abundance of 16S rRNA increased in "*Ca.* Nitrosoarchaeum spp." at IC low tide during winter and IC high tide during spring and in "*Ca.* Nitrosopumilus spp." at IC high tide during winter. The sites with the most abundant AOA in the 16S rDNA samples (HB low tide spring) also had the highest relative percent of active AOA.

"*Ca.* Nitrosoarchaeum spp." and "*Ca.* Nitrosopumilus spp." were the two most abundant AOA and were present in the communities in approximately equal proportions. Interestingly both were only active in the same community at HB low tide winter and spring, and in all other cases, one was active and the other was absent. The presence of

only "*Ca.* Nitrosoarchaeum spp." or "*Ca.* Nitrosopumilus spp." in the active communities often coincided with increases in the relative abundance of the active AOA relative to present communities (Figure 5A and 5B). Additionally, "*Ca.* Nitrosotalea spp." were present at three sites but was not detected among the active AOA.

The pattern for nitrifying bacteria was opposite of the archaea (Figure 5B). An interesting trend was made clear when the AOB and NOB were pooled and a separation of the two groups emerged as RNA:DNA in NOB showed greater shifts above the 1:1 line. There was only one instance, "*Ca.* Nitrotoga" at IC high tide during winter, where relative abundance of bacterial sequences decreased in the 16S rRNA when compared to relative abundance in the 16S rDNA (Figure 5A). The highest abundance of active nitrifiers was observed at high tide during winter at both IC and HB.

Despite its absence in several of the 16S rDNA samples, *Nitrosomonas* spp. were reliable members of the active nitrifying community and generally more abundant in the active communities than *Nitrosococcus* spp.. *Nitrosococcus* spp. were active at most of the sites with the exception of HB high tide spring (Figure 5A). An additional interesting discovery about HB high tide spring sample is that only *Nitrospira* sequences were detected at the 16S rDNA level; when the active community was examined, *Nitrospira* spp., *Nitrospina* spp., and *Nitrosomonas* spp. were active in equal proportions. "*Ca.* Nitrotoga spp." were only detected at one site in the 16S rDNA samples, but active in two different sites without an apparent trend. A higher abundance of nitrite oxidizers was observed, with *Nitrospira* spp. and *Nitrospina* spp. contributing to a greater relative abundance than the rest of the nitrifying genera. The relative abundance of active *Nitrospina* spp. was higher at HB than IC.

Co-variation among Nitrifying Communities

Co-variation among nitrifying genera as well as shifts in their distributions were observed based on a heat map analysis showing Spearman rank correlations in 16S rDNA and rRNA samples (Figure 6). "*Ca. Nitrotoga* spp.", *Nitrospira* spp., "*Ca. Nitrosoarchaeum* spp.", and *Nitrosomonas* spp. were positively correlated with each other (Figure 6A). The AOA, "*Ca.* Nitrosopumilus spp." co-varied with the NOB, *Nitrospina* spp. in the active and present communities (Figure 6B). "*Ca.* Nitrosopumilus spp." were negatively correlated with "*Ca.* Nitrosoarchaeum spp.". Negative correlations between *Nitrospina spp.* and AOB emerged, largely driven by the negative relationship with *Nitrospira* spp.. Active members of the genera *Nitrosococcus* were also negatively correlated with the co-varying "*Ca.* Nitrotoga spp.", *Nitrosomonas* spp. and *Nitrospira* spp..

The Influence of Environmental Factors on The Distribution of Nitrifying Communities

DNA community. The influence of environmental parameters varied depending on the functional group and among the different genera within the same functional group (Figure 6A). "*Ca.* Nitrosopumilus spp." the co-varying NOB, *Nitrospina* spp. were positively correlated with salinity, NH_4^+, and DOC. The relative abundance of *Nitrosococcus* spp. was strongly correlated with DOC, TDN, and porewater water NO_3^-. Negative correlations with temperature were observed in "*Ca.* Nitrotoga spp.", *Nitrosomonas* spp. and *Nitrosococcus* spp. The distribution of "*Ca.* Nitrosotalea spp." was positively correlated with turbidity and negatively correlated with salinity and NH_4^+. "*Ca.* Nitrotoga spp." and "*Ca. Nitrosoarchaeum spp.*" were positively correlated with

bottom water NO_3^-, sediment NH_4^+ and DO. And *Nitrospira* spp. distribution showed a strong positive correlation with sediment NH_4^+.

RNA community. The environmental factors associated with active nitrifying organisms were also examined though heatmap analysis (Figure 6 B). Salinity and DOC, and bottom water NH_4^+ were still important environmental factors associated with the active members of "*Ca.* Nitrosopumilus spp." and *Nitrospina* spp.. "*Ca.* Nitrosopumilus spp." were negatively correlated with turbidity while "*Ca.* Nitrosoarchaeum", "*Ca.* Nitrotoga spp.", and *Nitrosomonas* spp. positively correlated with turbidity and temperature. "*Ca.* Nitrosoarchaeum", "*Ca.* Nitrotoga spp.", and *Nitrosomonas* spp. sequences also negatively correlated with NH_4^+, and DOC. Additionally, positive correlations with DO, bottom water NO_3^-, and DOC and active *Nitrospira* spp. emerged.

DISCUSSION

Overall, the higher abundance of active AOB in the system suggests that bacteria primarily drive nitrification in the upper estuary and the role of AOA in the oligohaline reaches of the CFRE is minor. While AOA are generally more abundant in marine and estuarine systems, AOB were recently found in higher numbers and contributed to most of the nitrification (Puthiya Veettil et al. 2014, Li et al. 2015) as a result of higher NH_4^+ levels. Higher levels of NH_4^+ in the upper CFRE may reduce the competitive advantage of AOA over AOB for substrate, and allow AOB to successfully compete. Active archaea were most abundant at HB where salinity ranges from 0-14 and during the spring when lower NH_4^+ concentrations were measured in the water column and sediments and sediment %organics were higher. These findings are consistent with the studies that

support their use of small organic compounds and high affinity for NH_3 that enables AOA to outcompete AOB in oligotrophic environments.

