

Alterations in Nitrogen Cycling Resulting From Oyster Mediated Benthic-Pelagic Coupling

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## ABSTRACT

ASHLEY REBECCA SMYTH: Alterations in Nitrogen Cycling Resulting From Oyster Mediated Benthic-Pelagic Coupling  
(Under the direction of Michael F. Piehler)

Human activities have resulted in an array of stressors to coastal ecosystems. In the context of ecosystem function, two prominent changes have been nutrient enrichment and precipitous declines in the population of the eastern oyster, *Crassostrea virginica*. Although historically valued as a fishery, oysters provide broader ecological functions, which include filtering water thereby reducing turbidity as they feed and providing habitat for fish and crabs. Despite decades of oyster research, we lack a comprehensive understanding of how oysters influence nitrogen biogeochemistry in estuarine ecosystems. My research directly assessed the role of oysters in enhancing sediment denitrification and the efficacy of oyster reef restoration in alleviating nutrient pollution. I measured net N<sub>2</sub> fluxes from five major estuarine habitats: salt marshes, seagrass beds, oyster reefs and intertidal and subtidal flats. Given the current habitat distribution in this study system, denitrification (N<sub>2</sub> production) removed approximately 76% of the estimated watershed nitrogen load. Microcosm experiments were conducted to examine the direct effects of individual oysters on nitrogen dynamics. Results indicated that biodeposit production and excretion shifted sediments from a nitrogen source to a nitrogen sink. Experimental plots of live oysters, oyster shells and mud flats were used to distinguish between the effects of oyster feeding and reef structure on sediment denitrification. The production and accumulation of biotic material accounted for 60% of denitrification from oyster reef sediments while 40% was attributed to the abiotic

effects of the reef structure. Fluxes measured from restored intertidal oyster reef sediments demonstrated that oyster reefs prime sediments for enhanced denitrification in response to anthropogenic nitrogen loading; however, the magnitude of this effect is dependent on the habitat setting of the oyster reef. This research identified mechanisms by which oysters alter sediment nitrogen dynamics and enhanced our understanding of oyster reef impacts on ecosystem function. This information is critical for determining where to focus reef restoration and preservation efforts to produce the greatest benefit. Results from my research will inform management strategies, restoration projects and policies aimed at improving water quality and sustaining healthy estuarine ecosystems.

To my grandmother, Vola, for letting me play in the mud and encouraging my curiosity. She was a wonderful person and amazing grandmother, who I miss and love very much.

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## **PREFACE**

This thesis is organized in manuscript format and includes material that has been previously published and includes material that is co-authored. The text is divided into six sections, four of which are formatted for journal articles. The second chapter of my dissertation is a manuscript published in *Estuaries and Coasts* with authors A.R. Smyth, S.P. Thompson, K.N Siporin, W.S. Gardner, M.J. McCarthy, and M.F. Piehler, and the copyright owner (Springer-Verlag) granted permission for including this material in my thesis. Additional information regarding the methods used in this thesis can be found in Appendix A.



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## LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
°	Degree
±	Plus or minus
$^{15}\text{NH}_4^+$	Ammonium with $^{15}\text{N}$ stable isotope
1N	One equivalent weight of solute per liter of solution
A	Surface area
anammox	Anaerobic ammonium oxidation
ANOVA	Analysis of variance
Ar	Argon
C	Carbon
Chl. <i>a</i>	Chlorophyll <i>a</i>
CHN	Carbon, Hydrogen, Nitrogen
$C_i$	Inflow solute concentration
cm	Centimeter
$C_o$	Outflow solute concentration
DIN	Dissolved inorganic nitrogen
DNRA	Dissimilatory nitrate reduction to ammonium
Eff	Denitrification efficiency
F	Flow rate of water through the headspace of a core
HCl	Hydrochloric acid
hr	Hours
$i_{\text{inflow}}$	Concentration of solutes coming into each core

$i_{\text{outflow}}$	Concentration of solutes in the outflow of each core
$J$	Flux
km	Kilometer
LD	Detection limit
m	Meter
MIMS	Membrane inlet mass spectrometry
mmol	millimoles
N	nitrogen
N <sub>2</sub>	Di-nitrogen gas
N <sub>2</sub> O	Nitrous oxide
NH <sub>4</sub> <sup>+</sup>	Ammonium
NO	Nitric oxide
NO <sub>2</sub> <sup>-</sup>	Nitrite
NO <sub>3</sub> <sup>-</sup>	Nitrate
NO <sub>x</sub>	Nitrate plus Nitrite
O <sub>2</sub>	Oxygen
ppt	Parts per thousand
psu	Practical salinity units
SAV	Submerged aquatic vegetation
SOD	Sediment oxygen demand
SOM	Sediment organic matter
μM	Micro-molar
μmol	Micromoles



$\sigma$

Standard deviation

## **1. INTRODUCTION**

### **1.1 Importance of Estuarine Ecosystems**

Estuaries are at the critical transition zone between the terrestrial and coastal ecosystems. These areas provide a variety of ecosystem services including protection from storm events, storage and cycling of nutrients, and nursery habitat for commercially important species, while being heavily used for recreational activities (Costanza et al. 1997). With 38% of the world's population living within 100 km of the coast (Small and Nicholls 2003), estuarine ecosystems are among the most used and, consequently, the most degraded systems in the world (Jackson et al. 2001a, Lotze et al. 2006). Estuaries are experiencing multiple human impacts including dredging, pollution, introduction of invasive species, over-harvesting, watershed development, and anthropogenic climate change (Vitousek et al. 1997a, Lotze et al. 2006, Worm et al. 2006, Duarte et al. 2009, Beck et al. 2011). The interactions between these stressors have resulted in loss of several estuarine habitats, with multiple negative consequences for the services provided by these systems (Duarte 2009, Barbier et al. 2011, Boström et al. 2011). For example, the combined effects of over-harvesting and eutrophication have led to a drastic decline of oyster ecosystems (Lenihan and Peterson 1998, Lenihan et al. 1999), while sea-level rise and human induced land-use changes have affected salt marsh habitat (Bertness et al. 2002, Mattheus et al. 2010).

Excessive nutrient loading is a major cause of estuarine ecosystem degradation. Algal growth and primary production in estuarine ecosystems is generally limited by light and the availability of nutrients, particularly nitrogen (Paerl et al. 2006, Howarth 2008, Nixon 2009).

Additional nitrogen inputs from fertilizer use and runoff has lead to eutrophication, the formation of dead zones, harmful algal blooms, changes to energy flows and loss of biodiversity (Galloway et al. 2003, Paerl et al. 2006, Conley et al. 2009, Sharp et al. 2009). The impact of increased nitrogen inputs to the coast is far-reaching, affecting tourism, recreational water activities and fisheries. Accordingly, regulatory agencies have put in place policies regarding nitrogen inputs. However, the multiple sources and complexity of the nitrogen cycle make controlling nitrogen particularly difficult. As such, reduction in nutrient inputs alone is not enough to recover the lost services and restore ecosystem functions (Duarte et al. 2009). One solution to combat the effects of increased nutrient loading is to enhance the system's overall capacity to remove nitrogen (Brush 2009). Restoring areas that have high rates of denitrification, the microbially-mediated reduction of nitrate to  $N_2$  gas, is a solution that has promise. Denitrification permanently removes nitrogen from the ecosystem, thus counteracting eutrophication and reducing the effects of nitrogen pollution.

## **1.2 Nitrogen Dynamics in Shallow Coastal Ecosystems**

Two opposing processes, nitrogen fixation and denitrification help regulate the availability of nitrogen in estuarine systems. While nitrogen fixation converts atmospheric nitrogen to bioavailable nitrogen, denitrification releases nitrogen back to the atmosphere as nitrogen gas ( $NO$ ,  $N_2O$ ,  $N_2$ ). Denitrification is a dissimilatory nitrate reduction process performed by heterotrophic bacteria. Denitrifying bacteria are ubiquitous and denitrification can occur under anoxic conditions when there is ample supply of labile organic matter and nitrate (Seitzinger et al. 2006). When nitrate for denitrification is supplied through nitrification, the oxidation of ammonium to nitrate by chemoautotrophic bacteria, it is considered coupled nitrification-denitrification. Denitrification supported with nitrate from

the water column is referred to as direct denitrification. In most coastal systems, where the concentration of nitrate dissolved in the water is less than 10  $\mu\text{M}$ , the majority of denitrification is coupled to nitrification (Seitzinger et al. 2006).

### **1.3 Loss of Suspension Feeding Bivalves**

Oyster reef ecosystems have been reduced by 85% world-wide (Beck et al. 2011) and fisheries landings of the eastern oyster, *Crassostrea virginica* (Gmelin 1791) in North Carolina have declined by 90% in the last century (Lenihan et al. 2003, Beck et al. 2011). The dramatic decline in oyster reef ecosystems is the result of interactive effects between over-harvesting, destructive harvesting practices, increased spread of diseases and decline in water quality (Lenihan and Peterson 1998, Burreson et al. 2000, Beck et al. 2011). Oysters exert top down control on cultural eutrophication by removing phytoplankton biomass as they feed (Jackson et al. 2001a, Cerco and Noel 2007). This action reduces turbidity and allows light to penetrate deeper into the water column, which enhances the production of seagrass and benthic algae (Dame et al. 1984, Newell 2004). Oysters can also have bottom-up effects on eutrophication, where production and accumulation of biodeposits can change nutrient processing within the sediment and stimulate denitrification (Newell et al. 2002, 2005). Consequently, restoration of these filter-feeding bivalves has been suggested as a way to reduce phytoplankton biomass and mitigate nutrient loads (Jackson et al. 2001a, Cerco and Noel 2007, Coen et al. 2007, Fulford et al. 2010). However, oysters may also recycle nitrogen back to the water column, which may fuel primary production (Dame et al. 1984, 1989, Newell et al. 2002). Before oyster reef restoration can be included in nutrient management plans, it is necessary to understand and quantify the effects of oyster reefs on sediment nutrient dynamics in shallow water environments.

## **1.4 Linking Oysters to Sediment Nitrogen Dynamics**

Studies of oysters have focused on the effects of suspension feeding and metabolism by oyster reefs. As oysters feed and reduce phytoplankton biomass, water column concentration of ammonium increases as a result of excretion (Dame et al. 1989, 1992). This ammonium may be recycled back to the water column and used to further support phytoplankton production (Dame et al. 1989, Newell 2004). However, the unassimilated fraction of the nitrogen and carbon that was originally incorporated in phytoplankton is released as biodeposits, a mucus aggregate of feces and pseudo-feces (Newell and Jordan 1983). Biodeposits accumulate on the sediment as particulate organic matter and serve as a nutrient source for microbial metabolism (Newell et al. 2002, Giles and Pilditch 2006, Higgins et al. 2013). When biodeposits settle on aerobic sediments, nitrogen removal can be stimulated through increased coupled nitrification-denitrification (Newell et al. 2005). While increased denitrification from oyster biodeposits has been found in laboratory experiments (Newell et al. 2002), the effect of oysters on sediment nitrogen dynamics remains unclear because measurements of denitrification have not previously been conducted on sediments associated with oyster reefs. Figure 1.1 illustrates the interactions between oysters and the sediment nitrogen dynamics.

## **1.5 Significance of Research**

My dissertation research focused on understanding how the eastern oyster, *Crassostrea virginica*, affects biogeochemical processes in shallow water coastal systems. Research was conducted to evaluate the effects of the individual organisms and the interacting effects between the oyster, the reefs they form and the ecosystem on nitrogen exchanges at the sediment-water interface. Rates of  $N_2$  production (denitrification) and fluxes

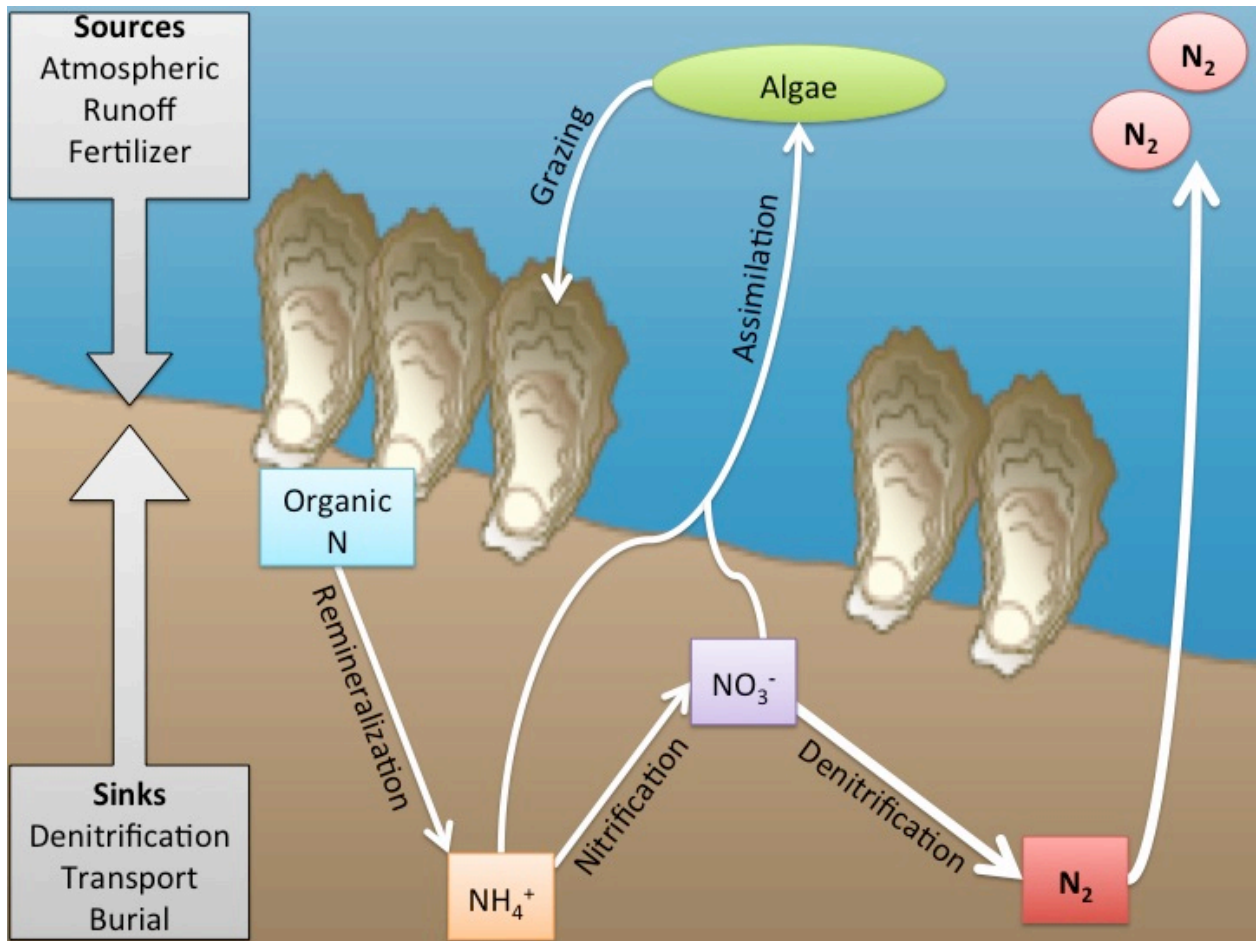
of nutrients were measured from natural and restored oyster reefs as well as reference sites and other estuarine habitats throughout North Carolina's estuaries and sounds. This information is critical for accurately assessing one of the most important ecosystem services provided by oysters and determining where to focus restoration and preservation efforts to produce the greatest benefit.

## **1.6 Study Objectives**

1. Spatial and Temporal Variability in Sediment Nitrogen Dynamics
  - a. Objective: Characterize spatial and temporal patterns of sediment nitrogen dynamics in shallow water estuarine habitats.
  - b. Hypothesis: Habitat type and temperature will affect rates of denitrification in shallow water coastal systems.
2. Linking Oysters to Biogeochemistry
  - a. Objective: Quantify the direct effects of an oyster on nitrogen removal and regeneration.
  - b. Hypothesis: Particulate organic matter from *Crassostrea virginica* will enhance rates of denitrification by providing a source of high quality organic matter and increasing availability of  $\text{NH}_4^+$  for coupled nitrification-denitrification.
3. The Influence of Ecosystem Engineering On Sediment Denitrification
  - a. Objective: Elucidate the relative importance of the accumulation of biotic and abiotic material to the production of  $\text{N}_2$  from oyster reef sediments.
  - b. Hypothesis: Interactions between the biological function and physical engineering of oyster reefs will result in the largest production of  $\text{N}_2$ .

#### 4. Importance of Landscape Position

- a. Objective: Determine how the habitat setting of oyster reef restoration affects oyster mediated sediment  $N_2$  production in response to nutrient pollution.
- b. Hypothesis: Restored oyster reefs will increase  $N_2$  production relative to reference habitats where oyster reefs have not been restored.



**Figure 1.1.** Conceptual diagram of how filter-feeding by oysters affects sediment nitrogen dynamics. Sources of nitrogen include, but are not limited to, the atmosphere (both deposition and nitrogen fixation), runoff and fertilizer. While denitrification, transport and burial are considered nitrogen sinks. Oysters repack nitrogen in phytoplankton and transfer it to the sediment as particulate organic nitrogen. The decomposition of the organic nitrogen produces ammonium and, in aerobic sediments, can support nitrate production via nitrification. Nitrate can diffuse back to the water column or fuel denitrification in anoxic sediments. This results in the release of N<sub>2</sub> gas back to the atmosphere.



## 2. ASSESSING NITROGEN DYNAMICS THROUGHOUT THE ESTUARINE LANDSCAPE<sup>1</sup>

### 2.1 Abstract

Assessing nitrogen dynamics in the estuarine landscape is challenging given the unique effects of individual habitats on nitrogen dynamics. We measured net N<sub>2</sub> fluxes, sediment oxygen demand and fluxes of ammonium and nitrate seasonally from five major estuarine habitats: salt marshes, seagrass beds (SAV), oyster reefs and intertidal and subtidal flats. Net N<sub>2</sub> fluxes ranged from  $332 \pm 116 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  from oyster reef sediments in the summer to  $-67 \pm 4 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  from SAV in the winter. Oyster reef sediments had the highest rate of N<sub>2</sub> production and the highest rates of ammonium release from the sediments of all habitats. Potential rates of dissimilatory nitrate reduction to ammonium (DNRA) were measured during the summer and winter. DNRA was low during the winter and ranged from  $4.5 \pm 3.0$  in subtidal flats to  $104 \pm 34 \mu\text{mol } ^{15}\text{NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$  in oyster reefs during the summer. Annual denitrification, accounting for seasonal differences in inundation and light, ranged from  $161.1 \pm 19.2 \text{ mmol N-N}_2 \text{ m}^{-2} \text{ yr}^{-1}$  for marsh sediments to  $509.9 \pm 122.7 \text{ mmol N-N}_2 \text{ m}^{-2} \text{ yr}^{-1}$  for SAV sediments. Given the current habitat distribution in our study system, an estimated  $28.3 \times 10^6$  mols of N are removed per year or 76% of estimated watershed nitrogen load. These results suggest that restoration has the

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<sup>1</sup> Chapter 2 is published as an article in the journal *Estuaries and Coasts* with authors with authors A.R. Smyth, S.P. Thompson, K.N Siporin, W.S. Gardner, M.J. McCarthy, and M.F. Piehler and is © by Springer. The text is reproduced here with permission from Springer. Substantive modifications to the published manuscript are denoted by a change in font.

potential to increase system-wide denitrification through selection of habitats with high rates of  $N_2$  production per  $m^2$  and areas with favorable inundation regimes.

## **2.2 Introduction**

Estuaries are complex ecosystems, influenced by marine, terrestrial and atmospheric inputs of material and energy. Complex interactions within these ecosystems are important determinants of the diversity and composition of the ecological community (Hosack et al. 2006). Dominant habitats in the temperate estuarine landscape include salt marshes, seagrass beds (SAV), oyster reefs and intertidal and subtidal flats. Each habitat has a unique effect on ecosystem function, such that the variety and areal extent of habitats influences the type and amount of services provided by the estuary (Correll 1978, Jones et al. 1994, Cloern 2007, Eyre and Maher 2010, Barbier et al. 2011).

Estuarine habitats provide many valuable ecosystem services, but these areas are often threatened by human activities (Vitousek et al. 1997b, Lotze et al. 2006, Brush 2009, Barbier et al. 2011, Beck et al. 2011). Eutrophication, which is accelerated by excessive nitrogen loading, has many negative consequences including loss of biodiversity, increased algal blooms, degradation of water quality, acceleration of species invasions and shifts in dominant biogeochemical pathways (Howarth et al. 1988, Nixon et al. 1995, Jackson et al. 2001a, Lotze et al. 2006, Paerl et al. 2006, Burgin and Hamilton 2007). Once nitrogen enters the estuary, it can be removed through burial, physical transport, or denitrification, the microbial conversion of inorganic nitrogen to  $N_2$  gas (Vitousek et al. 1997b).  $N_2$  production can result from the heterotrophic reduction of nitrate (classical denitrification) or through anaerobic ammonium oxidation (anammox) by chemolithoautotrophs (Burgin and Hamilton 2007). Although both pathways produce  $N_2$  gas, denitrification is more prevalent than

anammox (Dalsgaard et al. 2005, Koop-Jakobsen and Giblin 2009) and accounts for the majority of nitrogen loss in estuarine ecosystems (Seitzinger and Nixon 1985, Seitzinger 1988).

Unfortunately, anthropogenic activities have disrupted the balance between nitrogen inputs and exports, resulting in an increase in instances of eutrophication (Brush 2009). Nutrient recycling within estuaries helps to maintain water quality and supplies essential nutrients for the base of the food web. These services provide about \$21,100 ha<sup>-1</sup> (in 1994 US dollars) nitrogen removal in estuaries annually (Costanza et al. 1997). However, the overall amount of nitrogen removal depends on the distribution of specific habitats with some habitats conferring a greater ecosystem service than others (Valiela and Bowen 2002, Cardinale 2011). For example, the conversion of SAV to subtidal flat due to increased use of shoreline hardening structures could result in a loss of \$2,500 per acre per year worth of nitrogen removal (Piehler and Smyth 2011).

To improve water quality, many management and restoration strategies aim to enhance nitrogen removal within estuarine ecosystems (Galloway et al. 2003, Cerco and Noel 2007, Brush 2009, Barbier et al. 2011). However, landscape-scale assessment of nitrogen removal is complicated by the variety of estuarine habitats and temporal variability in denitrification rates (Cornwell et al. 1999, Groffman et al. 2006, Seitzinger 2008). While habitat type is a factor in determining transformations and exchanges of nitrogen in estuarine ecosystems (Gutierrez and Jones 2006, Eyre et al. 2011b, Piehler and Smyth 2011, Eyre et al. 2011a), the areal extent and location of these habitats relative to tidal regime will also impact nitrogen removal, influencing the time that sediments experience reduced conditions when denitrification can occur (Ensign et al. 2008, 2012). Thus, identifying and quantifying

landscape-scale water quality benefits of estuarine restoration requires knowledge of the location and elevation of habitats associated with rates of nitrogen removal.

### **2.2.2 Objectives**

This study investigated nitrogen dynamics in the major habitats of a temperate estuary. To evaluate the influence of habitat type on nitrogen dynamics, we examined multiple nitrogen cycling processes, seasonally, in sediments from intertidal oyster reefs, marshes, SAV and intertidal and subtidal flats. A model based on habitat area, elevation, water level and irradiance was created to extrapolate denitrification through both space and time. Using this approach, we were able to synthesize and adjust rate processes measured from individual habitats to the landscape scale.