This study was restricted to the oligohaline tidal reaches of the CFRE; therefore, an adequate representation of the role of AOA throughout the estuary was not obtained. Despite this, some insights into niche differentiation of AOA and AOB as well as their role in estuarine nitrification can be recognized. The presence of two co-varying groups, "*Ca.* Nitrosopumilus spp." with *Nitrospina spp.* and *Nitrosomonas spp.* with "*Ca.* Nitrotoga spp.", supports the formation of nitrifying aggregates in the CFRE (Figure 6A). Ammonia-oxidizing prokaryotes (AOP) and NOB commonly form dense clusters with specific zones of activity, an active ammonia-oxidizing zone on the outer perimeter of the micro-colonies and an inner nitrite-oxidizing zone (Okabe et al. 1999). Aggregation of "*Ca.*. Nitrotoga spp." with AOB was recently shown to support the active role of "*Ca.*. Nitrotoga spp." in nitrite oxidation (Lücker et al. 2014).

AOA. The alternating presence of "*Ca.* Nitrosoarchaeum spp." and "*Ca.* Nitrosopumilus spp." in the CFRE communities revealed an interesting pattern within the active AOA, which is consistent in other systems (Francis et al. 2005, Beman & Francis 2006). In the San Francisco Bay estuary, AOA phylogeny showed a clear distinction between fresh and salt water communities (Mosier & Francis 2008) as well as habitat (i.e. sediments, water column; Francis et al. 2005, Beman & Francis 2006) although it is important to note that this is not always the case (Bernhard et al. 2010). "*Ca.* Nitrosopumilus spp." are typically found in both marine and freshwater environments and were recently identified as the dominant member of the AOA in Lake Superior (Bollmann et al. 2014). Despite its reputation for being an "extreme oligophile" (Stahl &

de la Torre 2012b), "*Ca.* Nitrosopumilus spp." were present and active in the CFRE and positively correlated with bottom water NH_4^+, DOC and salinity. Alternatively, "*Ca.* Nitrosoarchaeum spp." were negatively correlated with bottom water NH_4^+, DOC and salinity suggesting a sensitivity salinity and perhaps NH_4^+ which might affect the distribution of "*Ca.* Nitrosoarchaeum spp.". AOA specific to oligohaline reaches of other estuarine systems were identified based on *amo*A gene sequences (Francis et al. 2005) and subsequently enriched "*Ca.* Nitrosoarchaeum limnia" BG20 from San Francisco Bay designating it as the "Low-Salinity" AOA (Mosier et al. 2012). The distinct presence of *Ca.* Nitrosoarchaeum spp. or "*Ca.* Nitrosopumilus spp.", with a few cases of overlap in the CFRE further supports niche differentiation among the AOA.

AOB. Surprisingly, there was a complete absence of *Nitrosospira* spp. and *Nitrosospina* spp. in the CFRE, despite their importance and abundance in other marine and estuarine systems (Ward et al. 2007). Instead *Nitrosomonas* spp. and *Nitrosococcus* spp. were present and active in the CFRE. *Nitrosomonas* spp. are generally associated with particles and dominant in some estuaries, perhaps due to a greater tolerance for irregular pulses of substrate in the environment (Phillips et al. 1999, Hollibaugh et al. 2002, Caffrey et al. 2003, Freitag & Prosser 2004, Bernhard et al. 2007). *Nitrosococcus* spp. were generally most active at IC during high tide, despite no increase in salinity, and showed no clear pattern at HB. *Nitrosococcus* spp. are generally restricted to marine and saline environments world-wide (Ward & O'Mullan 2002, Klotz et al. 2006). The finding is interesting, but it is unclear what environmental parameters might be associated with this pattern.

NOB. Nitrospira spp. were the most abundant active NOB at IC (Figure 4). *Nitrospira* spp. tend to dominate most of the NO_2^- oxidation in the natural and man-made worlds (Altmann et al 2003, Ehrich 1995) and their broad distribution implies their importance in nitrification in the CFRE. This group is capable of a mixotrophic lifestyle and adaptive to substrate limiting conditions (Lücker et al. 2010). The positive correlation with DOC observed in this study suggests use of simple organics that may support the active members of *Nitrospira spp.* in the CFRE. Despite the wide distribution and versatile lifestyle, *Nitrospira* spp. are also sensitive to environmental conditions such as oxygen limitation as they quickly disappear in sediments below the oxic layer (Altmann et al. 2004). The negative correlation with salinity and the decrease in abundance in both the 16S rDNA and rRNA communities at HB spring suggests sensitivity to salinity.

Interestingly, the disappearance of *Nitrospira* spp. at higher salinities coincided with the prevalence of *Nitrospina* spp. (Figure 4), specifically at HB where sediments experienced an increase in salinity but had similar levels of sediment %organics and NH_4^+. *Nitrospina* spp. were also positively correlated with DOC and NH_4^+, but this correlation does not necessarily support a mixotrophic metabolism in *Nitrospina* spp. (Abeliovich 2006). Instead, a correlation with both DOC and NH_4^+ considered in conjunction with the possible aggregation with *Ca Nitrosopumilus* spp. leads to a different conclusion. Remineralization of DOC may be supplying NH_4^+ for "*Ca Nitrosopumilus* spp.", often observed for AOA (Stahl & de la Torre 2012a), and subsequently providing NO_2^- to *Nitrospina* spp..

Nitrifying Members and Nitrification

The relative abundance of total archaea was strongly and negatively correlated with nitrification (Figure 6). Alternatively NOB and relative bacterial abundance were positively correlated with nitrification activities. Despite not being the most abundant active member of the CFRE sedimentary nitrifying communities, *Nitrospina* spp. demonstrated the strongest correlation with nitrification activities (Figure 6). The importance of *Nitrospina* spp. in marine ecosystems has been demonstrated, including oxygen minimum zones (OMZs) and been found to significantly correlate with nitrite oxidation rates with *Nitrospina* spp. in the Eastern Tropical Pacific (ETP) indicating its importance in the marine and estuarine N cycle (Suzuki et al. 2004, DeLong et al. 2006, Levipan et al. 2014). Overall, total active NOB and in particular, *Nitrospina* spp., are likely important in mediating the final step in sedimentary estuarine nitrification activities and controlling the rate at which NH_4^+ is oxidized to NO_3^-.

The importance of AOA cannot be ruled out in estuarine N cycling, even though elevated ratio of rRNA:rDNA is indicative of actively growing AOB and NOB (Campbell et al. 2011). One possible explanation may be that we captured the location in the tidal reaches of the estuary where the AOB and NOB are transitioning from the rare to the abundant .A next step would be to investigate the ratio of RNA:DNA along the estuarine salinity gradient and compare the distribution of active AOA and AOB throughout the system to determine the relative contribution of each group to nitrification activities.

CONCLUSIONS

Next generation sequencing analysis of 16S rDNA and rRNA revealed new findings in the distribution and role of AOA, AOB, and NOB in estuarine nitrification. We detected a higher abundance of AOA relative to their bacterial counterparts at the 16S rDNA level. However the pattern shifted when the active members of the nitrifying prokaryotes were examined, with NOB and AOB in higher abundance than AOA at the 16S rRNA level. Overall, NOB contributed to the highest relative abundance of the active nitrifiers. Decrease in relative abundance of *Nitrospira* spp. at higher salinities coincided with the prevalence of *Nitrospina* spp. *Nitrospira* spp. contributed to the greatest relative abundance of the active nitrifying communities. Salinity and NH_4^+ concentration were found to be important environmental variables in the development of niche differentiation of AOA, AOB and NOB communities.

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Table 1. Number of 16S rDNA and rRNA sequences filtered during different steps in bioinformatic analysis. Sediments from the west bank of Indian Creek (IC) and Horseshoe Bend (HB) were collected at high and low tides during the winter and spring in 2012 were used in the molecular analysis.

				Number of Sequences						Percent	
Nucleic Acid	Site		Season	Trimmed	Selected	Filtered	Total	Archaea	Bacteria	Archaea	Bacteria
16S rDNA	IC	Low	Winter	11955	8900	8665	3009	500	2509	16.6	83.4
		High		10609	8900	8752	1886	273	1613	14.5	85.5
		Low	Spring	12705	8900	8797	1767	392	1375	22.2	77.8
		High		11524	8900	8795	743	176	567	23.7	76.3
	HB	Low	Winter	9189	8900	8640	2057	278	1779	13.5	86.5
		High		9843	8900	8744	2022	417	1605	20.6	79.4
		Low	Spring	11554	8900	8787	1282	416	866	32.4	67.6
		High		12135	8900	8723	1183	550	633	46.5	53.5
16S rRNA	IC	Low	Winter	10093	8900	8684	2649	28	2621	1.1	98.9
		High		10468	8900	8745	1031	86	945	8.3	91.7
		Low	Spring	12066	8900	8718	2372	166	2206	7.0	93.0
		High		105128	8900	8746	1900	212	1688	11.2	88.8
	HB	Low	Winter	11750	8900	8742	2530	26	2504	1.0	99.0
		High		8914	8914	8571	2274	150	2124	6.6	93.4
		Low	Spring	9446	8900	8647	2264	289	1975	12.8	87.2
		High		11388	8900	8665	2018	236	1782	11.7	88.3

Table 2. Relative percent of present and active nitrifying prokaryotes. Sediments from Indian Creek and Horseshoe Bend were collected at high and low tides during the winter and spring in 2012 were used in the molecular analysis. Relative abundance of known nitrifying prokaryotes was calculated at the Levels of Domain and Genera for 16S rDNA and 16S rRNA sequences. Bold numbers indicate total values.

			Indian Creek				Horseshoe Bend			
			Winter		Spring		Winter		Spring	
Nucleic Acid	Domain	Genus	Low	High	Low	High	Low	High	Low	High
16S rDNA	Archaea	Ca. Nitrosoarchaeum	0.033	0.106	0.113	0.000	0.097	0.049	0.156	0.000
		Ca. Nitrosopumilus	0.033	0.053	0.057	0.000	0.097	0.099	0.078	0.085
		Ca. Nitrosotalea	0.033	0.000	0.057	0.000	0.000	0.000	0.078	0.000
	Bacteria	Nitrosococcus	0.033	0.000	0.000	0.000	0.049	0.000	0.078	0.000
		Nitrosomonas	0.000	0.053	0.057	0.000	0.000	0.099	0.000	0.000
		Ca. Nitrotoga	0.000	0.053	0.000	0.000	0.000	0.000	0.000	0.000
		Nitrospira	0.133	0.318	0.170	0.269	0.146	0.247	0.156	0.085
		Nitrospina	0.000	0.000	0.057	0.135	0.292	0.396	0.234	0.000
		AOA	**0.100**	**0.159**	**0.226**	**0.000**	**0.194**	**0.148**	**0.312**	**0.085**
		AOB	**0.033**	**0.053**	**0.057**	**0.000**	**0.049**	**0.099**	**0.078**	**0.000**
		NOB	**0.133**	**0.371**	**0.226**	**0.404**	**0.438**	**0.643**	**0.390**	**0.085**
		Total	**0.266**	**0.583**	**0.509**	**0.404**	**0.681**	**0.890**	**0.780**	**0.169**
16S rRNA	Archaea	Ca. Nitrosoarchaeum	0.038	0.000	0.084	0.105	0.040	0.000	0.088	0.000
		Ca. Nitrosopumilus	0.000	0.097	0.000	0.000	0.040	0.044	0.044	0.050
		Ca. Nitrosotalea	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Bacteria	Nitrosococcus	0.038	0.097	0.042	0.105	0.119	0.088	0.088	0.000
		Nitrosomonas	0.113	0.097	0.211	0.211	0.040	0.308	0.088	0.198
		Ca. Nitrotoga	0.000	0.000	0.084	0.000	0.000	0.044	0.000	0.000
		Nitrospira	0.529	0.776	0.464	0.316	0.237	0.396	0.353	0.248
		Nitrospina	0.000	0.097	0.084	0.158	0.356	0.396	0.265	0.297
		AOA	**0.038**	**0.097**	**0.084**	**0.105**	**0.079**	**0.044**	**0.133**	**0.050**
		AOB	**0.151**	**0.194**	**0.253**	**0.316**	**0.158**	**0.396**	**0.177**	**0.198**
		NOB	**0.529**	**0.873**	**0.632**	**0.474**	**0.593**	**0.836**	**0.618**	**0.545**
		Total	**0.717**	**1.164**	**0.970**	**0.895**	**0.830**	**1.275**	**0.928**	**0.793**

Figure 1. Sampling Sites in the Cape Fear River Estuary, NC, USA. Sampling sites in the upper, oligohaline portion of the estuary include Indian Creek and Horseshoe Bend.