## **2.3 Methods**

### **2.3.1 Site description**

Bogue Sound is a medium sized sound in the southeastern region of North Carolina (Figure 2.1). The mean water depth is 3m and semi-diurnal tides are approximately 0.7m. The sound has low levels of dissolved inorganic nutrients ( $0.90 \pm 1.32 \mu\text{M NO}_x^- + \text{NH}_4^+$ ,  $n=263$ ) and water-column chlorophyll *a* ( $4.5 \pm 2.3 \mu\text{g/l}$ ,  $n=261$ ; Thompson, unpublished). Bogue Sound is a diverse ecosystem located at the convergence of the South Atlantic and Mid-Atlantic biogeographic regions. A variety of habitats exist within the sound and include oyster reefs, SAV (dominated by *Halodule wrightii* in the spring and *Zostera marina* in the fall), salt marshes and intertidal and subtidal flats (Street et al. 2004). Habitats of each type were sampled for this study. Subtidal and intertidal flats sampled were relatively homogenous with no observable macrofauna.

Representative habitat sampling sites were located on the southern shoreline of Bogue Sound in the Roosevelt Natural Area and were sampled once per season during 2008 (January, March, July and November). *In situ* surface water temperature, salinity and dissolved oxygen were measured at each sampling (YSI 600 Series Sonde and Model 650 data logger, Yellow Springs Instruments, Yellow Springs, OH).

### **2.3.2 Sample collection**

Triplicate sediment cores were collected by hand from each habitat two hours prior to low tide in clear polycarbonate core chambers (6.4cm diameter X 30 cm). Core chambers were inserted directly into the sediment and pushed down so that each core contained 17 cm of sediment with minimal disturbance to the upper layer of sediment. Cores from SAV and marshes often contained roots and rhizomes as well as emergent vegetation. Cores were collected within each habitat with the exception of the oyster reef, where cores were collected immediately adjacent to the reef and did not include live oysters. In addition to sediment cores, ~30 l of sound water was collected for continuous flow core incubations.

### **2.3.3 Analytical methods**

Details regarding methods used in this study can be found in the appendix.

#### **2.3.3.1 Membrane inlet mass spectrometry**

Following collection, sediment cores and water were immediately (<1hr) transported to an environmental chamber (Bailey, Inc.) set to *in situ* water temperature at The University of North Carolina Institute of Marine Sciences (IMS) in Morehead City, NC. Dark conditions were maintained throughout the course of the incubation to minimize the effects of photosynthetic algae (An and Joye 2001, Tobias 2007, Hochard et al. 2010) and to prevent the formation of bubbles that would affect gas concentrations in water (Reeburgh 1969).

Cores were submerged in a water bath and sealed with gas tight lids equipped with an inflow

and outflow port with ~400ml of water overlying each core and incubated in a continuous flow system (Miller-Way and Twilley 1996, Lavrentyev et al. 2000, Ensign et al. 2008). Unfiltered, aerated water from the reservoirs was passed over the cores at a flow rate of 1.0 ml min<sup>-1</sup> (Miller-Way and Twilley 1996, Lavrentyev et al. 2000).

Following an 18-hour pre-incubation period (Eyre et al. 2002), samples were collected from the outflow port of each core three times over a 48-hour period to ensure steady state conditions were established (Miller-Way and Twilley 1996). A bypass line that flowed directly into sample vials was used to determine the concentration of dissolved constituents entering the cores was also sampled during each of the three times over the 48-hour period. This line accounted for changes in water chemistry associated with permeability of the tubing in the water entering the cores. Successive measurements from each core were averaged to give core specific values and reduce pseudo-replication associated with sample replication rather than treatment replication (Hurlbert 1984).

Samples were analyzed for N<sub>2</sub>, O<sub>2</sub> and Ar dissolved gases in water using a Balzers Prisma QME 200 quadruple mass spectrometer (MIMS; Pfeiffer Vacuum, Nashua, NH, USA; (Kana et al. 1994). Concentrations of O<sub>2</sub> and N<sub>2</sub> were determined using the ratio with Ar (Kana et al. 1994, Ensign et al. 2008). MIMS has a rapid analysis time, requires a small sample volume and little sample preparation, and has good precision (CV of N<sub>2</sub>/Ar <0.05%, CV of O<sub>2</sub>/Ar <0.04%). This method determines the net flux (production-demand) across the sediment-water interface such that a positive N<sub>2</sub> flux is assumed to be denitrification and a negative N<sub>2</sub> flux is assumed to be nitrogen fixation (An et al. 2001, Fulweiler et al. 2007). This method does not discern between N<sub>2</sub> production from denitrification, anammox or any other N<sub>2</sub> producing process. Fluxes of oxygen directed into the sediment were considered to

represent rates of sediment oxygen demand (SOD; (Kana et al. 1998, Piehler and Smyth 2011)).

### **2.3.3.2 Dissolved Nutrient Analysis**

Water samples (50ml) were collected for nutrient analysis from the bypass line and the outflow port of each core 24-hours after the incubation began. Water was filtered through Whatman GF/F filters (25 mm diameter, 0.7  $\mu\text{m}$  nominal pore size), and the filtrate was analyzed with a Lachat Quick-Chem 8000 (Lachat Instruments, Milwaukee, WI, USA) automated ion analyzer for nitrate ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) and ammonium ( $\text{NH}_4^+$ ) (detection limits: 0.04  $\mu\text{M}$ , 0.18  $\mu\text{M}$ , respectively).

### **2.3.3.3 Dissimilatory Nitrate Reduction to Ammonium**

Isotopic enrichment experiments were conducted to provide potential rates of dissimilatory nitrate reduction to ammonium (DNRA). DNRA experiments were performed on cores collected during summer 2007 in conjunction with Piehler and Smyth (2011) and during winter 2008 on cores collected as part of this study. In each case, after the initial 48 hours of sampling, the incubation water was enriched with  $^{15}\text{N-NaNO}_3$  to a final concentration of about  $100\mu\text{mol l}^{-1}$ . Incubations were continued and samples were collected at 24 and 48 hours after the enrichment for analysis. Concentration of  $^{15}\text{NH}_4^+$  was measured by HPLC (Gardner et al. 1995), and potential DNRA rates were calculated as production of  $^{15}\text{NH}_4^+$  (An and Gardner 2002). Total ammonium and nitrate concentrations were measured after enrichment using Lachat Quick-Chem 8000 (described above).

## **2.3.4 Calculations**

### **2.3.4.1 Flux calculations**

Flux calculations were based on the assumption of steady-state conditions and a well-mixed water column (Miller-Way and Twilley 1996). Benthic fluxes, including rates of potential DNRA, were calculated using the following equation:

$$J = ([i_{\text{outflow}}] - [i_{\text{inflow}}]) * \frac{F}{A}$$

**Equation 1:** Formula used to calculate a flux from the continuous flow core incubation method.

where  $[i_{\text{outflow}}]$  and  $[i_{\text{inflow}}]$  is the concentration ( $\mu\text{M}$ ) of any dissolved constituent leaving and entering the core, respectively,  $F$  is the peristaltic pump flow rate ( $1 \text{ hr}^{-1}$ ), and  $A$  is the surface area of the core ( $\text{m}^2$ ) (Miller-Way and Twilley 1996).  $[i_{\text{outflow}}]$  is the average of three measurements of samples collected over a 48-hour period;  $[i_{\text{inflow}}]$  is the average of the three measurements from the bypass line collected over a 48-hour period. For nitrogen species, a positive flux indicates an exchange from the sediment to the water column, and a negative flux indicates an exchange from the water column to the sediment. For  $\text{O}_2$ , a positive flux indicates an exchange from the water column and is denoted as SOD. Habitat specific fluxes were calculated as the mean of core specific values from replicates ( $n=3$ ). Errors presented here are the standard error of the means ( $n=3$ ).

#### **2.3.4.2 Determination of inundation time**

Average hours inundated per day were modeled based on water level and irradiance (adjusted based on the season) for each day of the year. Elevation surveys were conducted at three different individual habitats (including habitats where cores were collected) during low tide on 16 October 2009, 25 October 2009 and 15 November 2009 to obtain mean elevation for each habitat type ( $n=3$ ). Habitat elevation was determined using an automatic laser level (Model SAL24N, CST/Berger, Watseka, IL) with methods adapted from Storesund (2008). Water level was logged at 5-minute intervals in an adjacent subtidal creek with a HOBO



water level data logger (Model: U20-001-01, Onset Corporation, Pocasset, MA) from 25 September 2009 to 23 October 2009, to encompass one spring-neap tidal cycle. Water levels were corrected using *in situ* temperature and barometric pressure. Site-specific water level was indexed to water level data obtained from a NOAA monitoring gauge at Beaufort, NC collected during the same period. This relationship was then applied to NOAA water level data for December 1, 2007 to November 30, 2008 to hind cast levels at the Bogue Sound study site for the same period. Inundation was calculated as the duration when water levels were greater than sediment surface elevation. Total hours inundated between sunset and sunrise were calculated for each day and totaled for each season and each habitat. This value was used to scale the average hourly rate of N<sub>2</sub> production (positive N<sub>2</sub> flux) measured once during each season for each habitat under dark inundated conditions to annual rates. Annual rates were extrapolated for Bogue Sound using habitat maps from the North Carolina Division of Marine Fishers (NCDMF; (Chappell 2006)).

### **2.3.5 Data analysis**

Analysis of variance (ANOVA) and Tukey's post-hoc test were used to test whether net N<sub>2</sub> flux, SOD, ammonium flux, nitrate flux, and DNRA varied by site and season. If necessary, data were transformed to meet the assumptions of ANOVA. Linear regressions were used to assess the relationship between net N<sub>2</sub> flux and SOD. Analysis of covariance (ANCOVA) was used to determine if the regression lines were different between habitats. All analyses were considered significant at the  $p < 0.05$  level and were conducted in JMP 7.0.1 statistical software (SAS 2007).

## **2.4 Results**

### **2.4.1 Water chemistry**

The lowest water temperature occurred in January 2008 at 3.32 °C, and the highest was 30.1 °C in June 2008 (Table 2.1). Salinity ranged from 29.3 in July 2008 to 32.4 in January 2008 with a mean salinity of 31.2. Dissolved oxygen ranged from 5 mg/l in the July of 2008 to 12.3 mg/l in October 2008. Ambient nitrate concentration was consistently less than 0.5 µM. Ammonium ranged from 0.65 µM in October 2008 to 3.56 µM during July 2008.

#### **2.4.2 Dissolved Oxygen**

SOD was lowest in the winter and fall and highest in the summer for all habitats (Figure 2.2a). SOD ranged from  $33.61 \pm 16 \mu\text{mol O}_2 \text{ m}^{-2} \text{ hr}^{-1}$  during winter in SAV to  $2556 \pm 11 \mu\text{mol O}_2 \text{ m}^{-2} \text{ hr}^{-1}$  in the summer at oyster reefs. Significantly higher seasonal rates were measured for all habitats during the summer ( $p < 0.05$ ). Oyster reefs had significantly more oxygen demand than the other habitats, driven by rates measured during the spring and summer.

#### **2.4.3 Dissolved N<sub>2</sub>**

Net N<sub>2</sub> fluxes varied by site and season and the interaction was not significant (Figure 2.2b,  $p = 0.086$ ). N<sub>2</sub> fluxes ranged from  $332 \pm 116 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for the oyster reef in the summer to  $-67 \pm 4 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for SAV in the winter. All N<sub>2</sub> fluxes from the oyster reef were positive with high variability during the summer, including a rate of  $566.2 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  in one core. In general there were positive N<sub>2</sub> fluxes in the summer and negative N<sub>2</sub> fluxes during the other seasons. Negative N<sub>2</sub> fluxes were observed in sediments from the subtidal flat in spring and fall, intertidal flat in winter and spring, marsh and SAV in winter, spring and fall. Oyster reefs were the exception with positive N<sub>2</sub> fluxes occurring during each season, except fall. Overall, oyster reef sediments had significantly higher N<sub>2</sub> fluxes ( $107 \pm 48 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$ ,  $n = 12$ ) compared to the other habitats, driven mainly by

high production in the summer and marshes had the lowest flux ( $-4 \pm 18 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$ ,  $n=12$ ). Net  $\text{N}_2$  fluxes varied significantly with SOD (Figure 2.3,  $R^2=0.63$ ,  $p<0.001$ ). This relationship was observed for all habitats and all seasons, except fall, when variability among rates was the highest.

#### **2.4.4 Dissolved Inorganic Nitrogen**

Nitrate fluxes were not affected by habitat type but did vary by season (Fig 2.2c,  $p=0.0028$ ). There was no uptake or efflux of nitrate during the summer or fall for any habitat (i.e. no difference between concentration of nitrate leaving and entering the cores). All habitats exhibited an efflux of nitrate during the winter and demand during the spring, except SAV sediments, which had an efflux of nitrate during winter and spring. In general nitrate fluxes were low and highly variable, consistent with the low ambient nitrate concentration found in this study area.

Ammonium fluxes were also variable and exhibited no seasonal pattern (Figure 2.2d) but oyster reef sediments were significantly higher than other habitats ( $p=0.0002$ ). The interaction between season and habitat was also significant ( $p<0.0001$ ). The single largest efflux of ammonium occurred in oyster reef sediments during the summer ( $198 \pm 114 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$ ) when all oyster reef cores showed an efflux of ammonium greater than  $100 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$ ; the highest efflux was  $332.9 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$ . Seasonal differences were not detected for SAV or marsh habitats, while subtidal and intertidal flats had significantly more uptake of ammonium during the summer compared to the other seasons ( $p=0.008$  and  $0.0004$ , respectively).

#### **2.4.5 Dissimilatory Nitrate Reduction to Ammonium (DNRA)**

Potential DNRA rates were higher in the summer than the winter (Table 2.2,  $p<0.0005$ ). Oyster reef sediments had the highest average potential rate of DNRA ( $104.4 \pm$

34.3  $\mu\text{mol } ^{15}\text{NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$ ). On average, DNRA used 4.2% of the nitrate flux directed into the sediment during the summer. Given the small nitrate fluxes prior to the addition for DNRA, it was assumed that  $^{15}\text{NO}_3^-$  comprised the majority of the  $\text{NO}_3^-$  flux was compared to the  $^{15}\text{NH}_4^+$  fluxes (An and Gardner 2002). The percent of the nitrate flux for DNRA was calculated as the proportion of the added nitrate flux that was  $^{15}\text{NH}_4^+$ . Despite high DNRA rates in the oyster reefs, this process only accounted for 11.2% of the nitrate flux during this time, however a very large ammonium efflux was detected for each habitat after the addition.

#### 2.4.6 Extrapolations

Subtidal habitats (SAV and subtidal flats) were constantly inundated (Table 2.3). For habitats with variable inundation, intertidal flats were inundated the longest, followed by oyster reefs and marshes. Annual  $\text{N}_2$  production (adjusted for illumination and inundation) ranged from  $146.0 \pm 17.4 \text{ mmol N-N}_2 \text{ m}^{-2} \text{ yr}^{-1}$  from the marsh habitat to  $509.9 \pm 122.7 \text{ mmol N-N}_2 \text{ m}^{-2} \text{ yr}^{-1}$  from SAV (Figure 2.4). The low rates from the marsh habitat are the result of few instances of  $\text{N}_2$  production coupled with the high elevation relative to water level. Annual  $\text{N}_2$  areal production, **adjusted for inundation**, from oyster reef sediments was not significantly different from SAV sediments.

The annual rate of nitrogen removal ( $\text{mol N yr}^{-1}$ ) was determined by extrapolating the annual  $\text{N}_2$  production rates to the estuary using total area comprised by each habitat (Table 2.3). Assumptions of this extrapolation are that similar habitats exhibit similar effects on ecological processes and are affected by light and tide the same as the habitats used in this study. Results from this extrapolation indicated that subtidal flat habitats remove significantly more nitrogen per year than the other habitats because of the large area of these habitats in Bogue Sound (Fig 2.4;  $p < 0.0001$ ).

## **2.5 Discussion**

### **2.5.1 Net N<sub>2</sub> Fluxes**

Negative N<sub>2</sub> fluxes, which indicate nitrogen fixation, (Fulweiler et al. 2007) were found in sediments from SAV, marsh, subtidal flat and intertidal flat habitats during several seasons. Nitrogen fixation rates exceeding denitrification are not uncommon in estuaries (Joye and Paerl 1994, Currin et al. 1996, An and Joye 2001, Fulweiler et al. 2007, Fulweiler and Nixon 2011). Because incubations were conducted in the dark, heterotrophic bacteria were likely responsible for nitrogen fixation in these habitats (Howarth et al. 1988, Currin et al. 1996). High rates of nitrogen fixation are likely inversely related to ammonium concentrations; fixation is inhibited when sediments have high concentrations of extractable and soluble ammonium (Howarth et al. 1988). Although we did not measure porewater ammonium concentrations, there was low ammonium in overlying water and ammonium uptake by the sediments from these habitats. Therefore, in these N-limited systems, the additional nitrogen demand may be met through nitrogen fixation (Howarth and Marino 2006). In contrast, the high concentration of ammonium associated with oyster biodeposits (Newell et al. 2005, Higgins et al. 2011) was reflected in large ammonium fluxes and greater N<sub>2</sub> production from the oyster reef habitat.

Oyster reef sediments had the largest flux of ammonium from the sediment to the water column. High ammonium production from oyster reef sediments, especially during the summer, probably resulted from elevated oyster filtration rates (and thus organic matter deposition on the sediments) during this time (Grizzle et al. 2008, Pomeroy et al. 2006, Dame et al. 1992, Dame et al. 1985). High ammonium production suggests that heterotrophic bacteria were actively using the deposited organic matter during aerobic respiration or denitrification. Oyster excretion could

contribute to high ammonium production; however, this was not a source in this study since oysters were not included in the sediment incubations.

The largest positive  $N_2$  fluxes, (denitrification) occurred in the summer for all habitats. Seasonal differences in denitrification rates are common (Thompson et al. 1995, Eyre and Ferguson 2005, Piehler and Smyth 2011, Fulweiler and Nixon 2011), with higher rates in warmer months, when metabolism is higher (Brown et al. 2004). While the majority of  $N_2$  fluxes measured over the course of an annual cycle for SAV, subtidal flat and intertidal flat sediments were negative in cooler months; high positive  $N_2$  fluxes in the summer made average  $N_2$  fluxes positive. For the marsh habitat, positive  $N_2$  fluxes in the summer and large negative  $N_2$  fluxes in cooler months resulted in average fluxes that were not significantly different from zero, suggesting a balance between denitrification and nitrogen fixation. This result agrees with previous studies that have documented higher rates of nitrogen fixation relative to denitrification from sandy fringing marshes (Currin et al. 1996, Davis et al. 2004).

Nitrate required for denitrification can diffuse from the water column into the sediments (direct denitrification) or be produced in sediment through nitrification (coupled nitrification-denitrification). The low nitrate concentration in the overlying water (less than  $0.5 \mu\text{M}$ ) and small fluxes of nitrate into the sediment make direct denitrification unlikely in our study system (Seitzinger et al. 2006). Nitrate fluxes were low, but within the range of values measured from other oligotrophic systems with low ambient nitrate concentrations (Weston et al. 1996, Fear et al. 2005, Eyre et al. 2011a). The lack of a seasonal or habitat effect on nitrate fluxes suggests that coupled nitrification-denitrification was the dominant  $N_2$  production pathway for all habitats. We found nitrification to support on average about 98% of measured denitrification (positive  $N_2$  fluxes).

Oyster reefs had the highest rates  $N_2$  production per area. Studies of denitrification in oyster reef sediments are rare (Piehler and Smyth 2011), particularly when compared to SAV and marsh habitats. Current understanding of oyster-mediated denitrification stems from laboratory experiments using pelletized phytoplankton to simulate biodeposits, suggesting that the presence of oysters increases coupled nitrification-denitrification in the sediment (Newell et al. 2002). Our results support this hypothesis and suggest that denitrification was limited by the availability of nitrate. However, the elevated ammonium production and high SOD suggests that nitrification was limited by oxygen (Cornwell et al. 1999).

### **2.5.2 Sediment Oxygen Demand**

SOD was highest for the oyster reef sediments, indicating that the organic matter in this habitat is more rapidly metabolized compared to the other habitats. This difference is probably the result of the biodeposits associated with the oysters' feeding process (Newell et al. 2005). SOD from the marsh was low compared to other studies (Caffrey et al. 2007). Marshes in this study area were fringing marshes with sandy sediments (Mattheus et al. 2010). Habitats with sandy sediments have been associated with lower quality carbon despite large amounts of organic matter (Vance-Harris and Ingall 2005, Morgan et al. 2009), resulting in lower SOD.

We found a strong positive relationship between SOD and  $N_2$  fluxes for all habitats. In estuarine sediments, SOD is primarily from organic matter mineralization, nitrification and sulfide oxidation. High sediment SOD is often associated with high organic matter and decreased sediment oxygen penetration depth (Cornwell et al. 1999). Additionally, the positive relationship between SOD and denitrification has been found in coastal ecosystems where denitrification is coupled to nitrification and is controlled by the availability of organic carbon (Seitzinger 1994, Seitzinger and Giblin 1996, Piehler and Smyth 2011). We found

negative  $N_2$  fluxes when SOD was lowest, suggesting nitrogen fixation could occur despite the increase in sediment oxygen penetration depth (Paerl and CARLTON 1988, Fulweiler and Nixon 2011). Nitrogen fixation and denitrification have been found to co-occur in coastal systems (Joye and Paerl 1994, Fulweiler et al. 2007), but process-based links between oxygen demand and nitrogen cycling are still being developed (Burgin et al. 2010, Burgin and Groffman 2012).

### **2.5.3 Potential DNRA**

Previous studies that have examined DNRA in coastal ecosystems report that DNRA can account for 0% to 75% of the nitrate flux (Tobias et al. 2001, An and Gardner 2002, Ma and Aelion 2005, Gardner and McCarthy 2009). We expected that the high amount of organic matter and anoxic and sulfidic sediments in a nitrate-limited environment would create conditions favorable for DNRA over denitrification (Tiedje 1988, Kelso et al. 1997, Silver et al. 2001, Tobias et al. 2001, Gardner et al. 2006, Burgin and Hamilton 2007, Koop-Jakobsen and Giblin 2010). However, we found DNRA to be negligible during the winter and to account for 0.7% to 11.2% of the added nitrate flux in subtidal flats and oyster reefs, respectively, during the summer. This low percentage would leave a large portion of nitrate available for other processes, including denitrification.

We found higher rates of potential DNRA in oyster reef sediment compared to the other habitats. Oyster reef sediments had high SOD, indicating reduced conditions that may favor DNRA over denitrification. In a marine aquaculture system with significant and sustained organic matter production DNRA was also found to occur at relatively high rates than denitrification (Christensen et al. 2000). Previous studies suggest that denitrification is inhibited by sulfide accumulation associated with high rates of organic matter loading from



bivalve aquaculture (Carlsson et al. 2012). It is possible that a proportion of the ambient ammonium flux from the oyster reef could result from DNRA associated with  $^{14}\text{NO}_3^-$ , which was unaccounted for in this study. However, the low nitrate in the overlying water ( $< 1\mu\text{M}$ , Table 2.1) suggests that this is minor. To our knowledge, ours is the first study to measure potential DNRA in natural oyster reef sediments and more data are necessary to fully assess the pathways of nitrate reduction in these systems.

These values may underestimate actual rates because our method did not measure DNRA rates from  $^{14}\text{NO}_3^-$  that occurs naturally and did not consider losses of  $^{15}\text{NH}_4^+$  due to cation exchange reactions in the sediments. It is possible that  $^{15}\text{NH}_4^+$  produced through DNRA was exchanged in the sediments with  $^{14}\text{NH}_4^+$ . This exchange would cause an increase in  $^{14}\text{NH}_4^+$  release and an underestimate of DNRA (Gardner et al. 1991, Seitzinger et al. 1991, Gardner et al. 2006). Unfortunately, we have neither measurements of porewater ammonium nor ammonium affinity from these sediments. However, we observed an increase in total  $\text{NH}_4^+$  release after the enrichment of  $^{15}\text{NO}_3^-$ , suggesting a greater potential for DNRA than we detected (Gardner et al. 2006). Moreover, these data suggest that nitrogen retention through DNRA could increase in response to anthropogenic nutrient loading.