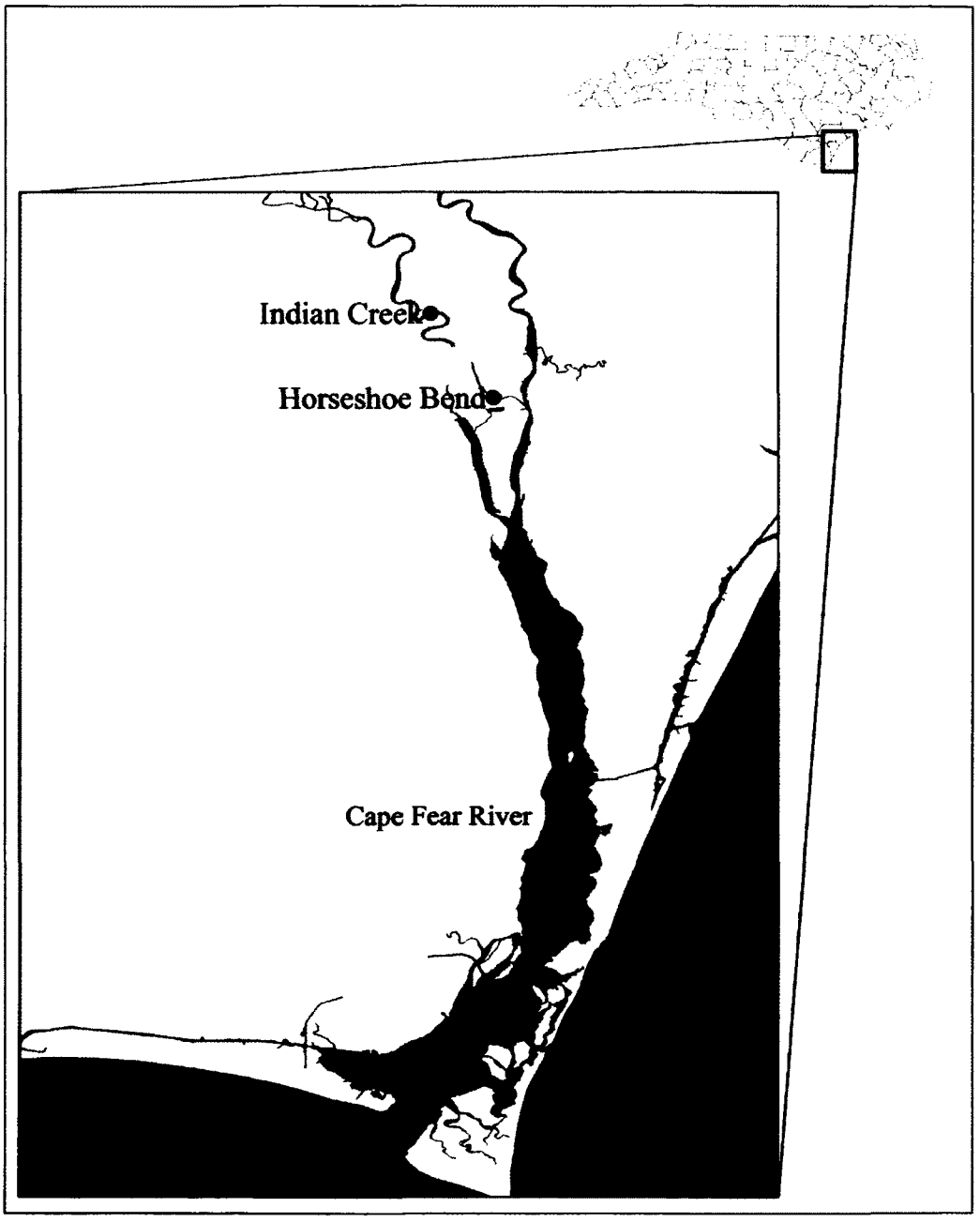

Indian Creek
Horseshoe Bend
Cape Fear River

Figure 2. Relative percent of present and active archaea. Sediments from Indian Creek (IC) and Horseshoe Bend (HB) were collected at high (H) and low (L) tides during the winter (W) and spring (S) in 2012 were used in the molecular analysis. Changes in community structure for 16S rDNA (A) and 16S rRNA (B) gene sequences were examined at the Phyla Level.

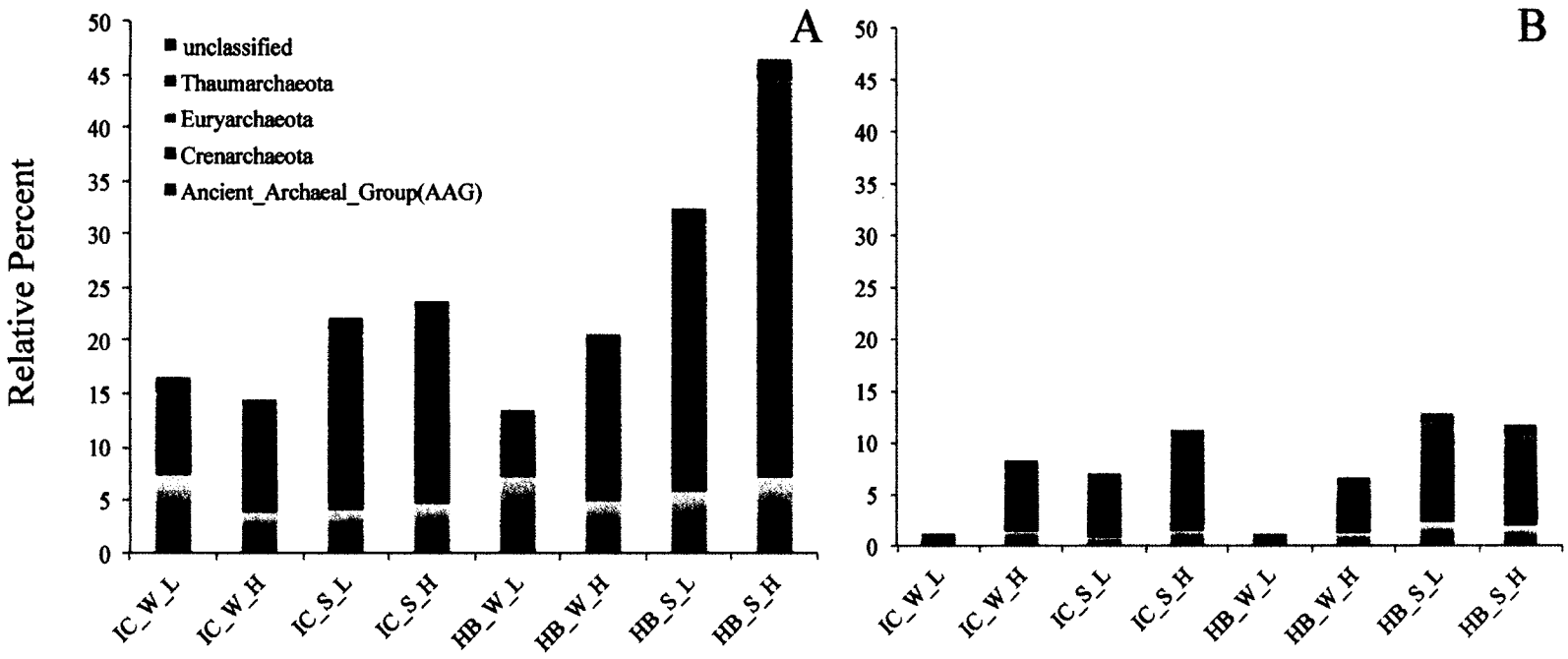

A
B
Relative Percent
unclassified
Thaumarchaeota
Euryarchaeota
Crenarchaeota
Ancient_Archaeal_Group(AAG)
0
5
10
15
20
25
30
35
40
45
50
IC_W_L
IC_W_H
IC_S_L
IC_S_H
HB_W_L
HB_W_H
HB_S_L
HB_S_H

Figure 3. Relative percent of present and active bacteria. Sediments from Indian Creek (IC) and Horseshoe Bend (HB) were collected at high (H) and low (L) tides during the winter (W) and spring (S) in 2012 were used in the molecular analysis. Changes in community structure for 16S rDNA (A) and 16S rRNA (B) gene sequences were examined at the Phyla Level.

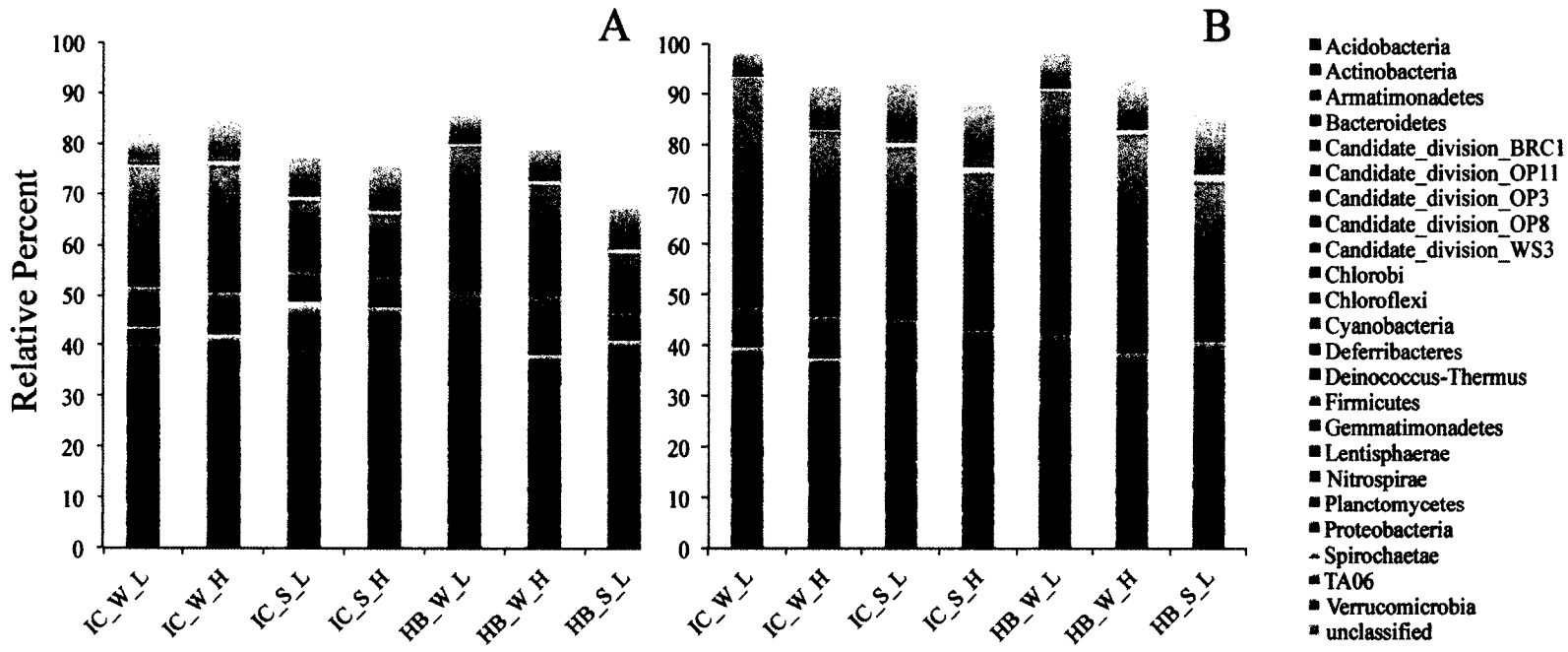

A
B
Relative Percent
100
90
80
70
60
50
40
30
20
10
0
IC_W_L
IC_W_H
IC_S_L
IC_S_H
HB_W_L
HB_W_H
HB_S_L
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Candidate_division_BRC1
Candidate_division_OP11
Candidate_division_OP3
Candidate_division_OP8
Candidate_division_WS3
Chlorobi
Chloroflexi
Cyanobacteria
Deferribacteres
Deinococcus-Thermus
Firmicutes
Gemmatimonadetes
Lentisphaerae
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetae
TA06
Verrucomicrobia
unclassified

Figure 4. Comparison of the relative percent of present and active nitrifying prokaryotes. Present (A) and active (B) members of known genera of nitrite oxidizing bacteria and ammonia-oxidizing archaea and bacteria and are presented for Cape Fear River sediment samples. Sediments from Indian Creek (IC) and Horseshoe Bend (HB) were collected at high (H) and low (L) tides during the winter (W) and spring (S) in 2012 were used in the molecular analysis.