### **2.5.5 Extrapolations**

Spatial and temporal variability in denitrification make it difficult to extrapolate rates to the landscape scale (Cornwell et al. 1999). In order for denitrification to be assessed at such a level, measurements must be made over many seasons, across a range of habitats, and account for tidal inundation (Seitzinger 2000). We used a model based on water level, elevation and light to scale rates of  $\text{N}_2$  production. These rates represented a lower limit because it was assumed that denitrification is limited to dark inundated sediments.

The purpose of our extrapolation was to assess nitrogen removal on the ecosystem scale; therefore, only positive fluxes were included.

Our results suggest that the amount of nitrogen removed by denitrification for an estuary depends on the amount and type of habitats located within the estuary, as each habitat has a unique effect on sediment nitrogen dynamics (Eyre and Maher 2010, Eyre et al. 2011b, Piehler and Smyth 2011, Eyre et al. 2011a). We found that oyster reefs and SAV provided disproportionately large amounts of nitrogen removal per unit area, while subtidal flats removed the largest amount of nitrogen within the ecosystem due to the area of these habitats within the estuary. Recent studies have examined how habitat area in tropical oligotrophic ecosystems affects nitrogen budgets and found seagrass beds to have the highest rates of nitrogen removal while flats served as important connectors (Eyre et al. 2011a). Results from our study also show that intertidal and subtidal flat habitats help to maintain the balance and function of estuarine ecosystems; however, these habitats do not provide the same quantity of ecosystem services as oyster reefs, SAV or marsh habitats and are generally considered to be of less value (Costanza et al. 1997, Barbier et al. 2011, Boström et al. 2011). Commonly, restoration strategies convert intertidal and subtidal flats to habitats that provide a greater number of services per area.

## **2.6 Conclusions**

Given the current habitat distribution of Bogue Sound, an estimated total of  $28.3 \pm 4.8 \times 10^6$  mols N are removed per year. Based on annual nitrogen load data from coastal streams within this area (Schwartz 2010), impervious surface coverage and watershed area (11.82%; USGS), we estimate the nitrogen load to Bogue Sound to be about  $37.3 \times 10^6$  mols N yr<sup>-1</sup>. Given these values, denitrification by the habitats in Bogue Sound removes about

76% of the total estimated nitrogen load and does not account for other sources of nitrogen (e.g. oceanic and non-point sources). This high nitrogen removal capacity of Bogue Sound contributes to maintaining water quality within this system. Although analyzing a complex system by adding rates from the individual compartments does not take into account the interactions, it is the first step in assessing landscape scale nitrogen removal. Rates of denitrification ( $N_2$  production only), which have been modified by the assumptions of the extrapolation, were not different; however, the mean of the measured net  $N_2$  fluxes, suggest that hourly areal fluxes are different by habitat type. Annual areal denitrification in oyster reefs is less than SAV and comparable to intertidal flats (Fig. 2.4); however oyster reefs have the highest areal denitrification in the summer (Fig. 2.2). Thus, restoration of habitats with positive mean net  $N_2$  fluxes and favorable inundation regimes have the potential to increase system-wide denitrification. This information will help in our understanding of how changes in the amount and types of habitats in the estuarine landscape impact ecosystem functions and services. Such knowledge is essential for management strategies aimed at mitigating the negative effects of increased nutrient inputs.

## **2.6 Acknowledgments**

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comments of 3 anonymous reviewers whose input significantly enhanced all aspects of this manuscript.

**Table 2.1** Physical and chemical properties of water at the sampling site for all nitrogen flux experiments (BD=Below Detection).

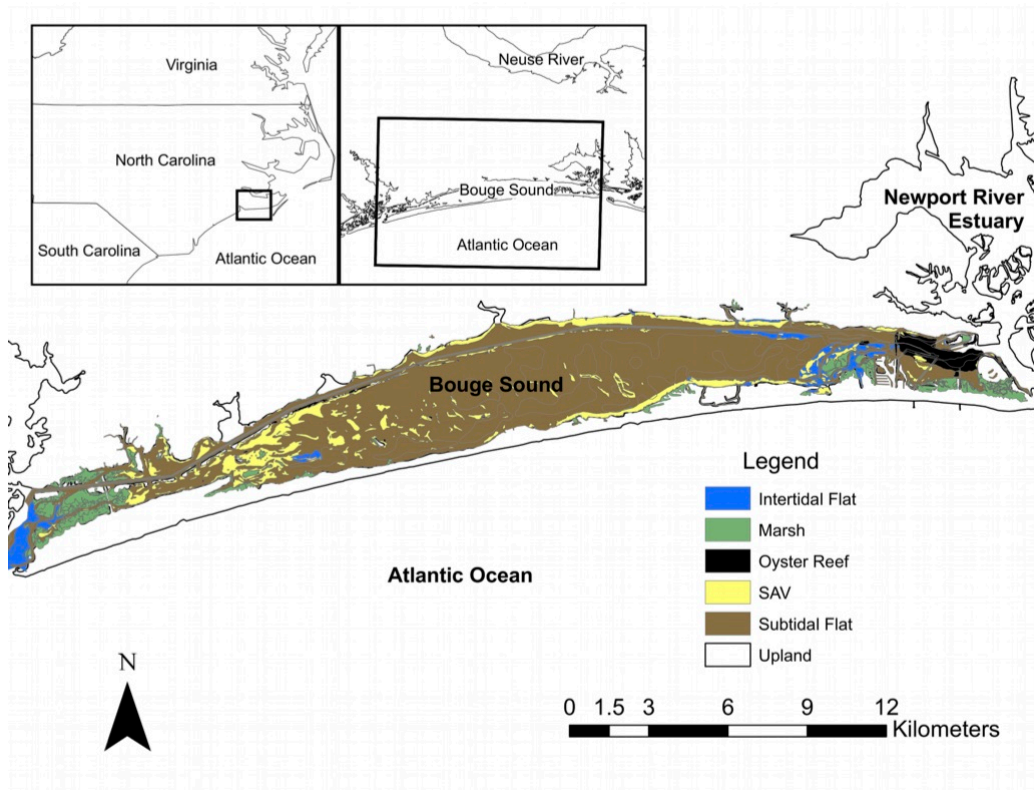
<b>Season</b>	<b>Month</b>	<b>Temp (°C)</b>	<b>Salinity</b>	<b>DO (mg/l)</b>	<b>NO<sub>x</sub><sup>-</sup> (µM)</b>	<b>NH<sub>4</sub><sup>+</sup> (µM)</b>
Winter	Jan.	3.32	32.4	11.4	0.33	1.28
Spring	March	19.0	31.6	8.84	0.49	0.91
Summer	July	30.1	29.3	5.00	BD	3.56
Fall	Nov.	7.44	31.5	12.3	BD	0.65

**Table 2.2** Mean rates of potential DNRA in two seasons and five habitats. Values are mean  $\pm$  1 SE. Significant differences are indicated by \*. There were no measures of ammonium and nitrate fluxes during the winter. Percent of nitrate flux was calculated as recovery of  $^{15}\text{NH}_4^+$  assuming that all of the  $\text{NO}_x$  flux was from the added  $^{15}\text{NO}_3^-$ .

<b>Season</b>	<b>Habitat</b>	<b>DNRA</b> ( $\mu\text{mol } ^{15}\text{NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$ )	<b>Ammonium Flux</b> ( $\mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$ )	<b>Nitrate Flux</b> ( $\mu\text{mol NO}_x \text{ m}^{-2} \text{ hr}^{-1}$ )	<b>% of Nitrate Flux that was <math>^{15}\text{NH}_4</math></b>
Summer 2007	Subtidal Flat	4.5 $\pm$ 3.00	46.5 $\pm$ 5.5	-688 $\pm$ 61.3	0.70
	Intertidal Flat	12.6 $\pm$ 5.40	124 $\pm$ 37.5	-737 $\pm$ 105	1.62
	SAV	26.3 $\pm$ 5.20	275 $\pm$ 87.7	-596 $\pm$ 81.2	4.54
	Marsh	11.4 $\pm$ 7.00	199 $\pm$ 14.0	-560 $\pm$ 21.8	2.94
	Oyster Reef	104* $\pm$ 34.3	443 $\pm$ 154	-1010 $\pm$ 281	11.2
Winter 2008	Subtidal Flat	None Detected			
	Intertidal Flat	None Detected			
	SAV	None Detected			
	Marsh	0.40 $\pm$ 0.30			
	Oyster Reef	0.80 $\pm$ 0.20			

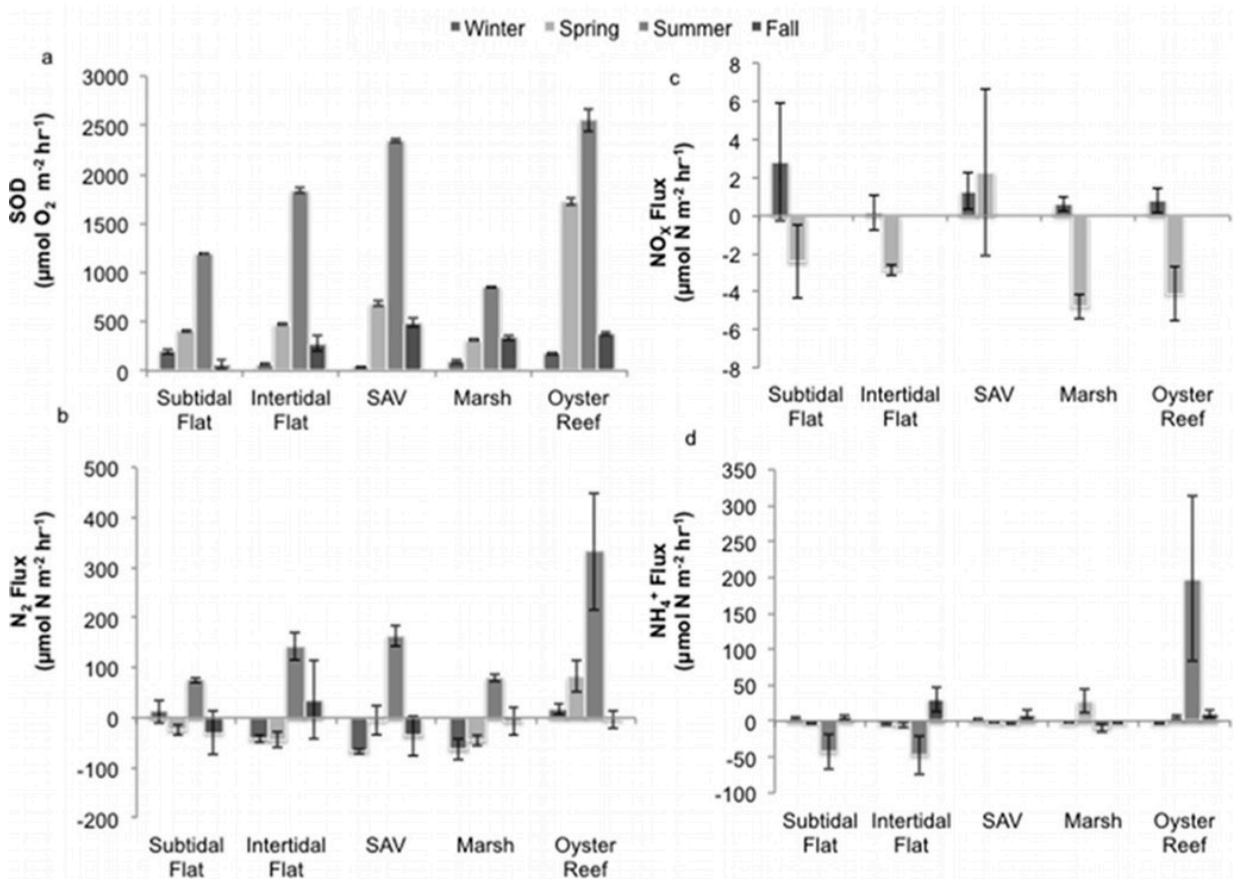
**Table 2.3** Hour inundation during the dark, based on seasonal differences in light and water level and total habitat area in the study system.

<b>Habitat</b>	<b>Average Hours Submerged per day in the Dark</b>			<b>Total Area (km<sup>2</sup>) in Bogue Sound</b>
Subtidal Flat	12	±	0.8	67.9
Intertidal Flat	8.2	±	0.8	3.7
SAV	12	±	0.8	21.1
Marsh	6.1	±	0.8	8.2
Oyster Reef	7.6	±	1.0	5.2

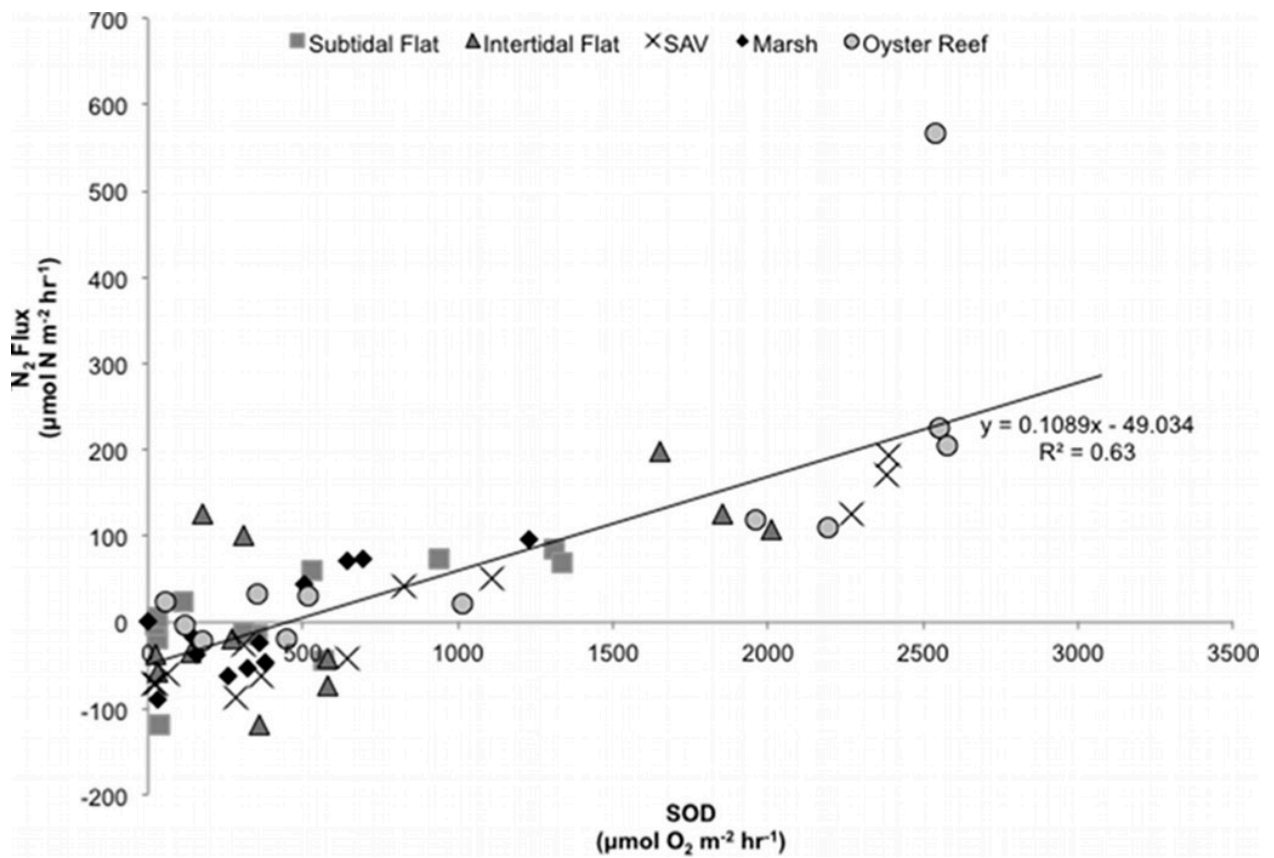


**Figure 2.1** Location and habitat map of the study area where extrapolations were performed.

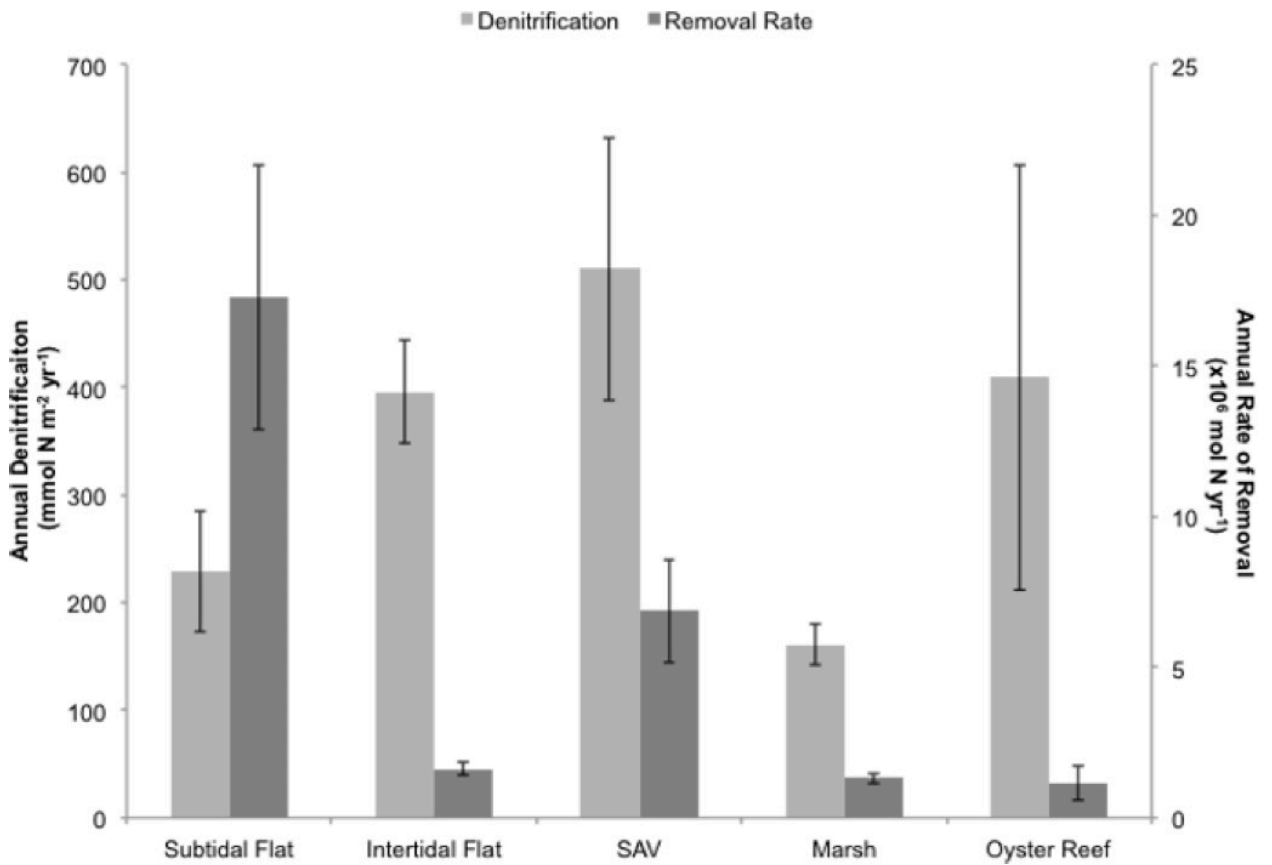




**Figure 2.2** Seasonal averaged ( $n=3$ ) sediment oxygen demand (a),  $\text{N}_2$  fluxes (b), Nitrate fluxes (c), and Ammonium fluxes (d) across the sediment water interface in different estuarine habitats. For figures b,c,d negative flux indicates uptake by the sediment and positive flux indicates release. Error bars represent standard error.



**Figure 2.3** Net N<sub>2</sub> flux as a function of sediment oxygen demand (SOD) for habitats in Bogue Sound, NC. Relationship of all data is plotted for reference.



**Figure 2.4** Annual areal rates of denitrification based on hourly rates that were adjusted for light and inundation for each habitat and annual rate of removal based on the total amount of habitat area in Bogue Sound.

### **3. OYSTER MEDIATED BENTHIC-PELAGIC COUPLING MODIFIES NITROGEN POOLS AND PROCESSES<sup>2</sup>**

#### **3.1 Abstract**

Anthropogenic nitrogen loading has led to eutrophication of many estuaries. Removal of nitrogen through enhanced denitrification has been identified as an ecosystem service provided by oysters. In this study, we assessed the effects of an individual oyster on nitrogen dynamics. Net fluxes of N<sub>2</sub>, O<sub>2</sub>, nitrate (NO<sub>x</sub>) and ammonium (NH<sub>4</sub><sup>+</sup>) were measured in continuous-flow microcosms that contained a live oyster, sediment, or a live oyster+sediment. Net N<sub>2</sub> fluxes were indicative of nitrogen fixation in the sediment treatment and denitrification in the oyster and oyster+sediment treatments. This difference probably resulted from increased organic matter deposition and ammonium production associated with excretion and biodeposit production. Our results suggest that oyster-mediated denitrification may be most apparent in carbon-limited systems. Despite high rates of ammonium production associated with the oysters, oyster-mediated denitrification accounted for 40% of the total inorganic nitrogen efflux in the oyster microcosms and 16% in the oyster+sediment microcosms. Despite high rates of ammonium production, the inclusion of the eastern oyster did not affect the pool of bioavailable nitrogen but shifted the microcosms from a nitrogen source to sink.

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<sup>2</sup> Chapter 3 is under review for the journal Marine Ecology Progress Series with authors A.R. Smyth, N.R. Geraldi, and M.F. Piehler.

### 3.2 Introduction

Human activities have drastically altered the structure and function of estuarine ecosystems. Two of the most troubling changes have been nutrient enrichment (Nixon et al. 1995) and loss of bivalves (Frankenberg 1995, Jackson et al. 2001a). Estuarine ecosystems are typically nitrogen limited (Paerl 1997, Howarth and Marino 2006) and phytoplankton growth stimulated by increased inputs of reactive nitrogen have led to eutrophication (Vitousek et al. 1997b). Nitrogen loss in estuaries occurs primarily through denitrification, the microbial conversion of nitrate or nitrite to  $N_2$  or  $N_2O$  (Seitzinger 1988, Nixon et al. 1995). However, development, deforestation and overharvesting have changed the estuarine landscape, reducing areas where conditions are favorable for denitrification (Lotze et al. 2006, Brush 2009). The loss of natural nitrogen sinks coupled with increased nitrogen inputs has exacerbated the imbalance in the estuarine nitrogen cycle further contributing to eutrophication.

Oysters and the reefs they form are ecologically and economically valuable habitats (Grabowski et al. 2012). However, 85% of oyster reefs have been lost globally due to overharvesting, pollution and disease (Lotze et al. 2006, Beck et al. 2011). Restoration of oysters has been suggested as a remedial tool to combat eutrophication and improve water quality while providing additional ecosystem services (Officer et al. 1982, Cerco and Noel 2007, Beck et al. 2011, Grabowski et al. 2012). Oysters filter large amounts of particulate matter from the water column, grazing as much as 12% of phytoplankton biomass (Grizzle et al. 2008). While a portion of this material is assimilated into oyster biomass (Carmichael et al. 2012), the undigested (pseudo-feces) and the unassimilated portions (feces) are transferred to the sediments as biodeposits (Newell and Jordan 1983). The transformation and transfer of material modifies conditions in the surrounding sediments and can affect biogeochemical

processes (Newell et al. 2002, Porter et al. 2004, Newell et al. 2005, Piehler and Smyth 2011, Smyth et al. 2013).

Oysters may enhance denitrification by modifying oxygen, carbon and/or nitrate availability. Oysters, like other bivalves, contribute to anoxic conditions favorable for denitrification through respiration and decomposition of organic material in biodeposits (Gelda et al. 2001, Bruesewitz et al. 2008). Biodeposition of carbon rich biodeposits could enhance denitrification, particularly in carbon-limited systems (Kimmel and Newell 2007, Higgins et al. 2013). When nitrate limits denitrification; increased ammonium associated with oyster excretion and remineralization of biodeposits may fuel nitrate production through nitrification (Dame et al. 1984, Lavrentyev et al. 2000, Newell et al. 2005). Additionally, bivalves may stimulate nitrification by filtering out bacterivorous protozoa that would otherwise consume nitrifying bacteria, leading to increased rates of nitrification because of decreased predation (Lavrentyev et al. 2000). However, ammonium produced in excess of nitrification probably returns to the water column, and is used to meet nitrogen demands for phytoplankton growth (Dame et al. 1984, 1985, Kemp et al. 1990).

Previous studies designed to examine oyster effects on nitrogen transformations have focused on adjacent sediments (Piehler and Smyth 2011, Smyth et al. 2013) or mimicked oyster-mediated biodeposition (Newell et al. 2002). We conducted a microcosm experiment to examine the direct effects of an individual oyster on nitrogen dynamics. The inclusion of live oysters in continuous-flow microcosms allowed us to quantify the extent to which oyster-mediated benthic-pelagic coupling influences the availability and processing of nitrogen. Understanding how an individual oyster modifies both nitrogen pools and processes is valuable for planning and evaluating restoration strategies to improve water quality.

### **3.3 Methods**

#### **3.3.1 Sample Collection**

The experiment was conducted in microcosms (polycarbonate 6.4 cm diameter X 30cm) that contained a live oyster, sediment, or a live oyster+sediment and incubated in a continuous-flow system. Intertidal oysters (*Crassostrea virginica*) were collected from Calico Creek, NC (34.728, -76.722), at low tide and stored in saltwater flow-through tanks for three days. Prior to the start of the incubation, the outside shell of each oyster was scrubbed with a brush to remove algae and biofilms and isolate the impacts of the oyster. Average oyster shell width in our experiment was  $9.34 \pm 0.45$  cm and the average weight of oyster tissue was  $1.0 \pm 2.5$  g.

Sediments samples (17 cm depth) were collected on 4 August 2009 during low tide from a homogenous intertidal flat in Bogue Sound, NC (a site suitable for oyster restoration) by pushing a microcosm chamber directly into the sediment. In addition, water (30l) was collected for use as reservoir water in the continuous-flow incubation. Surface water temperature, salinity and dissolved oxygen were measured prior to sample collection (YSI 600 Series Sonde and Model 650 data logger, Yellow Springs Instruments, Yellow Springs, OH).

#### **3.3.2 Benthic Flux Incubations**

Immediately after collection of sediment and water, all microcosm chambers were submerged in a water bath in an environmental chamber (Bally Inc.) set to *in situ* temperature (24.7 °C). Microcosms were randomly assigned a treatment (oyster, sediment, oyster+sediment) and each treatment was replicated 3 times. Microcosms were sealed with a

gas tight lid equipped with an inflow and outflow port and incubated in a continuous-flow system, where a peristaltic pump connected microcosms to the water reservoir. Aerated, unfiltered water was constantly passed through each microcosm at a flow rate of  $2\text{ml min}^{-1}$  with a turnover time of approximately 3 hours. After an initial 20-hour acclimation period in the dark, microcosms were incubated over 24-hrs in a 10:14 hr, dark:light cycle. A light intensity of approximately  $50 \mu$  einsteins was maintained using dual spectrum compact florescent lights. Oxygen in the reservoir water was monitored throughout the incubation and remained about 6 mg/l. During the incubation, oysters were actively feeding as indicated by a gap between the valves of the oyster shells. All oysters were alive at the conclusion of the experiment.

### **3.3.3 Analytical Techniques**

$\text{N}_2$ ,  $\text{O}_2$  and Ar were measured using a Balzers Prisma QME 200 quadruple mass spectrometer (MIMS; Pfeiffer Vacuum, Nashua, NH, USA) and concentrations of  $\text{N}_2$  and  $\text{O}_2$  were determined using the ratio with Ar (Kana et al. 1994, Ensign et al. 2008). Samples for nutrient analysis were filtered through Whatman GF/F filters (25 mm diameter,  $0.7 \mu\text{m}$  nominal pore size), and the filtrate was analyzed for nitrate (nitrate plus nitrite;  $\text{NO}_x$ ) and ammonium ( $\text{NH}_4^+$ ) with a Lachat Quick-Chem 8000 (Lachat Instruments, Milwaukee, WI, USA) automated ion analyzer (detection limits:  $0.04 \mu\text{M NO}_x$ ,  $0.18 \mu\text{M NH}_4^+$ ).

Samples for dissolved gas analysis were collected twice during the dark period and twice during the light period for each microcosm. Nutrient samples were collected once in the dark and once in the light. Flux calculations were based on the assumption of steady-state conditions (Miller-Way and Twilley 1996). Fluxes of dissolved nutrients and gasses were calculated as the difference between the concentration leaving and entering the microcosm



divided by the flow rate, and expressed relative to the area of the microcosm (Lavrentyev et al. 2000). A positive flux indicates production in excess of demand and a negative flux is a demand in excess of production within the microcosm. Fluxes from each microcosm were averaged for each treatment to calculate mean values and standard error (n=3). Daily fluxes were calculated as the sum of the light rate multiplied by 14 hours and the dark rate multiplied by 10 hours. For consistency, daily fluxes were divided by 24 hours and expressed per hour. Net dissolved inorganic nitrogen fluxes (DIN) were calculated as the sum of ammonium and nitrate fluxes plus nitrogen fixation minus denitrification.

### **3.3.4 Statistical Analysis**

The effect of treatment and illumination (dark or light) on fluxes of N<sub>2</sub>, O<sub>2</sub>, NO<sub>x</sub>, NH<sub>4</sub><sup>+</sup> and net DIN was tested separately using a mixed effect analysis of variance (ANOVA). Treatment was a fixed factor and illumination was nested within microcosm. Within factor significance was tested using Tukey's HSD. All analyses were considered significant at the p<0.05 level and were conducted using JMP 7.0.1 statistical software (SAS 2007).

### **3.4 Results**

Oxygen demand was lower in the sediment microcosms compared to the other treatments ( $F_{2,2}=23.11$ ,  $p<0.001$ ; Figure 3.1). We found no significant effect of light on oxygen demand ( $F_{3,3}= 0.8380$ ,  $p=0.4987$ ). Oxygen demand was higher in the light compared to the dark for the oyster and oyster+sediment treatments. Oxygen demand ranged from  $150 \pm 48 \mu\text{mol O}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for the sediment in the light to  $7270 \pm 352 \mu\text{mol O}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for the oyster in the light. Oxygen demand was significantly higher in treatments that contained an oyster compared to the sediment, probably attributable to oyster respiration.

N<sub>2</sub> fluxes were significantly different between each treatment ( $F_{2,2}=40.88$ ,  $p<0.0001$ ; Fig 3.1) and ranged from  $426 \pm 55 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for the oyster treatment in the light to  $-294 \pm 35 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for the sediment treatment in the light. The effect of light was not significant ( $F_{3,3}=0.1093$ ,  $p=0.8523$ ). Daily N<sub>2</sub> fluxes from the sediment treatment were negative, indicating net nitrogen fixation while fluxes from the oyster and oyster+sediment treatments were positive, indicating net denitrification.

NO<sub>x</sub> fluxes were highly variable and mostly negative (Fig 3.2). Fluxes ranged from  $-23 \pm 5.2 \mu\text{mol NO}_x \text{ m}^{-2} \text{ hr}^{-1}$  in the oyster treatment in the light to  $2.3 \pm 9.1 \mu\text{mol NO}_x \text{ m}^{-2} \text{ hr}^{-1}$  in the oyster+sediment treatment in the light. NO<sub>x</sub> fluxes were not affected by oyster presence ( $F_{2,2}=2.191$ ,  $p=0.154$ ) or light ( $F_{3,3}=0.3243$ ,  $p=0.808$ ).

The oyster and oyster+sediment treatments had positive fluxes of NH<sub>4</sub><sup>+</sup> while fluxes in the sediment treatment were negative (Figure 3.2). NH<sub>4</sub><sup>+</sup> fluxes ranged from  $-51 \pm 57 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$  in the light for the sediment treatment to  $588 \pm 83 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ hr}^{-1}$  in the light for the oyster treatment. Light did not affect NH<sub>4</sub><sup>+</sup> fluxes for either treatment ( $F_{3,3}=0.1840$ ,  $p=0.9052$ ). NH<sub>4</sub><sup>+</sup> fluxes were higher in the oyster and oyster+sediment treatments than the sediment treatment ( $F_{2,2}=19.634$ ,  $p=0.0002$ ).

Oyster mediated denitrification accounted for an average of 18% of the total dissolved inorganic nitrogen efflux, ranging from 16% from the oyster+sediment microcosm to 40% from the oyster only microcosm. Net DIN fluxes were not different between treatments ( $F_{2,2}=0.3272$ ,  $p=0.7272$ , Figure 3.2) or illumination ( $F_{3,3}=1.622$ ,  $p=0.2362$ ).

### 3.5 Discussion

A holistic understanding of oyster-mediated alteration to estuarine nitrogen dynamics

requires knowledge of the individual oyster's impact on nitrogen processing. Oyster presence caused a shift in the dominant nitrogen cycling pathway from nitrogen fixation to denitrification. Oyster mediated denitrification accounted for 40% of the total DIN efflux in the oyster microcosms and 16% in the oyster+sediment microcosms. Although the oyster treatments increased denitrification, these treatments also had significantly higher rates of ammonium production compared to the sediment microcosms. However, the net DIN flux was not different among treatments because of the magnitude of nitrogen fixation in the sediment only treatment.

Oyster biodeposits contain significant amounts of organic carbon, nitrogen and extractable ammonium that can supply fuel to the microbial community (Haven and Morales-Alamo 1966, Grenz et al. 1990, Giles and Pilditch 2006, Higgins et al. 2013). Oysters produce about 1.33 mg C to 16.8 mg C per gram oyster tissue per day as biodeposits (Haven and Morales-Alamo 1966, Higgins et al. 2013). Given the turn over time in our incubation and the clearance rates of an oyster, we estimate that oysters added  $2.66 \pm 6.65$  to  $33.6 \pm 84$  mg of particulate C to the microcosm. While we did not measure carbon quality or quantity, biodeposits were observed in the bottom of the microcosms. Oyster and oyster+sediment treatments also had high rates of ammonium production likely from direct excretion by the oyster and remineralization of organic matter in the biodeposits.

The oyster treatment had the highest rate of denitrification. It is probably that denitrifying bacteria found in the gut of oysters (Pujalte et al. 1999) were present in biodeposits, (Grenz et al. 1990, Azandégbé et al. 2012). Without competition from native benthic organisms, the majority of the carbon and nitrogen in the biodeposits could be used for heterotrophic metabolism leading to anoxic micro-zones and conditions favorable for

denitrification. Additionally, inputs of labile organic matter stimulate denitrification, whereas nitrogen fixation tends to dominate when organic matter is refractory (Fulweiler et al. 2013). It is probably that the combination of an added ammonium supply with a source of high quality organic matter lead to the shift from nitrogen fixation in the sediment microcosm to denitrification in oyster microcosms.

Quantifying the effects of oysters on ecosystem function is challenging, given methodological difficulty and the complexity of the reef ecosystems. Our results align with previous studies which concluded that oyster mediated denitrification occurs through coupling with nitrification stimulated by biodeposition and ammonium production from the oysters (Boucher and Boucher-Rodoni 1988, Newell et al. 2002, Piehler and Smyth 2011, Smyth et al. 2013). We found denitrification rates within the range of those reported for intertidal oyster reef sediments (Piehler and Smyth 2011, Smyth et al. 2013). However, oyster denitrification rates were higher than rates associated with the biodeposits alone (Higgins et al. 2013), probably attributable to oyster respiration altering O<sub>2</sub> dynamics. Oyster-mediated denitrification removed a similar percentage of nitrogen as when pelletized phytoplankton were used to mimic biodeposits (Newell et al. 2002).

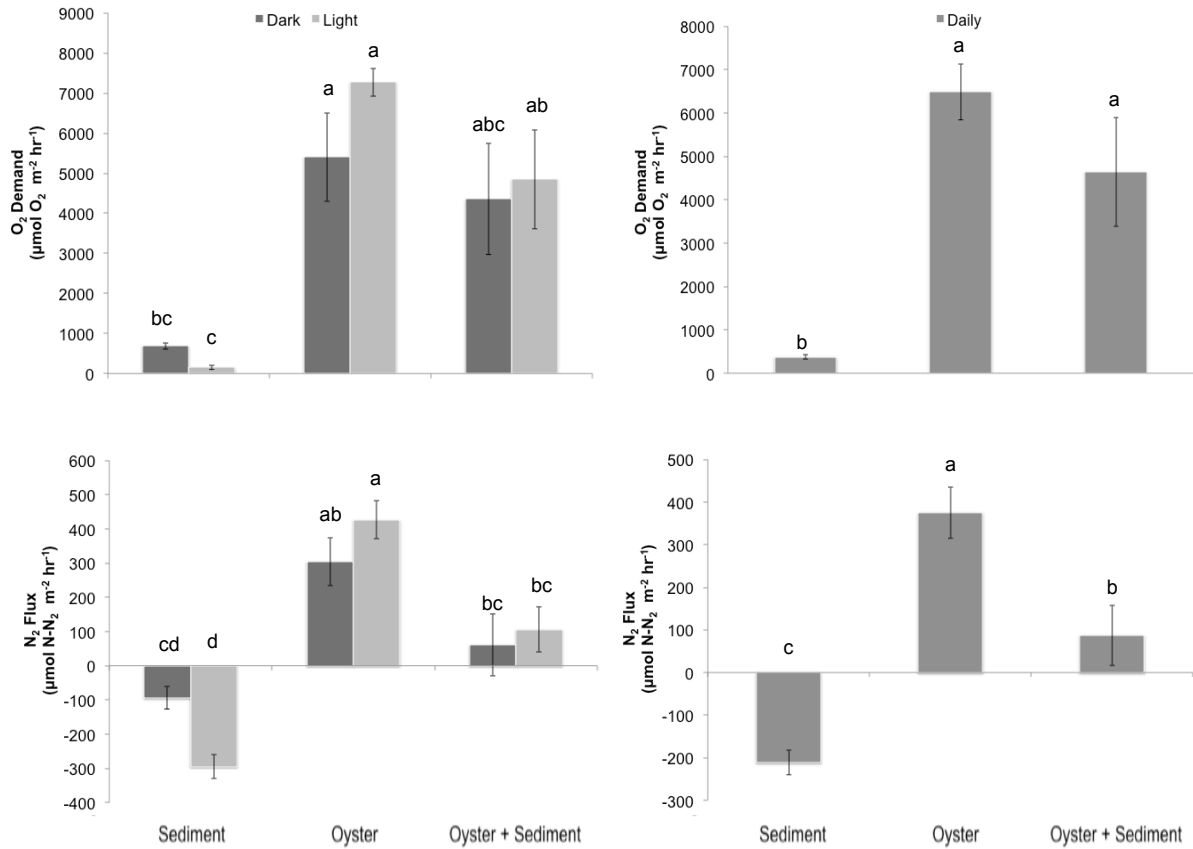
The inclusion of the oyster in the microcosm acted as an organic matter addition, which caused a shift from nitrogen fixation to denitrification. In systems with low levels of carbon loading, nitrogen fixation occurs at greater rates than denitrification (Fulweiler et al. 2008, 2011). Nitrogen and carbon inputs from oysters likely suppress nitrogen fixation and enhance denitrification. However, in carbon rich systems additional organic matter from the oysters may exacerbate reduced conditions, resulting in sulfide accumulation (Tenore and Dunstan 1973, Azandégbé et al. 2012) and increased anoxic microzones (Kemp et al. 1990)

that can inhibit nitrification (Joye and Hollibaugh 1995) and subsequently reduce rates of denitrification. At intermediate levels of carbon loading steep biogeochemical gradients persist, putting the zones for nitrification and denitrification in close proximity, which enhances coupling between these processes (Eyre and Ferguson 2009). Because oyster biodeposition adds organic matter, oyster mediated denitrification is more likely to occur when denitrification is limited by carbon availability.

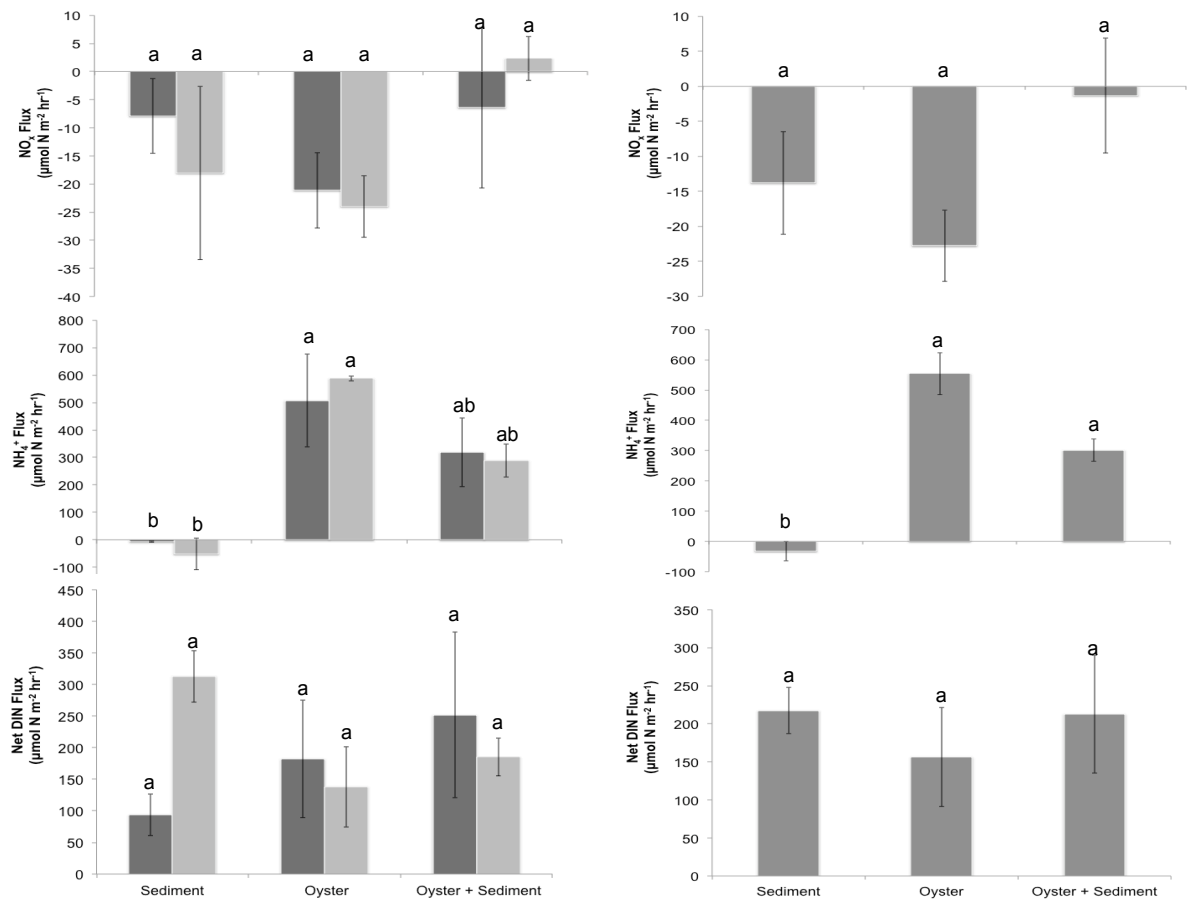
This study contributes to the growing body of evidence showing that oysters enhance denitrification (Newell et al. 2002, Piehler and Smyth 2011, Smyth et al. 2013). We found that although oyster presence increased the system's capacity to denitrify the net DIN flux was not different between treatments. In the absence of oysters sediments are a net source of reactive nitrogen whereas the addition of oysters increase organic matter deposition, alleviating carbon limitation and increasing denitrification. Thus, oyster restoration will not add additional nitrogen to carbon-limited systems but will provide valuable ecosystem services, including enhanced denitrification.

### **3.6 Acknowledgements**

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**Figure 3.1.** Mean dark (n=3), light (n=3) and Daily O<sub>2</sub> demand, N<sub>2</sub> gas flux from each treatment. Different letters indicate significant differences between the treatments. Error bars represent one standard error.



**Figure 3.2.** Mean dark (n=3), light (n=3) and Daily NO<sub>x</sub> flux, NH<sub>4</sub><sup>+</sup> flux and Net DIN flux from each treatment. Different letters indicate significant differences between the treatments. Error bars represent one standard error.

## **4. ENHANCED DENITRIFICATION IN OYSTER REEF SEDIMENTS IS A FUNCTION OF BOTH REEF STRUCTURE AND BIODEPOSIT PRODUCTION**

### **4.1 Abstract**

Anthropogenic activities have altered the structure and function of coastal ecosystems. Increased nutrient inputs have led to eutrophication and reduction in water quality. In addition, overharvesting and disease have reduced populations of suspension feeding bivalves that exert top down control on phytoplankton biomass. Oyster reef restoration has been proposed as a way to improve water quality and remove excess nitrogen. Biodeposits can fuel microbially mediated denitrification. However, the reef structure probably contributes to the accumulation of biodeposits and other organic matter on the sediments. We conducted a field experiment to distinguish between the effects of oyster feeding and reef structure on sediment denitrification. Experimental plots with live oysters, oyster shells and mud flats (control) were sampled for sediment organic matter, sediment C and N content, and fluxes of nitrogen ( $\text{NH}_4^+$ ,  $\text{NO}_x$  and  $\text{N}_2$ ) two weeks and four weeks after construction. Compared to the control, reefs with live oysters increased  $\text{N}_2$  production (denitrification) by 61% and reefs with shell only showed a 24% increase. These results indicate that biotic and abiotic interactions lead to enhanced biogeochemical activity in oyster reef sediments. Denitrification from experimental plots was equal to natural reefs after two weeks. Results from this experiment demonstrate the potential for restored reefs to improve water quality via nitrogen removal through a combination of physical and biological mechanisms soon after establishment. A mechanistic understanding of the influence of oyster



reefs on nitrogen biogeochemistry will improve management plans aimed at improving water quality.

## **4.2 Introduction**

Key processes in ecosystems are driven by interactions between organisms and their environment. Certain organisms act as engineers; modifying the supply of resources to other organisms through biotic or abiotic interactions (Jones et al. 1994, 2006). Ecosystem engineers change the environment through their own physical structure or by transforming material from one physical state to another. These organisms influence species richness, community composition and primary production (Wright and Jones 2004). Additionally, ecosystem engineers can contribute to the creation of biogeochemical hot spots, by changing the availability of resource to microbes or altering the environmental conditions in the sediment (McClain et al. 2003, Gutierrez and Jones 2006, Jones et al. 2006).

Changes in the physical and chemical environment are of particular importance for the nitrogen cycle, where slight alterations in conditions can change processing from nitrogen recycling to removal (Kemp et al. 1990, Fulweiler et al. 2013). Nitrogen is an important resource at the base of the food web and the limiting nutrient in coastal ecosystems (Nixon et al. 1996, Paerl 1997). Additional inputs of nitrogen from anthropogenic activities have led to increased instances of eutrophication and overall degradation of water quality (Vitousek et al. 1997b, Paerl et al. 1998, Galloway et al. 2003). While there are a variety of sources, nitrogen is removed through physical transport, burial, or denitrification (Vitousek et al. 1997b). Denitrification is a microbially-mediated reaction where carbon serves as the electron donor in the reduction of nitrate ( $\text{NO}_3^-$ ) to inert  $\text{N}_2$  gas. This reaction is an important nitrogen-sink in coastal systems but is restricted to anoxic sediments (Seitzinger 1988, Nixon et al. 1996,

Cornwell et al. 1999). Nitrate may come directly from the water column or through nitrification—the conversion of ammonium to nitrate (McClain et al. 2003, Seitzinger et al. 2006). However, nitrification is limited by ammonium availability, which is linked to organic matter deposition and is constrained to oxygenated sediments (Henriksen 1980, Kemp et al. 1990).