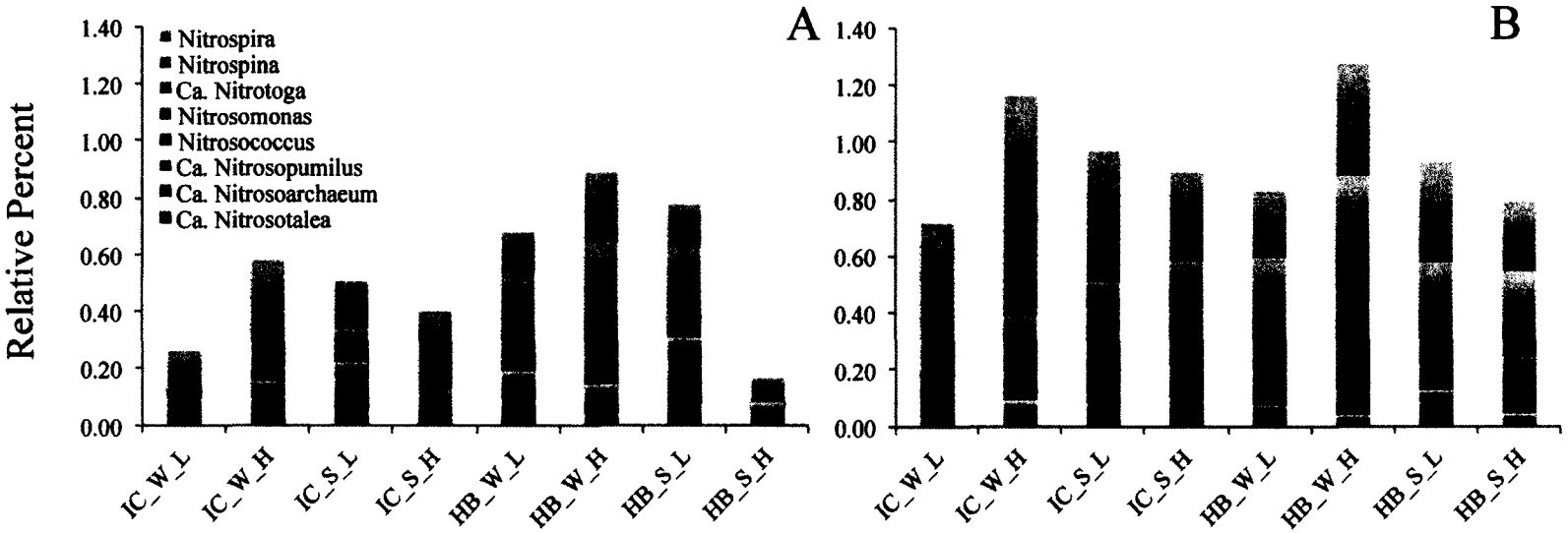
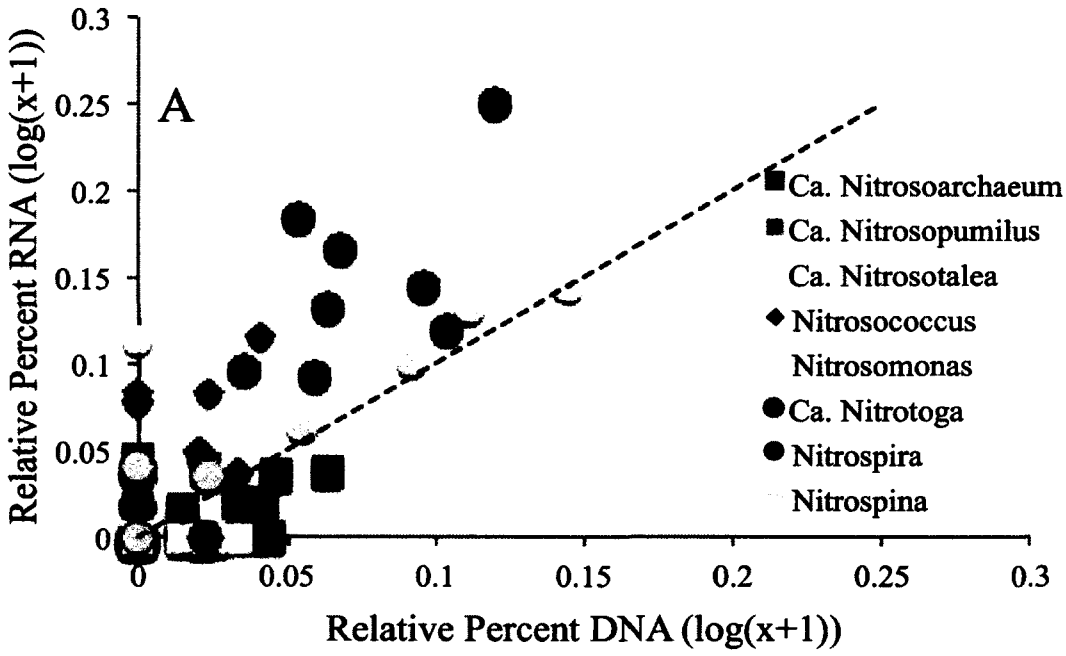

Relative Percent
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Nitrospira
Nitrospina
Ca. Nitrotoga
Nitrosomonas
Nitrosococcus
Ca. Nitrosopumilus
Ca. Nitrosoarchaeum
Ca. Nitrosotalea
A
B
IC_W_L
IC_W_H
IC_S_L
IC_S_H
HB_W_L
HB_W_H
HB_S_L
HB_S_H

Figure 5. Ratio of DNA:RNA for nitrifying prokaryotes. Sediments from Indian Creek and Horseshoe Bend at high and low tides during the winter and spring in 2012 were used in the molecular analysis. The ratios of rRNA:rDNA are shown for the specific nitrifying genera (A) and group total (B) for all Cape Fear River Estuary sediment samples. The dashed line represents 1:1 ratio.