Coastal ecosystems are associated with high rates of denitrification, removing nitrogen before it is transferred to the continental shelf (Seitzinger 1988, Nixon et al. 1996, Paerl 1997, Galloway et al. 2003). The quantity of nitrogen that is processed and removed is dependent on the amount and type of habitats in the landscape mosaic (Eyre et al. 2011a, Smyth et al. 2013). Unfortunately, destructive harvesting practices and degradation of water quality have led to biogenic habitat loss (oyster reefs, seagrass beds, marshes) and altered the structure and function of estuarine ecosystems (Lotze et al. 2006). Restoration of ecosystem engineers has been proposed as a way to recover ecosystem functions including denitrification (Byers et al. 2006, Brush 2009). However, incorporating ecosystem engineers into nutrient management plans requires an understanding of how abiotic structure and biotic processes change the availability of resources and conditions in the sediment (Jones et al. 2006).

The eastern oyster, *Crassostrea virginica*, is an ecologically and economically important ecosystem engineer (Lenihan and Peterson 1998, Lotze et al. 2006, Coen et al. 2007, Beck et al. 2011, Grabowski et al. 2012). The oyster fishery comprises a multi-million dollar industry, but over-harvesting and disease have led to an 85% reduction in reef ecosystems (Beck et al. 2011). Oyster mediated benthic-pelagic coupling shifts production from the water column to the sediment (Dame et al. 1984). Oysters feed on seston and

transfer the undigested and unassimilated fraction to the sediment surface as feces and pseudofeces, collectively biodeposits (Haven and Morales-Alamo 1966, Dame et al. 1984, Newell 2004).

In aerobic environments the deposition of organic matter as biodeposits stimulates coupled nitrification-denitrification by altering the redox environment of the surrounding sediments (Newell et al. 2002). Studies have shown intertidal oyster reef sediments to have high rates of denitrification relative to sediments without reefs (Piehler and Smyth 2011, Smyth et al. 2013). The primary explanation for these high rates is the accumulation of biodeposits on the sediment, absent from sediments from other habitats. However, the physical habitat structure may also be contributing to organic matter accumulation (Lenihan 1999, Lenihan et al. 2001, Gutierrez and Jones 2006, Falcao et al. 2009). Given the link between denitrification and carbon availability (Eyre and Ferguson 2009, Fulweiler et al. 2013), it is possible that a portion of the enhanced denitrification may result from allochthonous carbon loading attributable to the reef structure.

The goal of this study was to distinguish the relative importance of the physical structure of the oyster reef and biodeposit production of the oyster to sediment nitrogen dynamics. Based on our understanding of the factors that affect denitrification, we speculated that interactions between the biological function and physical engineering of oyster reefs would result in enhanced denitrification. To test the hypothesis, experimental plots were constructed containing either live oysters, oyster shell or sediment controls and nitrogen fluxes were monitored.

### **4.3 Methods**

### **4.3.1 Site Description**

The experiment was conducted at Hoop Pole Creek, in Bogue Sound, NC, USA (34.422, 76.455). Hoop Pole Creek is part of a wildlife refuge, located on the sound side of a barrier island. The area is closed to oyster harvest and contains natural and restored oyster reefs (O'Connor et al. 2008). Our experiment was conducted on intertidal mudflats located approximately 25m away from the edge of a fringing salt marsh.

### **4.3.2 Oyster Collection**

Clumps of intertidal oysters (*Crassostrea virginica*) were collected from Calico Creek, NC (34°728'N, 76°722'W), at low tide on 28 June 2010. Oyster clumps were transported back to The University of North Carolina- Institute of Marine Sciences (UNC-IMS) and stored in tanks with continuously flowing water from Bogue Sound. Oyster clumps were haphazardly placed in eight 5-gallon opaque buckets (42L of clumped shell) and each bucket was assigned to two groups. Oysters in four of the buckets were kept alive in experimental ponds exposed to natural light irradiation at UNC-IMS. The remaining four buckets of oysters were left outside in the sun for >2 weeks. This resulted in the removal of the oyster meat and the majority of all organic material while maintaining the structure of the oyster clumps. Prior to setting up the field experiment all oyster (>10mm) were counted and divided into four groups of each treatment.

### **4.3.3 Field Experiment**

To test the effects of reef structure on sediment nitrogen dynamics, we constructed replicate plots of 0.75 m<sup>2</sup> oyster reefs of live oysters or oyster shells on intertidal sediment flats. Each experimental plot had an oyster density of  $123 \pm 11$  individuals per plot (0.75m<sup>2</sup>).

The experiment was conducted from 16 July 2010 through 15 August 2010. On 16 July 2010 at low tide the intertidal mudflat was divided into 12-0.75m<sup>2</sup> plots (2 rows of 6 plots), each three meters apart. Plots were haphazardly assigned a treatment within each of the 2 rows: live oysters, oyster shell, or control (mud flat). Clumps of live oysters or oyster shell were positioned in an upright orientation in the water column to mimic a natural or fully restored and functioning oyster reef within their respective plots. A 10cm by 10cm area was left open in the center of each plot for sediment samplings. Plots were checked every three days to ensure oysters maintained an upright orientation and repositioned if necessary.

#### **4.3.4 Field Sampling**

Sediment samples were collected two weeks after construction of experimental plot. This allowed time for the sediments to establish equilibrium before sampling for biogeochemical analysis were collected (Porter et al. 2006).

Porewater was collected using “sippers” (McGlathery et al. 2001). Sippers were positioned to collect water in the pore space from 1cm-6cm depth. Water samples were filtered through Whatman GF/F glass fiber filters (25mm diameter, 0.7 μm nominal pore size) and frozen until dissolved inorganic nitrogen analysis. Sediment samples were collected for total carbon (C) and nitrogen (N) analysis from each plot. Sediment cores for C and N analysis were 3 cm in diameter and 5 cm in depth. Sediments in the upper 0.5 cm were assayed for chlorophyll *a* (Chl. *a*) as an estimate of benthic algal biomass with a 1.1 cm diameter-coring device. The upper 2 cm of sediment (2.5 cm diameter) from each plot was sampled for sediment organic matter (SOM) content. Upon collection, samples were kept in the dark on ice in a cooler and immediately (<1hr) transported back to UNC-IMS. All samples were stored frozen until analysis. Sediment cores (6.4cm diameter by 17 cm depth)

and sound water (~30l) were collected for use in continuous flow core incubations designed to measure exchanges across the sediment-water interface (Lavrentyev et al. 2000, McCarthy and Gardner 2003). Sediment samples for the flux experiment, SOM, and C and N analysis were collected again 4 weeks after construction. In situ surface water temperature and salinity were measured at each sampling (YSI 600 Series Sonde and Model 650 data logger, Yellow Springs Instruments, Yellow Springs, OH, USA). In response to the development of and oysters settlement (personal observation), the measurements were not continued beyond 4 weeks.

#### **4.3.5 Core Incubation**

Sediment cores and water for the flux incubation were immediately (<1hr) transported to an environmental chamber (Bailey, Inc.) set to *in situ* (30°C) temperature at UNC-IMS. Dark conditions were maintained throughout the course of the incubation to reduce the effects of photosynthetic algae (An and Joye 2001, Hochard et al. 2010) and to prevent the formation of bubbles that would affect gas concentrations (Reeburgh 1969). Sediment cores were submerged in a water bath and sealed with gas tight lids equipped with an inflow and outflow port and connected to a peristaltic pump (Miller-Way and Twilley 1996, Lavrentyev et al. 2000, McCarthy and Gardner 2003, Ensign et al. 2008). Unfiltered, aerated water collected from the site (37ppt) was continuously passed over the cores at a flow rate of 1ml per minute (Miller-Way and Twilley 1996, Lavrentyev et al. 2000).

Following a pre-incubation period of 24 hours, water samples were collected for dissolved gas and nutrient analysis from the outflow port of each chamber and a bypass that flowed directly into a sample vial, which represented the inflow concentrations (Miller-Way and Twilley 1996, Eyre and Ferguson 2002, McCarthy and Gardner 2003). Dissolved gases

were sampled and analyzed immediately after collection three times over a 48-hour period (24, 30, 48 hours) to ensure that steady state conditions were established, with respect to dissolved gasses, for each chamber. Samples for nutrient analysis were collected once after steady state had been established with respect to dissolved gas concentrations (~30 hours after the incubation began), filtered through Whatman GF/F glass fiber filters (25mm diameter, 0.7  $\mu\text{m}$  nominal pore size) and frozen until analysis. A bypass line that flowed directly into the sample vials was used to determine the concentration of dissolved constituents entering the cores and account for changes in the water column.

#### **4.3.6 Dissolved Gas Analysis**

Concentrations of dissolved gases in water were measured using a Balzers Prisma QME 200 quadruple mass spectrometer (MIMS; Pfeiffer Vacuum, Nashua, NH, USA). Concentrations of  $\text{O}_2$  and  $\text{N}_2$  were determined using the ratio with Ar (Kana et al. 1994, Ensign et al. 2008). This results in a net flux across the sediment-water interface and does not discern between the sources of  $\text{N}_2$ : therefore, denitrification refers to the net production of  $\text{N}_2$ . Fluxes of oxygen directed into the sediment were considered rates of sediment oxygen demand (SOD).

#### **4.3.7 Water Chemistry**

Nutrient samples from the porewater and the flux experiment were analyzed with a Lachat Quick-Chem 8000 automated ion analyzer for  $\text{NO}_x$  ( $\text{NO}_3^- + \text{NO}_2^-$ ) and  $\text{NH}_4^+$  concentrations using standard protocols (Lachat Instruments, Milwaukee, WI, USA:  $\text{NO}_3^- / \text{NO}_2^-$  (collectively  $\text{NO}_x^-$ ) Method 31-107-04-1-A;  $\text{NH}_4^+$  Method 31-107-06-1-A: detection limits: 0.04  $\mu\text{M}$   $\text{NO}_x$ , 0.18  $\mu\text{M}$   $\text{NH}_4^+$ ).

#### **4.3.8 Sediment Samples**

Sediments for C and N measurement were dried at 70°C, ground with a mortar and pestle, fumed for 48 hours with 1N HCl to remove inorganic C, and dried again. Fumed sediment samples were analyzed for organic C and N content with a Perkin Elmer CHN analyzer (Model 2400 Series II) standardized with acetanilide. Analysis for Chl *a* was performed according to Lorenzen (1967), modified to include the extraction of the sediment with 10ml of solvent (Pinckney et al. 1994). Cores for Chl *a* were placed in polypropylene centrifuge tubes with 10 ml of solvent, sonicated over ice for 30 seconds and extracted at 0°C for 18 hours. Chl *a* concentrations were determined using spectrophotometry (Lorenzen 1967). SOM content was assessed by loss on combustion. Sediment samples were dried, weighed, combusted at 525°C for 4 hours and weighed again. The difference between dried and combusted samples constituted the organic matter, which was expressed as a percentage of the total.

#### **4.3.9 Calculations**

Fluxes across the sediment-water interface were calculated as  $(C_o - C_i) \times f/a$ , where  $C_o$  is the outflow concentration, ( $\mu\text{mol L}^{-1}$ ),  $C_i$  is the inflow concentration (measured from the bypass line),  $f$  is the flow rate ( $0.06 \text{ L h}^{-1}$ ), and  $a$  is the sediment surface area ( $0.0032 \text{ m}^2$ ). Successive measurements from each core were averaged to give core specific values to prevent pseudo-replication. Denitrification efficiency was calculated as the percent of the dissolved inorganic nitrogen efflux that was  $\text{N}_2$  (Eyre and Ferguson 2009).

#### **4.3.10 Data Analysis**

A one-way repeated measures analysis of variance (ANOVA) was used to determine differences between treatments of fluxes, SOM and C:N that were measured from the same plots two weeks and four weeks after construction. A one-way ANOVA was used for Chl *a*



and porewater nutrients, which were only sampled once during the experiment. When effects were significant, Tukey's post-hoc test was used to determine differences between the treatments. The relationships between net N<sub>2</sub> fluxes and NH<sub>4</sub><sup>+</sup> fluxes, SOD, and SOM were analyzed using a linear regression. Results were considered statistically significant at an alpha level of 0.05. Statistical analyses were performed using R 2.13.1 (The R Foundation for Statistical Computing 2011).

## 4.4 Results

### 4.4.1 Flux Experiment

Water temperature (30°C) and salinity (37 ppt) were the same for both sampling events. The concentration of nitrate in water used for the flux incubations was 0.05 μM for the two week and 1.16 μM four week samplings. Ammonium concentration increased from 0.79 μM at the 2-week sampling to 1.10 μM after the 4-week sampling.

Net N<sub>2</sub> fluxes were not significantly different between sampling events (Table 4.1), but were between live oysters and other treatments (Fig 4.1). Fluxes of N<sub>2</sub> were lowest for the control treatment ( $166.72 \pm 17.48 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$ ) and highest for the live oyster treatment ( $269.06 \pm 29.43 \mu\text{mol N-N}_2 \text{ m}^{-2} \text{ hr}^{-1}$ ). Overall, N<sub>2</sub> production from the live oyster treatment was greater than the control by 61%. The shell treatment had a 24% increase in N<sub>2</sub> production compared to the control, but this increase was not significant (p=0.19). SOD was significantly different across all treatments (Table 4.1). SOD ranged from  $1240 \pm 271.9 \mu\text{mol O}_2 \text{ m}^{-2} \text{ hr}^{-1}$  for the control to  $2403 \pm 85.93 \mu\text{mol O}_2 \text{ m}^{-2} \text{ hr}^{-1}$  in the live oyster treatment.

Nitrate fluxes were directed from the water column to the sediment for all treatments and sampling periods (Table 4.2). The effect of time and treatment were significant for nitrate fluxes (Table 4.1). Nitrate fluxes from the live oyster treatment were significantly

different from the control ( $p=0.03$ ), while the shell treatment was not different from either the control ( $p=0.16$ ) or the live treatment ( $p=0.60$ ). Nitrate demand was greater at the four-week sampling compared to the two-week sampling period (Table 4.1).

Ammonium fluxes were significantly different among treatments and between sampling periods. The interaction between these terms was also significant (Table 4.1). Ammonium fluxes were directed from the sediment to the water column for all treatments (Table 4.2). Overall, ammonium production decreased between the two sampling events. Ammonium production from the sediment to the water column was significantly higher for the live oyster treatment compared to the control ( $p=0.02$ ) but the shell treatment was not different the control ( $p=0.76$ ) or the live treatment ( $p=0.06$ ) after two weeks. There were no significant differences between the treatments during at the four-week sampling. Although highly variable, the live oyster treatment had more ammonium flux from the sediment to the water column relative to other treatments.

Denitrification efficiency was significantly different between treatments and sampling events but the interaction was not significant (Table 4.1). Denitrification efficiency was lower at the two-week sampling compared to the four-week sampling ( $p=0.012$ ). Denitrification efficiency was lower in the live oyster treatment compared to the control ( $p<0.05$ ). The shell treatment was not different from the control ( $p=0.41$ ) or the other live treatment ( $p=0.10$ ).

#### **4.4.2 Sediment Properties**

C:N was not different among treatments or sampling events (Table 4.1). C:N ranged from  $9.58 \pm 0.26$  in the live oyster treatment to  $10.9 \pm 0.36$  in the shell treatment (Figure 4.2). SOM ranged from  $3.89 \pm 0.66\%$  in the live oyster treatment to  $1.19 \pm 0.19\%$  in the control.

The live oyster treatment had more SOM compared to the control ( $p < 0.05$ ) and shell treatment ( $p < 0.05$ ; Table 4.1, Figure 4.2).

Benthic algal biomass (Chl. *a*) and porewater nutrients were only collected after two weeks (Fig 4.3). Concentration of dissolved inorganic nitrogen in the porewater ranged from  $46.19 \pm 15.28 \mu\text{M}$  in the control to  $103.2 \pm 43.60 \mu\text{M}$  in the live oyster treatment.

Ammonium constituted the majority of the DIN pool for all treatments. A difference could not be detected between treatments for porewater DIN ( $p = 0.15$ ); however, there was a trend, where concentration increased with the degree of reef complexity (e.g: control, abiotic, biotic and abiotic effects) associated with the treatment. Benthic algal biomass ranged from  $51.17 \pm 17.13 \text{ mg m}^{-2}$  in the shell treatment to  $88.96 \pm 17.14 \text{ mg m}^{-2}$  in the live treatment but differences were not significant ( $p = 0.23$ ).

#### **4.4.3 Correlations**

Net  $\text{N}_2$  fluxes had strong, and significant positive correlations with SOD, ammonium and SOM (Fig 4.3). These relationships were significant when all the data was included in the analysis. The observed positive relationship between net  $\text{N}_2$  fluxes and ammonium flux and SOM were driven by two samples from the live oyster treatments.

### **4.5 Discussion**

#### **4.5.1 Summary of Results**

This field experiment was designed to determine the effects of oyster reef structure and of oyster processes on sediment nitrogen dynamics. We found the net  $\text{N}_2$  flux to be dominated by denitrification for all treatments. The significantly higher rate of  $\text{N}_2$  production and SOM content at the live oyster treatment compared to the control and the shell treatment

suggest that oysters have the greatest effect on denitrification when the biological and physical attributes of the reef are coupled. Oysters remove particulate organic matter from the water column as they feed and produce biodeposits, which increases the organic and fine-particle content of the sediment surface (Thrush et al. 2006, O'Connor et al. 2008). Oyster reefs, as a physical structure in the water column, reduce water velocity and increase sedimentation (Lenihan 1999, Widdows and Brinsley 2002, Pietros and Rice 2003). Organic matter that accumulates adjacent to oyster reefs can be used for heterotrophic metabolism leading to increased denitrification. Our results indicate that the abiotic structure and biotic processes alter the physical and chemical environment of the sediment, resulting in the formation of biogeochemical hot spots. Understanding the relationships between reef habitats and biogeochemical cycling will aid in determining the functional value of these organisms in estuarine ecosystems.

#### **4.5.2 Treatment Effects**

The live oyster treatment had significantly more SOD and N<sub>2</sub> production compared to the shell treatment and the control, these differences lead to the conclusion that the quantity and/or quality of organic matter delivered to the sediment contributed to differences in rates of denitrification. Autochthonous carbon is more labile, contains more energy and is more easily assimilated than allochthonous organic matter (Christensen et al. 1990, McClain et al. 2003). The live oyster treatment, with feeding oysters caused the accumulation of both autochthonous and allochthonous sources of organic matter, while the shell treatment had no internal source of organic matter. We found that the live oyster treatment increased denitrification over the control by 61%. Of this increase, 40% was due to allochthonous inputs from the reef structure and 60% was attributable to production and accumulation of

oyster derived autochthonous material. Results from our experiment support the hypothesis that areas where organic matter accumulates are primed for enhanced biogeochemical activity (Guenet et al. 2010).

Denitrification was linked to organic matter remineralization and nitrification, as indicated by the positive relationship found between denitrification and SOM, as well as SOD and ammonium production (Seitzinger 1994). Organic matter content of oyster reef sediments is positively correlated to oyster density and production of biodeposits (Haven and Morales-Alamo 1966, O'Connor et al. 2008). Biodeposits are highly concentrated, labile organic matter with low C:N ratios that have a high sinking velocity and are easily retained on the sediments (Widdows et al. 1998, Newell et al. 2002). While wave action and tidal currents may distribute biodeposits, the reef structure enhances sedimentation of these particles on and adjacent to the oyster reef (Widdows et al. 1998, Widdows and Brinsley 2002). Biodeposits are therefore incorporated into the sediments within a very small region around the bivalve population, increasing dissolved nutrient concentrations within that zone (Newell et al. 2005, Giles and Pilditch 2006, Giles et al. 2009, Borsje et al. 2011). Consequently, differences detected in our study are most likely attributable to the production and accumulation of biodeposits. Thus, the effects of the oyster are the result of the combination of structure and vital processes of living oysters. However, further studies will need to be conducted to quantify the rate of biodeposit accumulation on sediments from oyster reefs.

The differences in SOD between the treatments is attributable to variations in the labile carbon pool (Ferguson et al. 2003). Higher SOD is indicative of more organic matter loading and higher rates of carbon mineralization (Ferguson et al. 2003, Ferguson and Eyre

2012). Thus, the live oyster treatment, which had the highest SOD, probably had more a higher quantity and quality of organic matter than the other treatments. The significantly lower rate of SOD from the control suggests that more recalcitrant organic matter accumulated here relative to the shell and reef treatments. Although, SOM content was not different between the control and shell, the organic matter that accumulated at the shell treatment was more labile, as suggested by the higher SOD. These results indicate that carbon loading and mineralization rates were enhanced by the reef structure.

Many studies have found a strong positive relationship between SOD and denitrification (Seitzinger and Giblin 1996, Fennel et al. 2009, Piehler and Smyth 2011). For that reason, the significant difference in SOD among treatments suggests denitrification rates would also be affected by treatment. While the shell treatment added some labile carbon to the sediment relative to the control, the lack of differences in denitrification suggests that denitrification was limited by nitrate. This limitation was alleviated in the live oyster treatment through the biotic production and abiotic accumulation of biodeposits. The accumulation of organic matter on the sediment surface and typically does in quiescent conditions decrease the oxygen penetration depth, putting the oxygenated zone for nitrification closer to the anoxic zone for denitrification (Kemp et al. 1990, Caffrey et al. 1993, Cornwell et al. 1999). Additionally, the nitrogen fraction in biodeposits fuels denitrification (Newell et al. 2002). An effect of increased denitrification has been demonstrated in laboratory experiments with induced accumulation of biodeposits on the surface of sediment cores (Newell et al. 2002, Higgins et al. 2013). The reef structure appears to enhance the accumulation of biodeposits which otherwise would be diffusely distributed

with less effect on nitrogen removal. Thus, the reef structure and production of biodeposits by the oysters act synergistically to enhance denitrification.

#### **4.5.3 Efficiency**

Sediments from bivalve reefs are often associated with high concentrations of ammonium that can be recycled back to the water column and used for primary production (Dame et al. 1984, 1989, Lavrentyev et al. 2000, Pietros and Rice 2003, Bruesewitz et al. 2008). Despite this, oyster mediated denitrification can reduce the amount of nitrogen available for recycling by 16- 40% (Smyth et al. 2013). Since oysters can affect nitrogen retention and removal, we compared denitrification efficiency (the percent benthic efflux of inorganic nitrogen that is  $N_2$ ) among treatments (Eyre and Ferguson 2009). The highest efficiency was associated with the control because remineralization of the refractory organic matter produces less ammonium. The decrease in resources to the microbial community results in low fluxes but high efficiency. While high rates of carbon loading in the live oyster reef treatment enhanced microbial activity it also may inhibit nitrification resulting in a higher release of ammonium (Kemp et al. 1990). Denitrification efficiency associated with these newly restored reefs was lower than efficiencies from sediments associated with natural reefs (Piehler and Smyth 2011). Denitrification efficiency from restored oyster reefs less than four weeks old in the Chesapeake Bay were lower than control sites without reefs (Kellogg et al. 2013). Given the high denitrification efficiencies associated natural reefs, restored reefs greater than 10 years old (Chapter 5) as well as the increase between the two week and four week sampling events, efficiency likely increases as the reef matures and a fully functioning reef community which consumes nutrients and carbon and reduces the efflux of ammonium back to the water column is established (Peterson et al. 2003).

#### **4.5.4 Benthic Algal Biomass**

The colonization of benthic algae on sediments can affect rates of denitrification (An and Joye 2001). Benthic algae can provide a source of nitrogen and labile C to denitrifiers and lead to an increase in coupled nitrification-denitrification (Risgaard-Petersen 2003). While our experiments were conducted in the dark to reduce these effects, the impact of the algae may have persisted and affected denitrification rates in our treatments (Sundbäck and Miles 2000, Ferguson et al. 2007). Benthic algae can contribute to the SOM content, which may have led to the lack of differences in SOM detected between the control and the shell treatment. The high benthic algal biomass from the live oyster reef may have been associated with phytoplankton pigments in biodeposits (Haven and Morales-Alamo 1966). However, the lack of a difference in benthic algae biomass between treatments suggests that a relatively minor contribution to enhanced denitrification in the live oyster reefs.

#### **4.5.5 Limitations and Assumptions**

We recognize that the patchiness of our experimental setup may have led to edge effects. The small plot size likely had exaggerated reef-sediment boundaries relative to nature, where reefs exist in long continuous patches (Micheli and Peterson 1999), resulting in greater interactions between the reef, material in the water, and the sediment. In mussel patches the growth of individuals near the edge is greater than those in the middle due to increased food availability (Svane and Ompi 1993). Thus, the increase in edges per reef area might have resulted in optimal feeding by the oysters and increased biodeposit production. However, oyster reef patches are a common configuration of oyster reefs in this area (Macreadie et al. 2012). Patchiness of reefs can affect the amount of sediment that is retained within the reef (van Leeuwen et al. 2010). Larger continuous reefs have many more



individual oysters leading to increased organic matter loading and more retention of sediment (Lenihan 1999, Borsje et al. 2011). Additionally the size and patchiness of the reef affects bed roughness and friction velocity which in turn affects the transport of biodeposits to and retention on the sediments (Newell et al. 2005). Nevertheless, we maintained a density similar to natural reefs in the area (Lenihan and Peterson 1998, O'Connor et al. 2008). In addition, sediment characteristics including ambient organic matter, sediment porosity and grain size probably affected our results (Jones et al. 2011). Understanding how the physical attributes of the reef ecosystem including environmental variables, spatial arrangement, patchiness and size of the reef affect sediment biogeochemistry requires further investigation.

#### **4.5.6 Functional Equivalency Trajectories**

The loss of suspension feeding bivalves can affect nutrient fluxes, sediment characteristics and community composition, although oyster reef restoration may recover these lost functions (Thrush et al. 2006, Coen et al. 2007). However, it can take many years for restored systems to achieve the functional value of natural systems (Simenstad and Thom 1996). For example, restored salt marshes require 15-25 years to be functionally equivalent to a natural marsh (Craft et al. 2003). Biogeochemical cycles can take even longer to establish since newly restored salt marshes sequester nitrogen but lack a stored pool of internally recycled nitrogen (Craft et al. 1989, 1999).

Restoration of oyster reefs can begin to recover lost oyster populations but the recovery of associated services may take 2-14 years depending on environmental factors (Schulte et al. 2009, Grabowski et al. 2012). We found denitrification from our restored oyster reefs to be equivalent to rates from natural oyster reef sediments within a month of construction (Piehler and Smyth 2011, Smyth et al. 2013). While restoration of reefs with

live oysters is sometimes feasible (Geraldi et al. 2009), it is more common to use oyster shell for restoration (Coen and Luckenbach 2000). We found that the addition of oyster shell alone can increase denitrification and carbon mineralization but that the nitrogen removal benefit of oyster reef restoration occurs once a community of actively filtering bivalves is established.

#### **4.6 Conclusions**

The two-part influence of oyster engineering on nitrogen dynamics results from a combination of abiotic structure and accumulation of biotic material. Oyster reefs provide many ecosystem services, including enhancement of fisheries and maintenance of biodiversity (Lenihan 1999, Grabowski et al. 2005, 2012). Increased nitrogen removal is often considered a benefit of oyster reef restoration; however, difficulty associated with measuring denitrification and complexity of the reef ecosystem have made it challenging to incorporate this benefit into restoration and management plans (Grabowski 2004, Groffman et al. 2006, North et al. 2010, Jones et al. 2010). We found that while physical engineering is important, production and accumulation of biodeposits drives the greater portion of denitrification in oyster reef ecosystems. The impact of oysters and their reefs on sediment biogeochemistry and benthic community structure underscores their importance as both organisms and structure within the large ecosystem.

#### **4.7 Acknowledgments**

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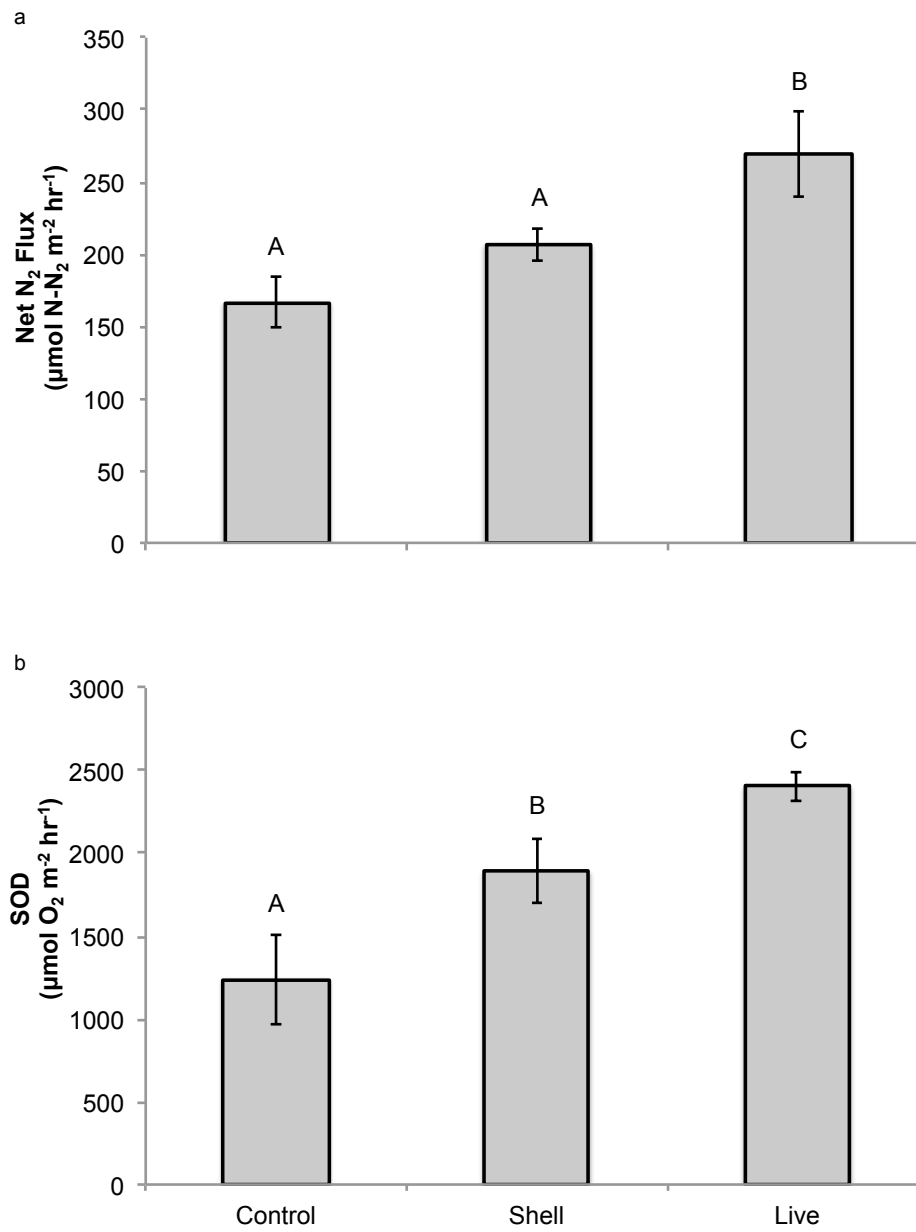
by funding from North Carolina Sea Grant and the National Science Foundation (NSF EAR-0815627 and NSF OCE-0961929).

**Table 4.1** Results from one-way repeated measures ANOVA used to test for differences in fluxes across the sediment-water interface, denitrification efficiency and sediment properties for each treatment over the four-week sampling period. Significant p values ( $p < 0.05$ ) are indicated in bold.

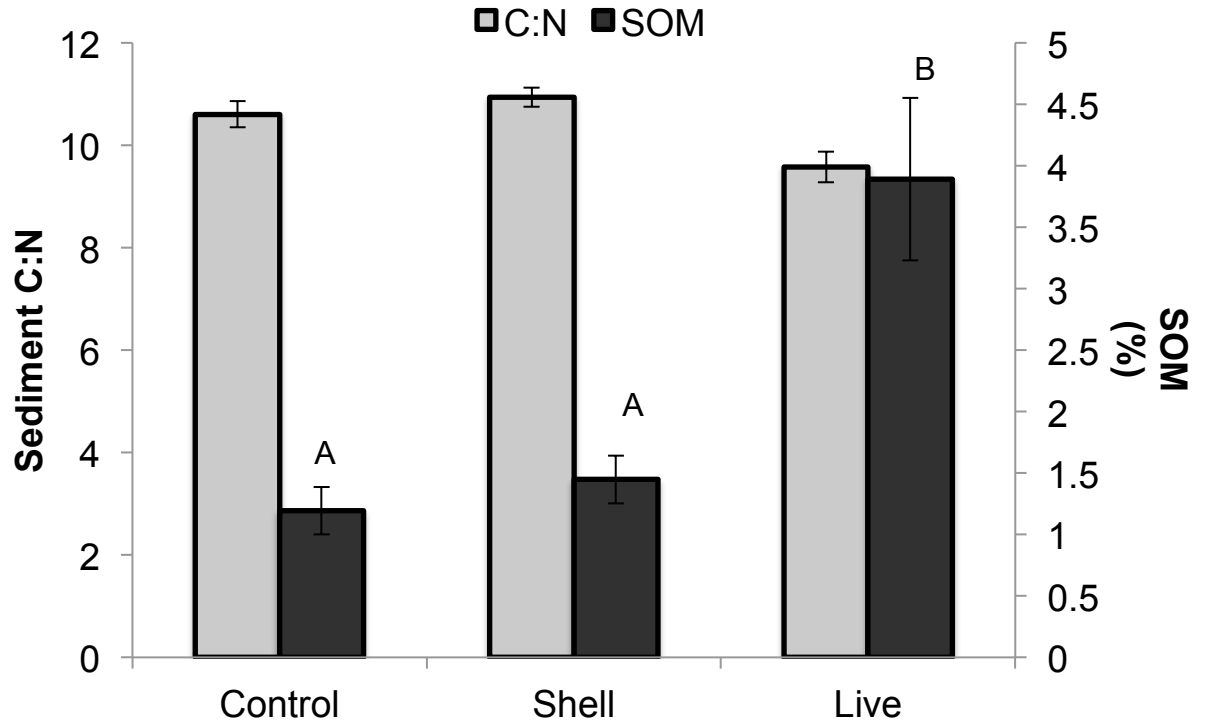
Effect Tests		Net N <sub>2</sub> Flux		SOD		NO <sub>x</sub> Flux		NH <sub>4</sub> <sup>+</sup> Flux		Efficiency		SOM		C:N		
Source	<i>N</i>	<i>DF</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
<b>Treatment</b>	2	2	11.1	<b>0.00</b>	19.1	<b>0.00</b>	4.58	<b>&lt;0.05</b>	6.38	<b>0.02</b>	6.42	<b>0.02</b>	13.5	<b>0.00</b>	2.26	0.16
<b>Time</b>	1	1	1.16	0.31	4.98	0.05	15.6	<b>0.00</b>	8.81	<b>0.02</b>	8.55	<b>0.02</b>	2.18	0.17	0.24	0.63
<b>Treatment*Time</b>	2	2	0.01	0.99	0.94	0.43	2.33	0.16	5.84	<b>0.03</b>	3.16	0.10	0.42	0.67	0.41	0.68

**Table 4.2** Fluxes of nitrate and ammonium across the sediment-water interface and denitrification efficiency for each treatment from the sampling periods 2 weeks and 4 weeks after construction and the experimental mean. A positive value indicates a flux from the sediment to the water column and a negative value indicates a flux from the water column to the sediment. Values are mean  $\pm$  1 standard error (n=4).

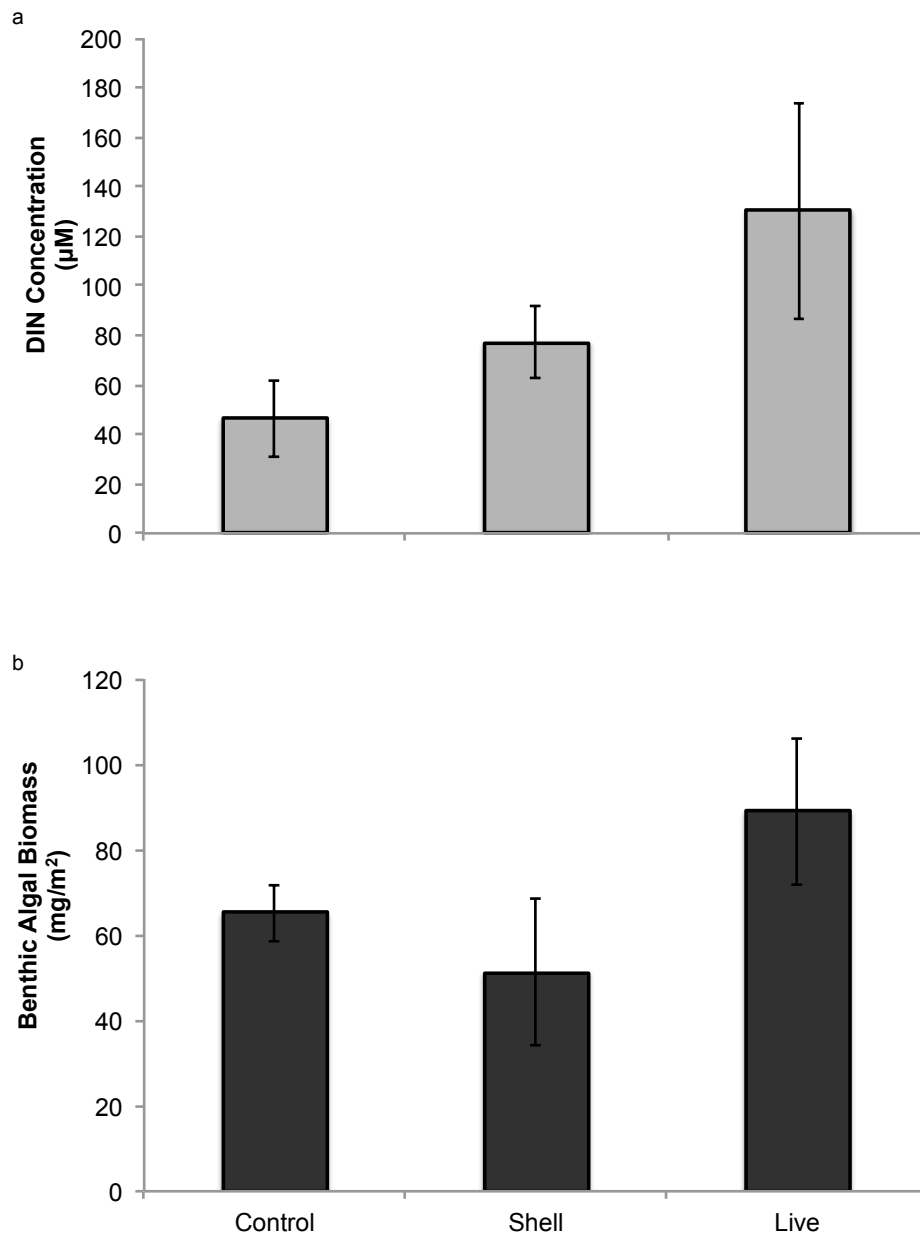
		Two Weeks			Four Weeks			Mean		
<b>NO<sub>x</sub> Flux</b>										
	Control	-0.01	$\pm$ 0.57	-3.32	$\pm$ 5.60	-1.67	$\pm$ 3.79			
	Shell	-0.39	$\pm$ 0.17	-14.61	$\pm$ 4.29	-7.50	$\pm$ 4.73			
	Live	-0.33	$\pm$ 0.14	-18.18	$\pm$ 1.40	-10.53	$\pm$ 4.87			
<b>NH<sub>4</sub><sup>+</sup> Flux</b>										
	Control	20.24	$\pm$ 22.75	24.03	$\pm$ 15.65	22.13	$\pm$ 18.11			
	Shell	197.47	$\pm$ 73.73	5.34	$\pm$ 9.64	101.40	$\pm$ 70.76			
	Live	956.08	$\pm$ 342.37	157.70	$\pm$ 66.72	499.86	$\pm$ 294.67			
<b>Denitrification Efficiency</b>										
	Control	91.40	$\pm$ 8.44	88.19	$\pm$ 5.86	89.79	$\pm$ 6.78			
	Shell	57.72	$\pm$ 14.46	95.72	$\pm$ 3.06	76.72	$\pm$ 14.03			
	Live	32.65	$\pm$ 12.94	69.84	$\pm$ 11.02	53.90	$\pm$ 14.67			



**Figure 4.1** Net N<sub>2</sub> flux (a) and SOD (b) for each treatment. Significant treatment differences are denoted with different letters ( $p<0.05$ ). Error bars represented one standard error of the mean ( $n=4$ ).

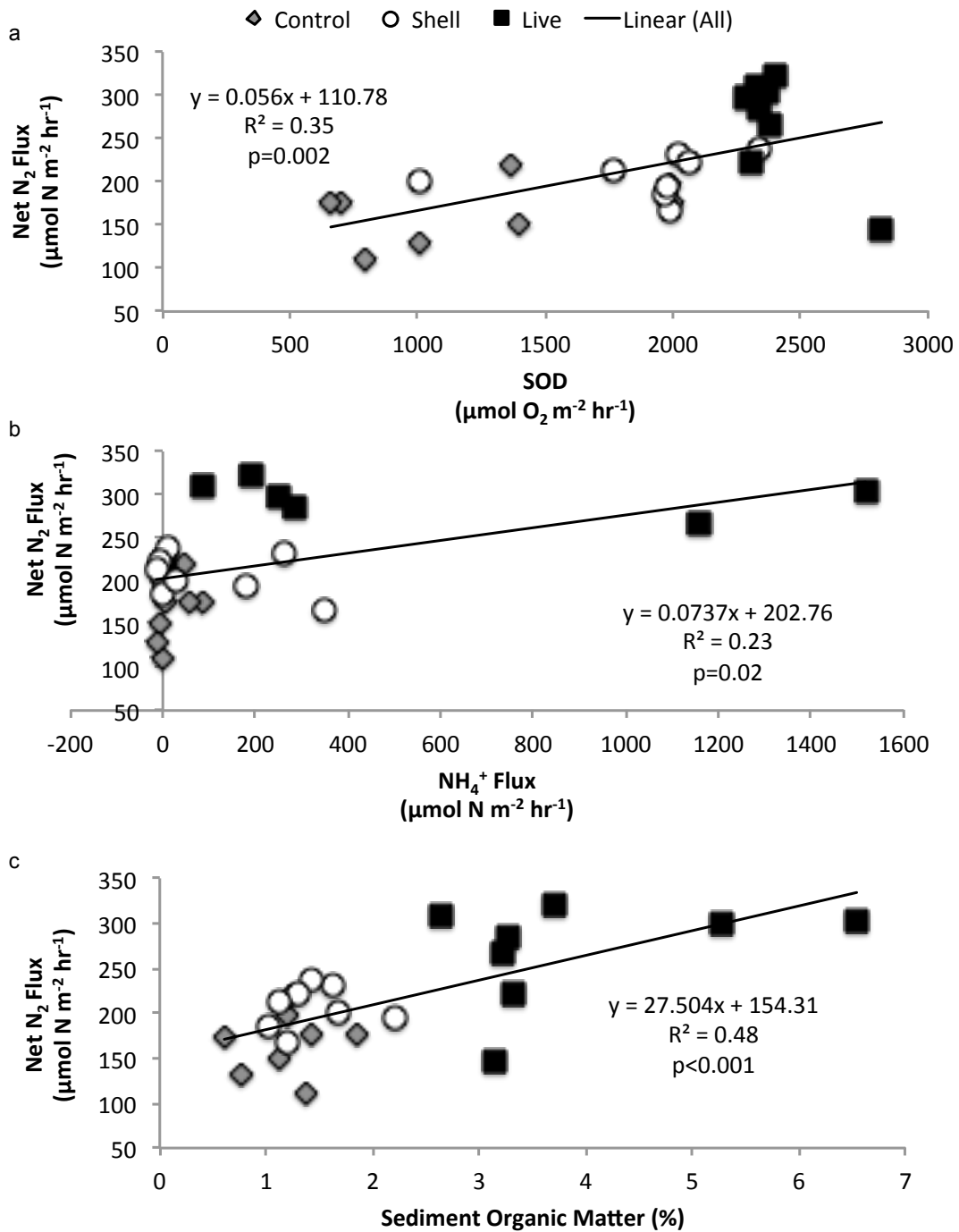


**Figure 4.2** Sediment carbon: nitrogen ratios and SOM for each treatment. Treatments with different letters are significantly different ( $p < 0.05$ ). Error bars represent one standard error ( $n=4$ ).



**Figure 4.3** Mean porewater DIN ( $\text{NO}_x + \text{NH}_4^+$ ) concentration from the upper 7cm of sediment (a) and benthic chlorophyll biomass (b) for each plot. Errors bars are one standard error ( $n=4$ ).





**Figure 4.4** Sediment net N<sub>2</sub> flux as a function of SOD (a), Ammonium Flux (b), and SOM (c) for each treatment during each sampling period. Different treatments are indicated by symbols.

## **5. HABITAT SETTING INFLUENCES NITROGEN REMOVAL BY RESTORED OYSTERS REEFS**

### **5.1 Abstract**

Coastal ecosystems have a complex mosaic of habitats, and the arrangement of these habitats influences ecosystem processes. However, little is known about the influence of habitat configuration on nitrogen biogeochemistry. We investigated how the habitat setting of restored intertidal oyster reefs affects fluxes of nitrate plus nitrite ( $\text{NO}_x^-$ ), ammonium ( $\text{NH}_4^+$ ) and  $\text{N}_2$ . Fluxes were measured from oyster reef sediments adjacent to salt marshes, seagrass beds, and mudflats, as well as analogous control habitats without reefs under both ambient and experimental nitrate levels. All reef and control habitats were net denitrifying. Reefs enhanced sediment denitrification by 18-275% over the controls, with the largest increase occurring in the mudflat habitat. Denitrification significantly increased in the marsh-reef and mudflat-reef under experimental nitrate levels. These results suggest that oysters prime the sediment for enhanced denitrification in response to nutrient pollution. Oyster reef sediments also had higher rates of  $\text{NH}_4^+$  production compared to control habitats, but denitrification efficiency was not different between the reef and control habitats. Under elevated nitrate levels, oyster density at first increased and then slightly decreased denitrification rates. Nitrogen dynamics in the mudflat habitat were most affected by reef presence due to relative isolation from other biogenic habitats and highest densities of oysters. Our results indicate that oyster-mediated denitrification is dependent on the habitat setting and that oyster density is a likely a driver for this pattern. These results suggest that the landscape setting of a

restored oyster reef can largely impact the delivery of services it provides, and thus should be considered carefully in restoration and management plans.

## **5.2 Introduction**

Ecosystems are often comprised of complex habitat landscapes, where interactions among patches influence function of the system as a whole. Models of heterogeneity classify systems as “homogeneous”, consisting of one patch; “mosaic”, with no interaction between distinct patches; and “interactive”, where exchanges occur between patches (Lovett et al. 2005). While knowledge of the individual components provides valuable information, the connection between habitats sustains complex biological communities (Noss 1983). Thus, understanding the causes and consequences of habitat configuration on ecosystem processes is becoming increasingly important for developing species-recovery plans (Bond et al. 2005, Kouki et al. 2011), constructing protected areas (Leathwick et al. 2008) and for designing conservation strategies (Pastor et al. 1999, Grabowski 2002, Lovett et al. 2005, Turner and Chapin 2005).