A
Relative Percent RNA (log(x+1))
Relative Percent DNA (log(x+1))
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.05
0.1
0.15
0.2
0.25
0.3
Ca. Nitrosoarchaeum
Ca. Nitrosopumilus
Ca. Nitrosotalea
Nitrosococcus
Nitrosomonas
Ca. Nitrotoga
Nitrospira
Nitrospina

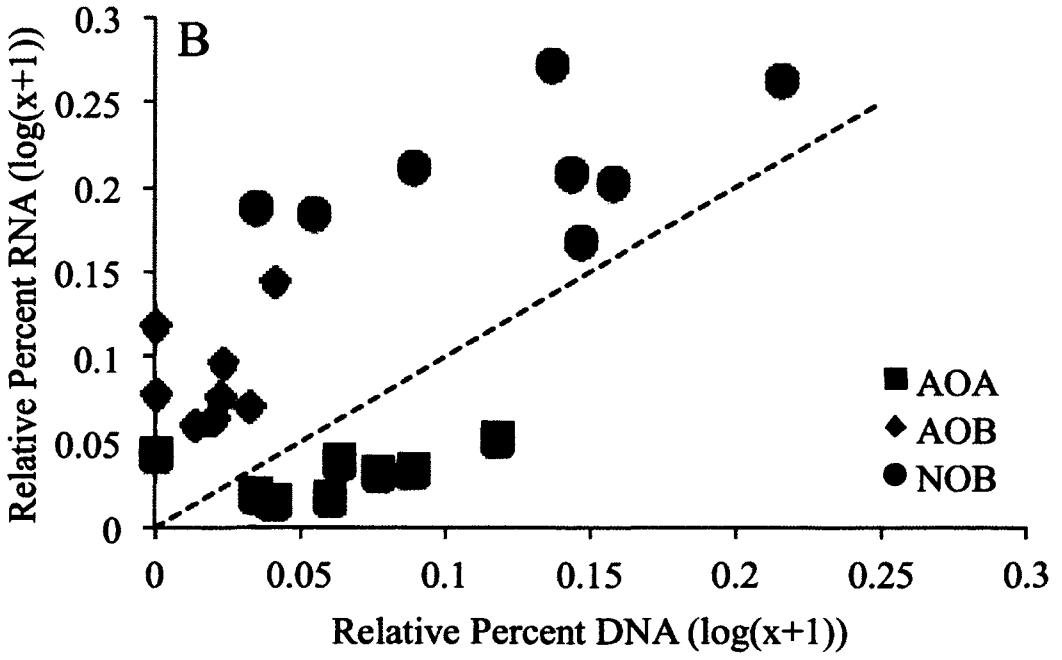
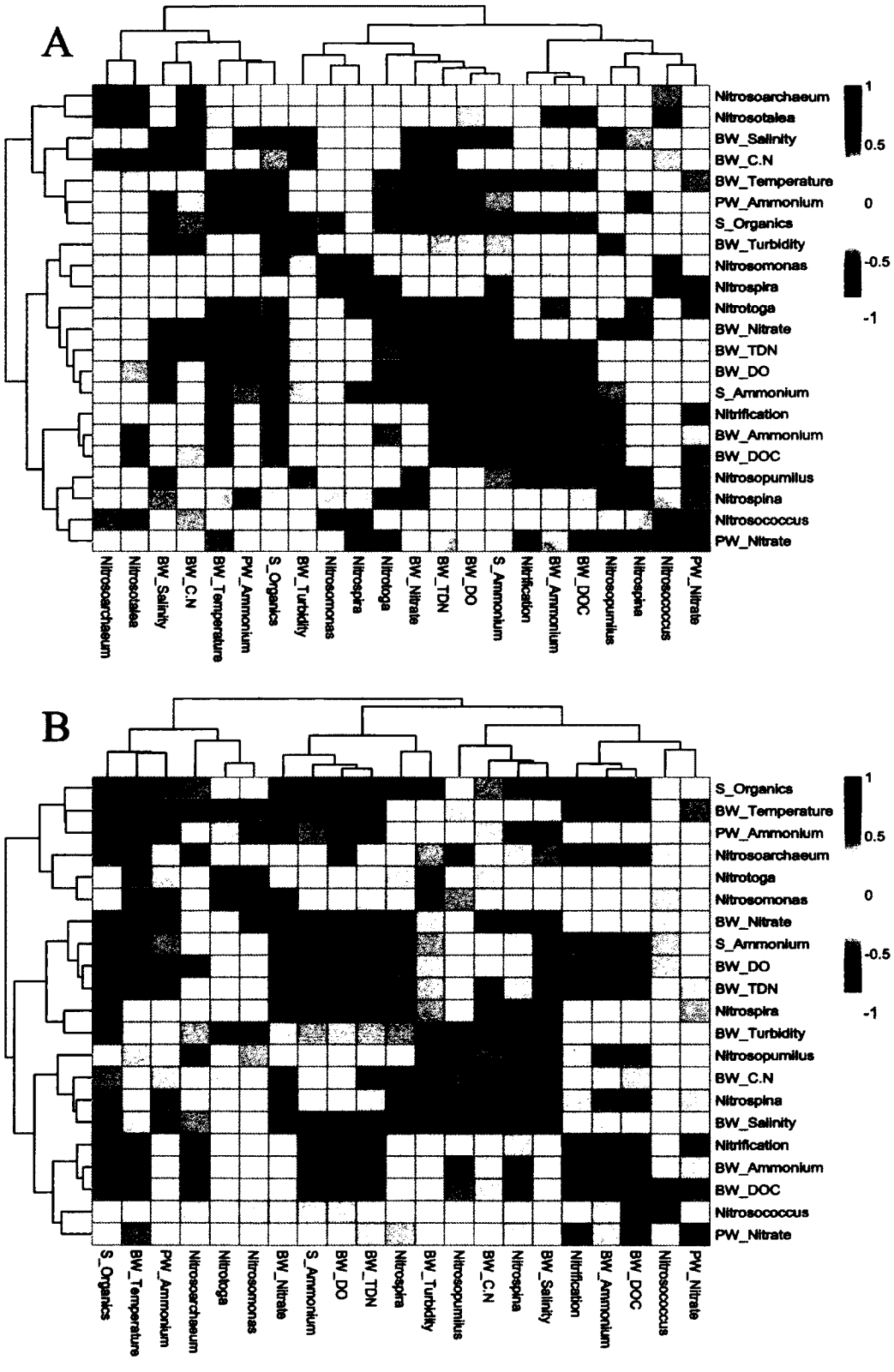

B
Relative Percent RNA (log(x+1))
Relative Percent DNA (log(x+1))
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.05
0.1
0.15
0.2
0.25
0.3
AOA
AOB
NOB

Figure 6. Heatmap analysis showing Spearman's Correlations for biological and environmental parameters. Sediments from Indian Creek and Horseshoe Bend at high and low tides during the winter and spring in 2012 were used in the molecular analysis. Correlations between environmental factors, nitrification rate measurements and the relative percent of present (16S rDNA; A) and active (16S rDNA; B) nitrifying communities were each run separately. Environmental parameters include bottom water (BW), porewater (PW), and sediment (S) parameters. Colors depict r-values returned by correlation analyses. Eculidean distance measures were used to cluster columns based on similarities of r-values with dark red indicating strong positive correlations and dark blue indicating strong negative correlations.