Coastal ecosystems are among the most degraded ecosystems in the world (Noss 1983, Lotze et al. 2006, Beck et al. 2011). Biogenic habitats are being lost at rates of 1-9% per year (Bond et al. 2005, Duarte et al. 2008, Kouki et al. 2011). Deterioration of these habitats, resulting from nearby fish trawling, destructive harvesting practices, dredging and eutrophication, has led to significant changes in the structure and function of coastal ecosystems (Lenihan and Peterson 1998, Jackson et al. 2001b, Lotze et al. 2006, Leathwick et al. 2008, Duarte 2009, Deegan et al. 2012). The traditional approach for designing management and restoration strategies has been to focus on single habitats (Thayer 1992). However, the composition and configuration of the habitats can influence animal movement,

population dynamics, species interactions, and ecosystem functions (Micheli and Peterson 1999, Cardinale et al. 2002, Lovett et al. 2005, Grabowski et al. 2005, Lopez et al. 2006).

The ability to cycle, process and remove nutrients is among the most valuable benefit humans receive from the environment (Costanza et al. 1997). Nutrient cycling controls the availability of essential elements at the base of the food webs. However, excessive nutrient inputs from fertilizer use and runoff have led to eutrophication of coastal systems (Nixon 1995, Carpenter et al. 1998, Galloway et al. 2003). As anthropogenic nutrient loading has accelerated, the balance between nutrient inputs and exports has shifted, affecting growth, composition and biomass of primary producers (Smith et al. 1999, Conley 2000). Once nitrogen enters the system it can be removed through physical transport, burial or denitrification (Vitousek et al. 1997b). Denitrification, the microbially-mediated conversion of bioavailable nitrogen to N<sub>2</sub> gas, accounts for the largest nitrogen sink in estuarine ecosystems (Seitzinger and Nixon 1985, Seitzinger 1988). Unfortunately, habitat modification and loss have reduced the denitrification capacity of these ecosystems (Brush 2009). In order to recover this lost service and help reverse eutrophication, it is necessary to restore and enhance habitats with high rates of denitrification. While knowledge of the effects of habitat interaction on trophic dynamics (Grabowski et al. 2005) and ecosystem stability (Callaway et al. 2003) is mounting, little is known about how the design of restoration projects affects ecosystem processes such as organic matter transformations and nutrient cycling (Franklin and Forman 1987, Irlandi 1994, Lovett et al. 2005, Turner and Chapin 2005, Dobson et al. 2006).

Oyster reefs were once a prominent habitat within the estuarine ecosystem. However, oyster reefs have declined by about 85% worldwide in the last century (Beck et al. 2011).

Oysters have been exploited as a fishery and neglected as a biogenic habitat (Lenihan and Peterson 1998). The importance of oyster reefs can be assessed on the ecosystem services they perform including mitigating erosion, providing habitat and nursery grounds for fish, enhancing biodiversity associated with hard substrate and improving water quality through filtration and enhanced denitrification (Grabowski and Peterson 2007). One of the most valuable of these services is the benefit to water quality through nitrogen removal via denitrification (Grabowski et al. 2012). Because of these economic and ecological values, significant efforts are currently underway to restore and enhance oysters in estuarine ecosystems.

Intertidal oyster reefs occur in three distinct habitat settings: 1) between salt marshes and seagrass beds, 2) adjacent to fringing salt marshes lacking seagrass beds, or 3) in isolation on mudflats (Lanier 1981). The position of oyster reefs affects predator-prey dynamics (Micheli and Peterson 1999) and community structure (Grabowski et al. 2005). However, the effect of habitat setting on the ability for oyster reef restoration to improve water quality remains unknown. This information is critical to maximize services as oyster reef restoration research and practice moves forward. We tested whether oyster-mediated denitrification was affected by proximity to other habitats. In addition we asked whether habitat setting of restored reefs influenced the ability of the sediment microbial community to remove nitrogen in response to anthropogenic nutrient loading. To address these questions we conducted experiments on sediments from restored oyster reefs in each of the three habitat settings.

### **5.3 Methods**

### **5.3.1 Study Site**

Oyster reefs selected for this study were located in the sound between Beaufort and Shackleford Banks on the central North Carolina coast. This area contains seagrass beds, salt marshes, oyster reefs and intertidal mudflats, and is located within the Rachel Carson National Estuarine Research Reserve. Intertidal oyster reefs restored, some in 1997 and some in 2000 by Grabowski and colleagues (2002, 2005) were used in this study. Reefs were either isolated on mudflats or adjacent to salt marshes alone or both salt marsh and seagrass beds (hereafter: mudflat-reefs, marsh-reefs, and seagrass-reefs). Salt marshes, seagrass beds or mudflats without reefs present were used as controls (Figure 5.1). Three reefs and three controls from each habitat were sampled. Reefs were compared to controls to determine how habitat setting of restored oyster reefs affects sediment nitrogen dynamics.

### **5.3.2 Sample Collection**

Continuous-flow core incubations were used to determine rates of nitrogen exchanges at the sediment-water interface. Sediment cores (contained in 6.4cm diameter by 17 cm long polycarbonate tubes) were collected by hand from reefs and controls at low tide on June 28, 2010. For reef samples, cores were collected at the edge of the reef and did not contain live oysters. In addition to sediment cores, ~ 100 L of water from the study location were collected for use in core incubations. Surface water measurements of dissolved O<sub>2</sub>, salinity and water temperature (YSI 600 Series Sonde and Model 650 data logger, Yellow Springs Instruments, Yellow Springs, OH, USA) were also collected.

### **5.3.3 Core Incubations**

Following collection, sediment cores and water were immediately (<1hr) transported to an environmental chamber (Bailey, Inc.) at The University of North Carolina's Institute of

Marine Sciences (IMS) in Morehead City, NC. Sediment cores were submerged in a water bath and sealed with gas tight lids equipped with an inflow and outflow port and connected to a peristaltic pump (Lavrentyev et al. 2000). Unfiltered, aerated water collected from the site was continuously passed over the cores at a flow rate of 1-2 ml per minute (Miller-Way and Twilley 1996, Lavrentyev et al. 2000). All incubations took place in the dark and at 30°C.

Following an 18-hour pre-incubation period, samples were collected from the inflow and outflow port of each core for dissolved gas and nutrient analysis. Dissolved gases were sampled and analyzed several times over a 48-hour period to ensure steady state conditions were established (Miller-Way and Twilley 1996). Steady state was established when the slope of concentration vs. time for each microcosm was not different from zero. Samples for nutrient analysis were collected once during this period after steady state was established with respect to the dissolved gas samples. A bypass line that flowed directly into the sample vials was used to determine the concentration of dissolved constituents entering the cores.

To experimentally examine the importance of habitat setting for oyster reef restoration in response to nutrient pollution, water was enriched with  $\text{NaNO}_3$  (~800  $\mu\text{M}$ ) after 48 hours of sampling. Dissolved gas and nutrient samples were then collected for an additional 48 hours as described above.

#### **5.3.4 Sediment Organic Matter**

Upon completion of the core incubations (and associated dissolved gas and nutrient sampling), the upper 2 cm of sediment in each core was sampled for organic matter content. Sediment organic matter (SOM) was calculated by mass difference from dried sediments (125 °C for 6h) before and after ignition at 525 °C for three hours.

#### **5.3.5 Density Measurements**

All oyster reefs were sampled on 17 October 2012 for oyster density. Oyster density was determined by placing a 0.25 m<sup>2</sup> quadrat on each reef (one quadrat per reef) and all oysters with a shell length greater than 25 mm were counted (Powers et al. 2009). Additional samples were not collected for oyster density due to the long term monitoring that occurs at this site and small size of the reefs.

### **5.3.6 Membrane Inlet Mass Spectrometry**

Dissolved gas samples were analyzed for concentrations of N<sub>2</sub>, O<sub>2</sub> and Ar using a Balzers Prisma QME 200 quadrupole mass spectrometer (Kana et al. 1998). Concentrations of N<sub>2</sub> were determined using the ratio with Ar for each salinity and temperature (Kana et al. 1994, Ensign et al. 2008). This technique results in a net N<sub>2</sub> flux (gross denitrification-gross nitrogen fixation) across the sediment-water interface and does not distinguish between the sources of N<sub>2</sub>. Consequently, “denitrification” refers to N<sub>2</sub> production from heterotrophic denitrification, anammox and any other N<sub>2</sub> producing process. Previous studies in shallow water coastal ecosystems have shown that anammox contributes only a small portion to the total N<sub>2</sub> flux, and it is assumed that denitrification comprises the major production pathway of N<sub>2</sub> in this study (Koop-Jakobsen and Giblin 2009).

### **5.3.7 Water Chemistry**

Water samples were filtered through Whatman GF/F glass fiber filters (25mm diameter, 0.7 μm nominal pore size) and frozen until analysis. Filtrate was analyzed with a Lachat Quick-Chem 8000 automated ion analyzer for NO<sub>x</sub> (NO<sub>3</sub><sup>-</sup>+NO<sub>2</sub><sup>-</sup>) and NH<sub>4</sub><sup>+</sup> concentrations using standard protocols (Lachat Instruments, Milwaukee, WI, USA: NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> Method 31-107-04-1-A, NH<sub>4</sub><sup>+</sup> Method 31-107-06-1-A; detection limits: 0.04 μM NO<sub>x</sub>, 0.18 μM NH<sub>4</sub><sup>+</sup>).



### 5.3.8 Calculations

Fluxes across the sediment-water interface were calculated as  $(C_o - C_i) \times f/a$ , where  $C_o$  is the outflow concentration ( $\mu\text{mol L}^{-1}$ ),  $C_i$  is the inflow concentration,  $f$  is the flow rate ( $0.06 \text{ L h}^{-1}$ ), and  $a$  is the sediment surface area ( $0.0032 \text{ m}^2$ ). Successive measurements from each core were averaged to give core-specific values and prevent pseudo-replication.

Denitrification efficiency was calculated as the percent of the dissolved inorganic nitrogen efflux that was  $\text{N}_2$  (Eyre and Ferguson 2009).

### 5.3.9 Statistical Analyses

The effects of habitat (mudflat, marsh or seagrass), reef presence (reef or control), and nutrients on fluxes, and denitrification efficiency were tested using a three-way analysis of variance model (ANOVA). A two-way ANOVA, testing the effects of habitat and reef presence was used for SOM content. A one-way ANOVA was used to determine significant differences in oyster density across habitat setting. An alpha level of 0.05 was used for all analyses. Student-Newman-Keuls (SNK) post-hoc test was chosen for multiple comparisons after the ANOVA. SNK adjusts the Type I error to increase the power of individual comparisons (Day and Quinn 1989), and offers a compromise between Type I error and per comparison power (Underwood 1997). In addition, regression analyses were performed to investigate the effect of oyster density on denitrification. Models with the lowest Akaike's Information Criterion (AIC) were chosen. Statistical analyses were performed using R 2.13.1 (The R Foundation for Statistical Computing 2011).

A power analysis was employed for  $\text{N}_2$  fluxes, the main variable of interest, comparing the four groups and three samples per group with a significance level of 0.05. Unfortunately, the small sample size ( $n=3$ ) caused our experiment to have very low power

(power of 0.26,  $\beta=0.74$ ). In order to increase our power to 0.80 ( $\beta=0.20$ ), we would have to increase our sample size to about 8 reefs or controls per habitat. However, we were unable to process more samples because of methodological limitations. While retrospective power analysis has little benefit for the current experiment it does provide insight into the design of future experiments.

## **5.4 Results**

### **5.4.1 Flux Experiment**

ANOVA results on the effects of habitat, reef presence and nutrients and their interactions on  $N_2$ ,  $NO_x$ ,  $NH_4^+$  and denitrification efficiency are presented in Table 5.1. Post hoc analysis was used to explore the interactions among habitats within each level of reef presence and nutrients (Table 5.2), among nutrients within each level of reef presence and habitat (Table 5.3), and among reef presence within each level of habitat and nutrients (Figure 5.2).

### **5.4.2 Net $N_2$ Flux**

Net  $N_2$  fluxes were positive for all habitats, indicating denitrification in excess of nitrogen fixation. Denitrification ( $N_2$  production) rates were not significantly different between habitats, but were affected by reef presence and nutrients (Table 5.1). The interactions between habitat and reef presence as well as reef presence and nutrients were also significant (Table 5.1). Differences in  $N_2$  flux among habitats for the controls or reefs were not detected prior to the addition of nitrate (Table 5.2). After the nitrate addition, there were no differences in denitrification between the control habitats, while denitrification rates in mudflat-reefs were significantly higher than for the seagrass-reefs and marsh-reefs (Table

5.2). Denitrification rates tended to increase in response to the addition of nitrate; however, this increase was only significant for mudflat-reefs and marsh-reefs (Table 5.3). Reefs increased denitrification over the controls by 18% in the marsh, 71% in the seagrass and 275% in the mudflat. Pairwise comparisons indicated that this increase was only significant in the mudflat setting (Fig 5.2a). After the nitrogen addition, denitrification rates were significantly higher for the reef compared to the control for the marsh and mudflat habitats (Fig 5.2b).

#### **5.4.3 Nitrate Flux**

The effects of habitat, nutrients, and reef presence, habitat and nutrients, and the interaction between habitat, reef presence and nutrients were significant for fluxes of nitrate (Table 5.1). Dissimilarities among habitats were not detected prior to the addition of nitrate for controls or reefs (Table 5.2). After the nitrate addition, nitrate fluxes in the mudflat-control were distinctively higher from the seagrass-control and marsh-control. The nitrate flux in seagrass-reef was different from the marsh-reef (Table 5.2). Nitrate fluxes changed before and after the addition of nitrate for the marsh-control, seagrass-control and marsh-reef (Table 5.3). Nitrate fluxes did not vary between the controls and reefs for any habitat before the nitrate addition (Figure 5.2c). Nitrate fluxes were significantly different between the reef and control for mudflat and seagrass habitat after the addition of nitrate (Figure 5.2d). The mudflat-control had a positive nitrate flux while the mudflat-reef had a negative flux. The seagrass-control had more nitrate demand than the seagrass-reef.

#### **5.4.4 Ammonium Flux**

There was a significant effect of nutrients on ammonium fluxes (Table 5.1). Ammonium fluxes were not different among habitats for the controls or reefs prior to the

nitrate addition (Table 5.2). Ammonium fluxes in reefs were not dissimilar among habitats after the addition of nitrate, while the flux in the marsh-control was higher compared to the mudflat-control (Table 5.2). Ammonium fluxes were significantly different before and after the addition of nitrate except for the mudflat-control (Table 5.3). Ammonium fluxes were not distinguishable between the control and reef for any habitat before the nitrate addition (Figure 5.2e). After the nitrate addition, the ammonium flux from the mudflat-reef was significantly higher than the mudflat-control (Figure 5.2f).

#### **5.4.5 Denitrification Efficiency**

The effect of nutrients was significant on denitrification efficiency (Table 5.1). Denitrification efficiency decreased after the nutrient addition for the control and reef in each habitat (Figure 5.3). However, denitrification efficiency was not different among habitats for the controls or reefs before or after the addition of nitrate (Table 5.2).

#### **5.4.6 Sediment Organic Matter**

The effects of habitat, reef presence, and the interaction between habitat and reef presence were significant for SOM content (Figure 5.4). Overall, the seagrass-reef had the highest SOM content. SOM content in the mudflat habitat and marsh habitat was not affected by reef presence. The seagrass-reef increased SOM content relative to the seagrass-control. The marsh-control and seagrass-control had significantly higher SOM compared to the mudflat-control.

#### **5.4.7 Density**

Oyster density was significantly higher on mudflat-reefs than on marsh-reefs and seagrass-reefs. Oyster density was lowest on marsh-reefs, averaging  $157 \pm 79$  ind.  $m^{-2}$ . Density on seagrass-reefs was  $204 \pm 90$  ind.  $m^{-2}$ , while mudflat-reefs had densities of  $673 \pm$

81 ind. m<sup>-2</sup>. There was no pattern between density and spatial location of the reefs in the study system. Before the nitrate addition, a linear regression model best explained the relationship between density and denitrification; however, this relationship was not significant (Figure 5.5a, R<sup>2</sup>= 0.23, p=0.11). The relationship between denitrification and density after the experimental nitrate addition was best explained by a second-order polynomial relationship (Figure 5.5b, R<sup>2</sup>= 0.65, p=0.009).

## **5.5 Discussion**

### **5.5.1 Oyster Reef Habitat Setting and Denitrification**

Our experiment investigated the effects of habitat setting of restored oyster reefs on sediment nitrogen dynamics, including denitrification. We examined nitrogen dynamics in sediments from nine restored reefs (each > 10 years old) in three different habitats settings and analogous habitats without reefs. Determining the benefits of restored areas relative to reference areas lacking restoration aids in identifying restoration sites for maximal benefit (Vitousek et al. 1997b, Palik et al. 2000). We found positive net N<sub>2</sub> fluxes from the controls and reefs in each habitat, indicating that denitrification dominates the N<sub>2</sub> flux. Denitrification (net N<sub>2</sub> production) tended to be higher in reefs compared to controls without reefs in all habitats, though the difference was only detectable statistically in the mudflat habitat at ambient nutrient levels. Evidence of higher SOM and an increase in N<sub>2</sub> production after nitrate limitation was alleviated by the addition of nutrients leads to the conclusion that benthic-pelagic coupling facilitated by the oyster increases the supply and quality of sediment organic matter, which enhances denitrification in response to anthropogenic nitrogen loading.

### **5.5.2 Mechanisms for Denitrification**

Oyster-mediated increases in denitrification probably results from the production and accumulation of biodeposits, which supply organic nitrogen and carbon to the sediment microbial community (Newell et al. 2005, Higgins et al. 2013). The effect of the reef may be functionally redundant when there are other biogenic habitats present (Heck et al. 2003, Grabowski et al. 2005, Geraldi et al. 2009). The lack of difference in denitrification between the reef and control in the marsh and seagrass habitats suggests that neither resources nor substrate limited the microbial community in these habitats. However, differences in denitrification rates between the marsh-control and marsh-reef after addition of nitrate indicate redundancy only in the seagrass habitat.

Bacteria capable of denitrification are ubiquitous and denitrification can occur when three conditions are met: low oxygen concentration, and sufficient nitrate and organic matter (Seitzinger et al. 2006). In most estuarine and coastal environments nitrate availability generally limits denitrification. Nitrate used for denitrification is produced by nitrification or supported directly by nitrate in the water column. Oyster biodeposits can increase coupled nitrification-denitrification in sediments with an oxic surface layer (Newell et al. 2002). However, organic matter deposition can change the oxygen penetration depth and minimize the zone where nitrification can occur. Consequentially, organic matter loading can hinder coupled nitrification-denitrification, but enhance direct denitrification when nitrate is available in the overlying water (Caffrey et al. 1993, Cornwell et al. 1999). The increase in denitrification detected after the addition of nitrate suggests that the increase in organic matter from the oysters primed the sediments for denitrification.

The effect of priming, when the addition of nitrate increased processing of organic matter through enhanced denitrification, was not evident in the seagrass habitat, possibly due to the high SOM content. Remineralization of the organic matter may have caused the sediments to become reduced, resulting in sulfide accumulation that could inhibit nitrification and denitrification (Joye and Hollibaugh 1995). In addition, nitrate reduction may have occurred via dissimilatory nitrate reduction to ammonium (DNRA) instead of denitrification in the seagrass-reef. DNRA is favored over denitrification in systems with high carbon availability and becomes increasingly important with elevated nitrate loading (Tiedje 1988, Tobias et al. 2001, Koop-Jakobsen and Giblin 2010). These results suggest that oyster reef restoration may be a tool for water quality management in reducing the amount of nitrogen that is received by the coastal zone; however, denitrification appears to be affected by the habitat setting of the reef.

### **5.5.3 Denitrification Efficiency**

In shallow coastal systems denitrification efficiency (the proportion of the total inorganic nitrogen efflux that is  $N_2$ ) may be more important for eutrophication management than the actual rate of denitrification (Eyre and Ferguson 2009). Denitrification efficiency indicates the likelihood of the nitrogen in organic matter being converted to  $N_2$  gas. Denitrification efficiency decreases as carbon loading accelerates because of increased ammonium recycling to the water column and inhibition to nitrification/denitrification (Caffrey et al. 1993, Joye and Hollibaugh 1995, Eyre and Ferguson 2009). We found that denitrification efficiency was not different among habitats though the reefs had slightly higher efficiencies compared to the controls. Efficiencies from restored reefs in this study were similar to those of natural oyster reefs (Piehler and Smyth 2011). There was a decrease

in denitrification efficiency after the nitrogen addition. Under this scenario, DNRA may have become more important than denitrification, resulting in additional ammonium production (An and Gardner 2002, Gardner et al. 2006, Burgin and Hamilton 2007, Fulweiler et al. 2008). In order for oyster restoration to be effective at managing nutrient pollution in coastal systems, efficiency must be maintained or enhanced. We found that oyster reefs did not increase efficiency over the controls. However, the higher rate of denitrification from the reefs suggests that reefs process more organic matter than the controls, reducing the amount of organic matter within the system and preventing eutrophication in the coastal zone.

#### **5.5.4 Oyster Density and Nitrogen Removal**

Oyster density can be used as a measure of ecosystem services (Peterson and Lipcius 2003, Luckenbach et al. 2005). As oyster density increases water filtration, habitat complexity, fish production, nutrient bioassimilation and invertebrate refuge also increase (Peterson and Lipcius 2003, Soniat et al. 2004, Luckenbach et al. 2005, Rodney and Paynter 2006, Higgins et al. 2011, Ermgassen et al. 2013). However, relationships between oyster density and ecosystem processes are not always linear (Dame et al. 2002). Our model indicates that the relationship between denitrification and oyster density before the addition of nitrate (ambient conditions) is not significant. Under experimental nitrate levels, oyster density at first increased and then slightly decreased denitrification rates. The lack of relationship under ambient conditions suggests that denitrification was limited by nitrification, which was alleviated when nitrate was available directly from the water column.

The significant relationship between denitrification and oyster density after the nitrogen addition supports our hypothesis that oyster reefs prime sediment for enhanced denitrification. However, the relationship suggests the possibility of a threshold, where at



high oyster densities the large volume of biodeposits may cause sediments to become anoxic resulting in nutrient regeneration rather than removal (Tenore and Dunstan 1973, Kemp et al. 1990, Newell et al. 2005). Studies from bivalve aquaculture sites have found increasing biodeposition with density and reduced conditions in sediments (Christensen et al. 2000, 2003, Nizzoli et al. 2005, Minjeaud et al. 2009, Higgins et al. 2013). Under this scenario, nitrate is reduced through DNRA rather than denitrification (Tiedje 1988, An and Gardner 2002). Although the high oyster density at the mudflat-reefs is driving our relationships, our data indicate that maximum denitrification is reached with a density of about 600 ind/m<sup>2</sup>. While our model suggests the possibility of a threshold, it is feasible that the characteristics of the mudflat habitat, rather than density, are driving this relationship. Understanding the interaction between oyster density and ecosystem services requires further investigation, but will help inform restoration and conservations efforts such that the maximum benefits are achieved.

#### **5.5.6 Implications for Restoration and Conservation**

Improved water quality through enhanced denitrification is often cited as a benefit of oyster reef restoration. However, managers and scientists lack comprehensive measurements regarding the effect of restoration on recovering this service. Results from our study suggest that oyster restoration will enhance denitrification; however, the amount of nitrogen that can be removed appears to depend on the habitat setting of the restored reef. Our study indicates that oyster reef restoration sited on mudflats will yield the greatest nitrogen removal. The mudflat-reef had the highest rates of denitrification of all the habitats, demonstrated increased denitrification with nutrient pollution and maintained denitrification efficiency under ambient and elevated levels of nitrogen. In addition, oyster reefs restored in mudflat

habitats provide additional ecosystem services. Mudflat-reefs are associated with an increase in juvenile fish abundance, macroinvertebrates and provide refuge from predation, which contributes to the higher density of bivalves (Micheli and Peterson 1999, Grabowski et al. 2005). Thus, restoring reefs in the mudflat habitat likely has many advantages.