A
Nitrosoarchaeum
Nitrosotalea
BW_Salinity
BW_C.N
BW_Temperature
PW_Ammonium
S_Organics
BW_Turbidity
Nitrosomonas
Nitrospira
Nitrotoga
BW_Nitrate
BW_TDN
BW_DO
S_Ammonium
Nitrification
BW_Ammonium
BW_DOC
Nitrosopumilus
Nitrospina
Nitrosococcus
PW_Nitrate
1
0.5
0
-0.5
-1
B
S_Organics
BW_Temperature
PW_Ammonium
Nitrosoarchaeum
Nitrotoga
Nitrosomonas
BW_Nitrate
S_Ammonium
BW_DO
BW_TDN
Nitrospira
BW_Turbidity
Nitrosopumilus
BW_C.N
Nitrospina
BW_Salinity
Nitrification
BW_Ammonium
BW_DOC
Nitrosococcus
PW_Nitrate
1
0.5
0
-0.5
-1

CHAPTER 7

Conclusions

We examined the linkage between the structure and function of microbial communities responsible for sedimentary nitrogen processes in the New River Estuary (NRE) and Cape Fear River Estuary (CFRE). The integration of sophisticated geochemical techniques with various modern molecular techniques allowed for a better understanding of the nitrogen cycling microbial community structure, community responses to changes in environmental parameters, and the recycling and removal of nitrogen in estuarine sediments.

In all seasons, denitrification was the dominant N_2 producing pathway in the NRE and CFRE and anammox contributed up to 14% of total N_2 production. Both anammox and denitrification rates measured were consistent with other studies while sedimentary nitrification and DNRA rates were within the lower quartile of published values. We found evidence to support the coupling of the carbon, nitrogen, and sulfur cycles revealed through positive correlations between porewater H_2S concentrations and %organics with anammox, denitrification, and DNRA activities (Chapters 2, 3, 4). Phylogenetic analysis of Hzo sequences revealed that Jettenia-like organisms were dominant throughout the NRE (Chapter 2). A freshening of the estuary during the winter distributed upstream anammox communities to downstream sties and increased anammox activities. This provides evidence that episodic flushing events may have influenced the widespread distribution of Jettenia-like organisms.

Biogeochemical hotspots were identified as statistical outliers using our newly established quantitative method (Chapter 3). We found denitrification and anammox hotspots accounted for 35.6% and 60.9% of total N_2 production while comprising only 7.3% and 10.6% of the total sampling sites, respectively. An increase in functional gene

abundance could not account for the occurrence of exceptionally high denitrification and anammox activities at the identified hotspots. We proposed alternative biological and environmental mechanisms governing these areas of elevated activities; the presence and interactions of denitrifiers and anammox bacteria with higher substrate affinity under optimal environmental conditions allowed for the elevated activities observed at hotspots. Successful application of *nir*S microarray provided support for this proposal and revealed that the abundance of specific archetypes were associated with increased potential for denitrification (Chapter 4). Additionally, co-variation of archetypes allowed for the formation of two different denitrifier assemblages associated with different environmental conditions. Together these findings highlight the importance of the environmental and microbial features of a system in the composition of denitrifying communities and the essential role of individual members within the community function.

The CFRE study focuses on sediment community responses to small-scale (tidal) and large-scale (seasonal) changes under different tidal regimes. Denitrification activities were highest among the measured nitrogen cycling processes in the CFRE while bacteria capable of DNRA were most abundant (Chapter 5). The strong centrality of sediment NH_4^+ levels and the potential for coupled nitrification-denitrification and nitrification-anammox supports the role of substrate availability as a driver of these processes, while salinity was found to positively influence DNRA activities. We saw significant and positive correlations between denitrification activities and abundance of denitrifying bacterial communities. Similar trends occurred with nitrification activities and *amo*A

gene abundance in the AOB and AOA, suggesting that both bacteria and archaea are equally important to NH_4^+ oxidation in the CFRE.

The role of AOA and AOB in estuarine nitrification was further explored in Chapter 6 through next generation sequencing of 16S rRNA gene (rDNA) and rRNA of archaea and bacteria. We found higher abundance of AOA at the rDNA level, but AOB and NOB were more abundant in the active nitrifying communities. *Nitrospira spp.* contributed to the greatest relative abundance of the active nitrifying communities. The presence of specific AOA, AOB, and NOB changed in response to salinity and NH_4^+ concentration. The formations of nitrifying consortia in estuarine sediments were proposed based on the co-variation of AOP and NOB. Together, our study highlighted changes in relative abundance of the nitrifying prokaryotes at the rDNA and rRNA along with changing environmental conditions to support niche differentiation within the nitrifying prokaryotes in sediments of the CFRE.

Examination of microbial community structure, measurements of biogeochemical rates and assessment environmental conditions enabled us to further elucidate some of the interactions that occur within the nitrogen cycling microbial communities. Knowledge of community structural response to varying environment conditions is essential for understanding the microbial regulation of nitrogen cycling processes in estuarine systems. Information obtained from these studies enable us to better understand the role of microorganisms in the mitigation of increased nitrogen loading in estuaries.

VITA

JESSICA ANNE LISA

Born in Teaneck, New Jersey, USA on June 10, 1983. Grew up on the Jersey Shore. Graduated from Long Branch High School in 2001. Received a B.S. in Biology with a concentration in Marine and Environmental Biology (*Magna Cum Laude*) from Monmouth University in 2007. Worked as a research assistant for Monmouth University's Urban Coast Institute under the guidance of Professor John Tiedemann. Entered into the M.S. program at the University of North Carolina at Wilmington under graduate advisor Dr. Bongkeun Song in 2008. Worked as a Visiting Research Student Collaborator, Geosciences, Princeton University in the fall of 2010 under Dr. Bess Ward. Bypassed into the Ph.D. program in 2011. Transferred to the Virginia Institute of Marine Science, College of William and Mary in 2013 as a student of Dr. Bongkeun Song.