### **5.5.7 The Estuarine Ecosystem**

Estuarine ecosystems are among the most used and degraded systems in the world (Jackson et al. 2001a, Lotze et al. 2006). As these ecosystems are impacted by humans, ecosystem services, such as fisheries, maintenance of water quality and resistance to disturbance, are also lost (Worm et al. 2006). Excessive nutrient loading contributes to the decline in water quality and is linked to eutrophication, dead zones, harmful algal blooms and loss of biodiversity (Galloway et al. 2003, Paerl et al. 2006, Conley et al. 2009, Sharp et al. 2009). Reduction in nutrient inputs alone is not enough to recover the lost services (Duarte et al. 2009). Restoration of oyster reefs has been proposed as a solution to reduce phytoplankton biomass and to improve water quality (Dame et al. 1984, Newell 1988, Jackson et al. 2001a, Dame et al. 2002, Cerco and Noel 2007). However, restoring reefs on mudflats may increase the denitrification capacity of the estuary more than reef restoration near salt marshes or seagrass beds. Effective management of estuarine and coastal ecosystems requires consideration of how interactions between habitats impacts ecosystem function and services. Many restoration projects are often designed to enhance one service, but when consideration for the habitat setting is integrated into the design, these projects will probably provide many benefits.

## **5.6 Acknowledgments**

Many thanks for S.P. Thompson and L. Dodd for help in the field and laboratory. Comments from S.P. Thompson, B. VanDusen and L. Brown greatly improved this manuscript. North Carolina Sea Grant and the National Science Foundation provided financial support for this project.

**Table 5.1** Results of a 3-way ANOVA testing the effects of oyster reef presence, habitat setting, nutrient load, and their interactions on dissolved gasses and nutrients. “Efficiency” denotes denitrification efficiency.

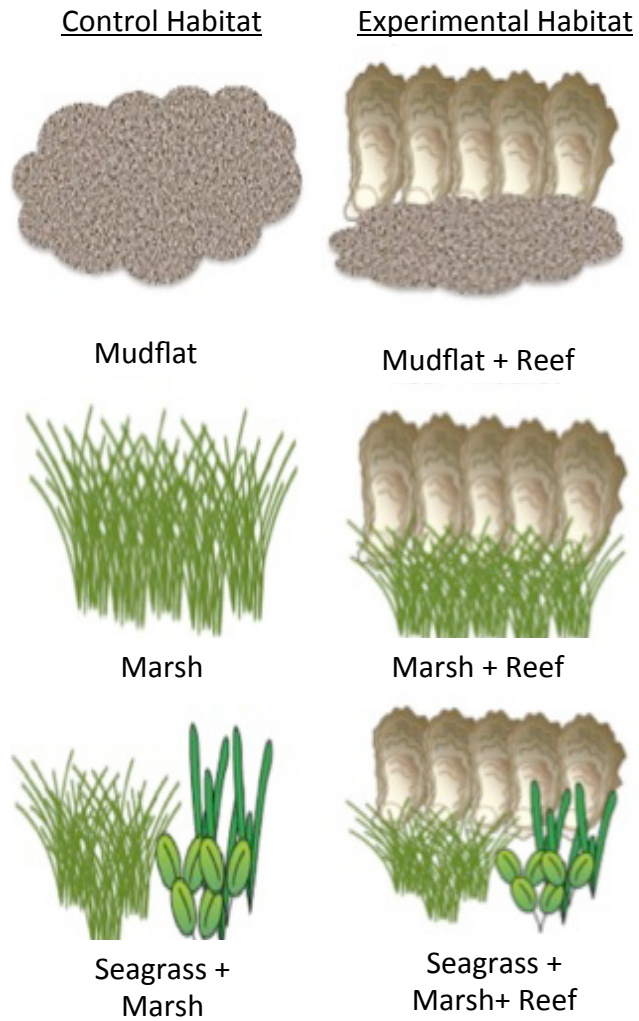
<b>Effect Tests</b>				<b>N<sub>2</sub> Flux</b>		<b>NO<sub>x</sub> Flux</b>		<b>NH<sub>4</sub><sup>+</sup> Flux</b>		<b>Efficiency</b>	
<i>Source</i>	<i>Nparm</i>	<i>DF</i>	<i>F Ratio</i>	<i>Prob &gt; F</i>	<i>F Ratio</i>	<i>Prob &gt; F</i>	<i>F Ratio</i>	<i>Prob &gt; F</i>	<i>F Ratio</i>	<i>Prob&gt;F</i>	
Habitat	2	2	1.38	0.27	6.25	<b>0.01</b>	1.22	0.31	1.34	0.28	
Reef Presence	1	1	42.18	<b>&lt;0.001</b>	0.39	0.54	2.42	0.13	0.99	0.33	
Nutrients	1	1	38.41	<b>&lt;0.001</b>	39.85	<b>&lt;0.001</b>	35.99	<b>&lt;0.001</b>	72.54	<b>0.00</b>	
Habitat*Reef Presence	2	2	7.57	<b>&lt;0.001</b>	4.74	<b>0.02</b>	0.62	0.55	0.419	0.66	
Habitat*Nutrients	2	2	1.48	0.25	6.28	<b>0.01</b>	0.75	0.48	0.272	0.76	
Reef Presence*Nutrients	1	1	9.69	<b>&lt;0.001</b>	0.38	0.54	1.92	0.18	0.022	0.88	
Habitat*Reef Presence*Nutrients	2	2	2.57	0.10	4.73	<b>0.02</b>	0.76	0.48	0.216	0.81	

**Table 5.2** Results from post hoc comparison among levels of Habitat within each level of Reef Presence\*Nutrients conducted after three-way ANOVA for nutrient and dissolved gas fluxes as well as denitrification efficiency (denoted here as “Eff.”). Significance was assessed at p=0.05.

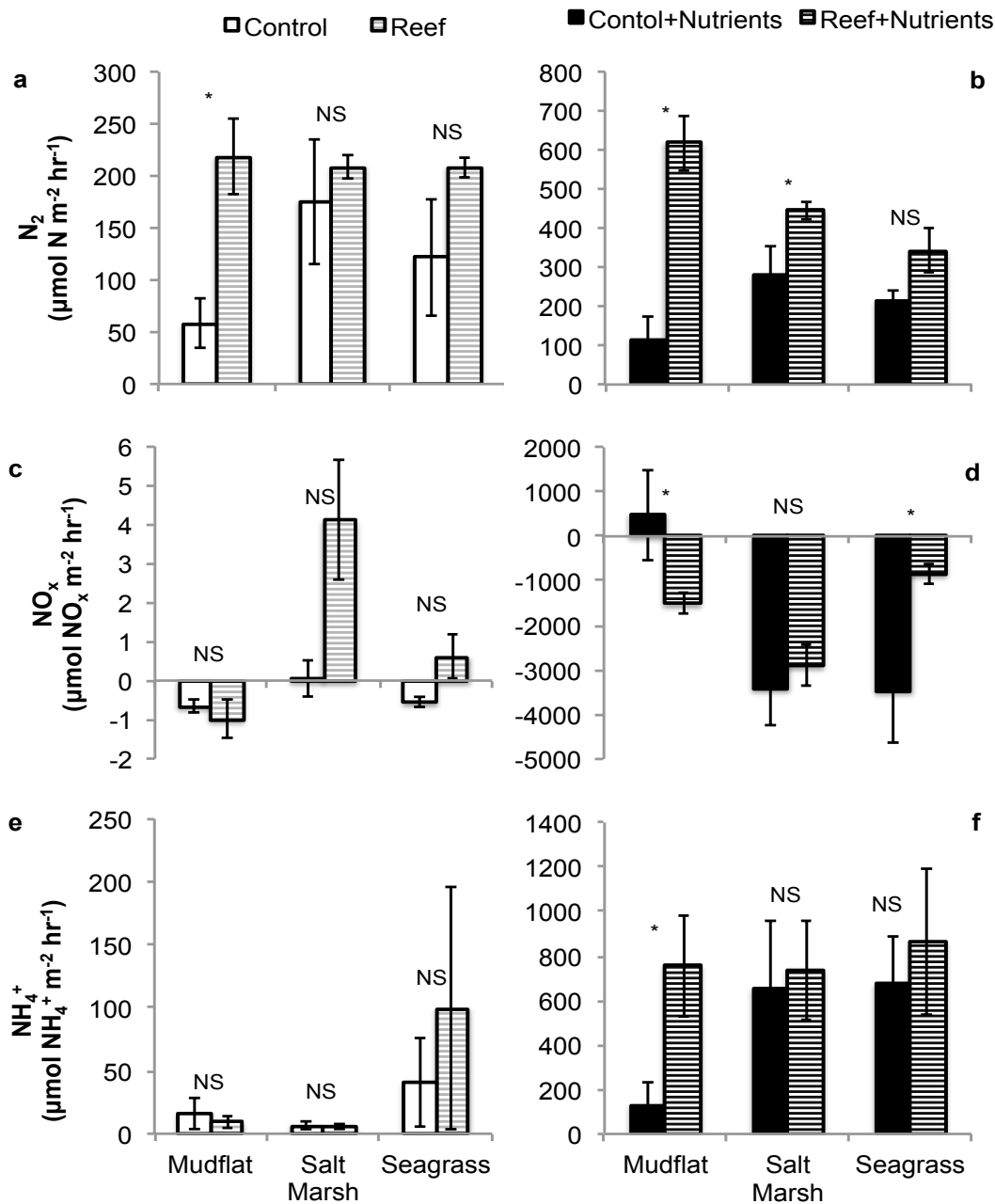
<b>Reef Presence</b>	<b>Nutrients</b>	<b>N<sub>2</sub> Flux</b>	<b>NO<sub>x</sub> Flux</b>	<b>NH<sub>4</sub><sup>+</sup> Flux</b>	<b>Eff.</b>
Control	Ambient	NS	NS	NS	NS
Control	Experiment	NS	Mudflat>Seagrass Seagrass=Marsh	Marsh>Mudflat Seagrass=Marsh Mudflat=Seagrass	NS
Reef	Ambient	NS	NS	NS	NS
Reef	Experiment	Mudflat>Seagrass Mudflat>Marsh Marsh=Seagrass	Seagrass>Marsh Mudflat=Marsh Mudflat=Seagrass	NS	NS

**Table 5.3** Results from post hoc comparison among levels of Nutrients within each level of Reef Presence\*Habitat conducted after three-way ANOVA for nutrient and dissolved gas fluxes as well as denitrification efficiency (denoted here as “efficiency”). Significant pairwise contrasts before and after the addition of nitrate are distinguished (NS= $p>0.05$ , \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ).

<b>Reef Presence</b>	<b>Nutrients</b>	<b>N<sub>2</sub> Flux</b>	<b>NO<sub>x</sub> Flux</b>	<b>NH<sub>4</sub><sup>+</sup> Flux</b>	<b>Efficiency</b>
Control	Mudflat	NS	NS	NS	**
Control	Marsh	NS	***	*	***
Control	Seagrass	NS	***	*	***
Reef	Mudflat	***	NS	**	**
Reef	Marsh	**	***	**	**
Reef	Seagrass	NS	NS	**	**

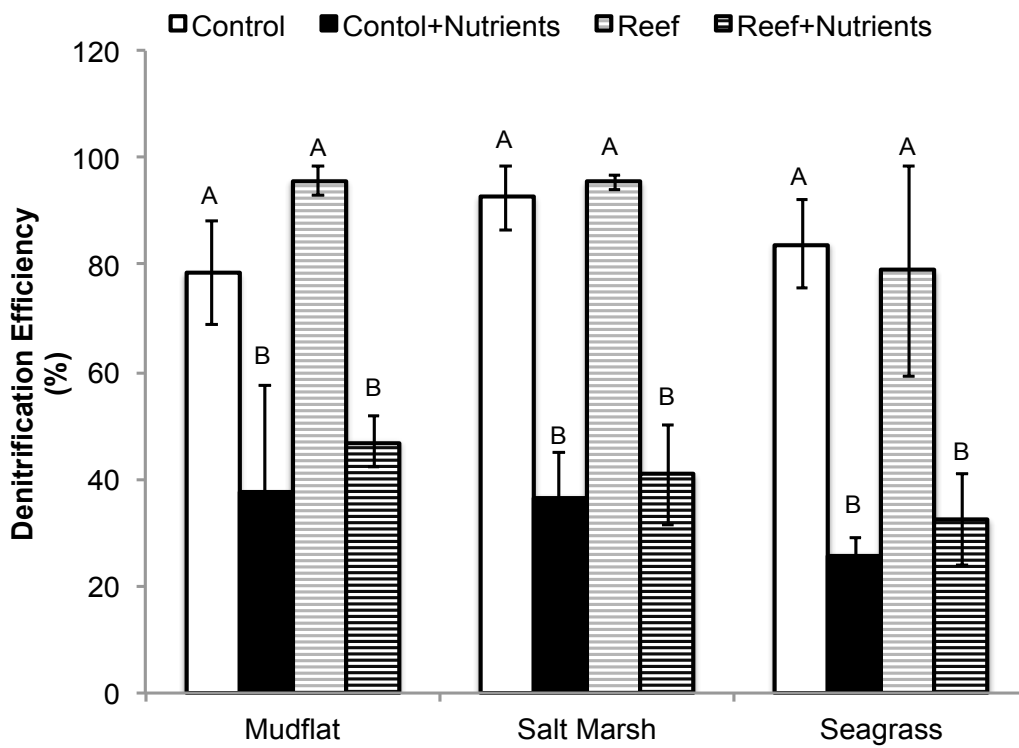


**Figure 5.1** The experimental setup of this study. Each control and reef was replicated three times for each habitat. The marsh+seagrass habitat is termed seagrass in the text.

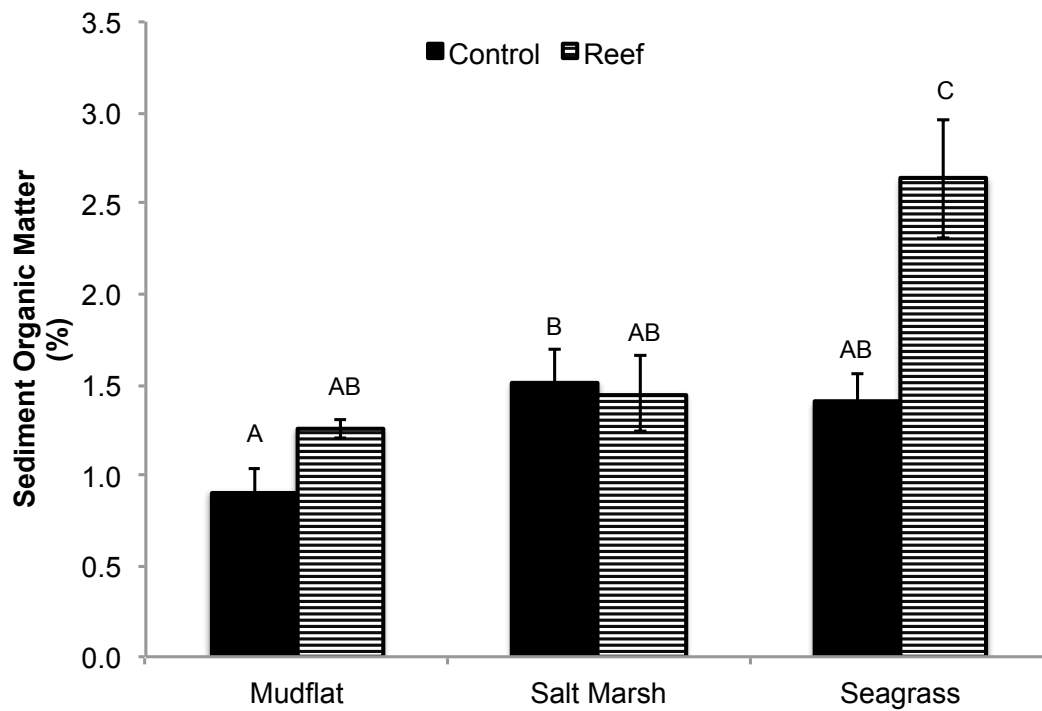


**Figure 5.2** Mean net fluxes of  $N_2$ ,  $NO_x$ , and  $NH_4^+$  before ((a) (c) (e)) and after ((b) (d) (f)) experimental nitrogen additions for reef and control areas in each habitat ( $n=3$ ). A positive value indicates flux out of the sediment and negative value indicates flux from the water column to the sediment. Significant differences between control and reefs for each habitat are indicated with asterisks. NS indicates no significant difference. Error bars present one standard error of the mean.

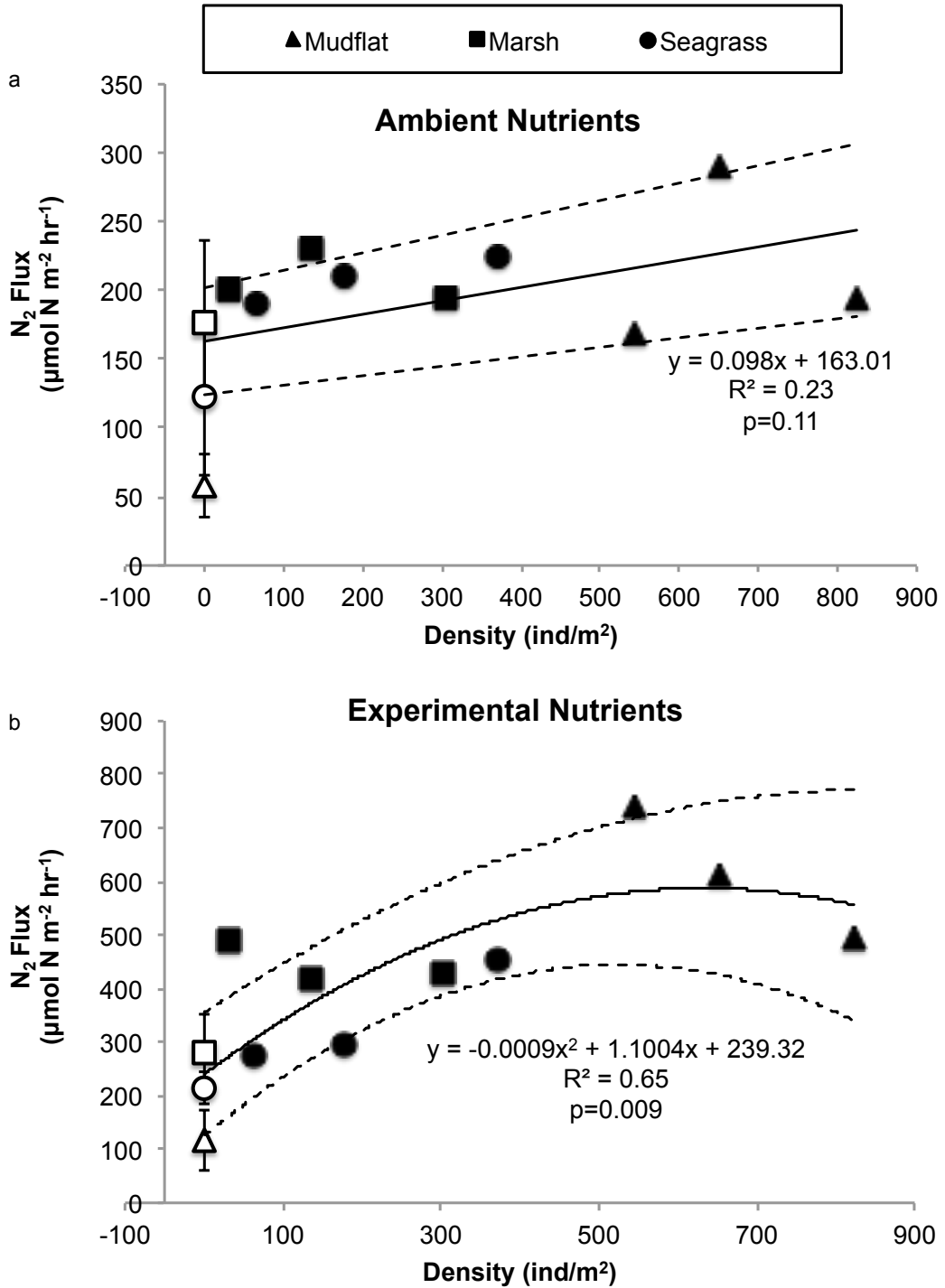




**Figure 5.3** Mean denitrification efficiency (percent of the total dissolved inorganic nitrogen efflux that is N<sub>2</sub>) for the control and reef in each habitat before and after the addition of nutrients. Errors are one standard deviation. Histograms not sharing letters are significantly different from each other ( $p < 0.05$ ).



**Figure 5.4:** Mean sediment organic matter from the upper 2cm of each sample during both sampling events. Errors are one standard error of the mean. Histograms not sharing letters are significantly different from each other ( $p < 0.05$ ).



**Figure 5.5** Relationship between denitrification and oyster density under before (a) and after (b) nitrogen addition. Solid line is the model, dashed lines are 95% prediction intervals. Regression includes the mean value of the control habitats in each habitat (open) and all reefs (solid).

## 6. CONCLUSION

The nitrogen cycle is a complex and challenging biogeochemical cycle to study as a consequence of the various forms for nitrogen. Nitrogen can be found in particulate, dissolved and gaseous phases and nitrogen compounds can occur in oxidation states from -3 to +5. Nitrogen is integral to nucleic and amino acid synthesis, making it essential for life. However, the majority of nitrogen on Earth is found in the atmosphere as N<sub>2</sub> gas.

Two opposing processes help control the availability of nitrogen: nitrogen fixation and denitrification. Nitrogen fixation converts atmospheric nitrogen to biologically available forms of nitrogen, conversely denitrification returns nitrogen to the atmosphere by transforming nitrate to N<sub>2</sub>. Variation between these processes has resulted in nitrogen limitation to primary production in marine ecosystems (Vitousek and Howarth 1991). When supplied in excess, nitrogen can have deleterious effects (Conley et al. 2009). Anthropogenic activities have at least doubled the amount of bioavailable nitrogen in the environment (Vitousek et al. 1997b). Because denitrification removes dissolved nitrogen, this process has received much attention in recent years as a way to remediate cultural eutrophication (Galloway et al. 2003, Brush 2009). However, our understanding of denitrification is far from complete, in part because of the difficulty associated with measuring this biogeochemical process and the variety of factors that control it (Cornwell et al. 1999, Groffman et al. 2006, 2009). Though the goal of this dissertation was to examine nitrogen dynamics in estuarine systems, it was not my initial intent to study the nitrogen dynamics of oyster reefs. Rather that focus grew out of my desire to gain a better understanding of what

contributes to the creation of oyster reefs as hot spots for denitrification activity that my early research highlighted.

## **6.1 Summary of Results**

Research for this dissertation started with the objective of characterizing the spatial and temporal patterns of sediment nitrogen dynamics in shallow water coastal systems. Previous studies in estuarine systems have focused on one habitat and have used a variety of different methods. As a consequence, it is challenging to integrate measurements of denitrification over larger scales (Cornwell et al. 1999). Ecosystem assessments of denitrification are further complicated in tidal systems where biotic and abiotic conditions are in constant flux. In Chapter 2, I examined nitrogen dynamics in a variety of different estuarine habitats over an annual cycle. Daily rates accounting for light and water level were extrapolated to the estuary based on habitat area. I found that given the current spatial arrangement, denitrification removed 76% of the estimated watershed nitrogen load (Smyth et al. 2013). These results suggest that changes in the area and distribution of habitats in the estuarine landscape will impact ecosystem functions and services. For example, restoration of oyster reefs on intertidal flats would increase the denitrification capacity of the system and increase nitrogen removal benefits by \$1,400 per acre per year (Piehler and Smyth 2011).

This finding led me to further investigate nitrogen dynamics in oyster reefs. I realized that although enhanced denitrification is often cited as a benefit of oyster reef restoration (Grabowski and Peterson 2007), this claim was based on measurements from a laboratory experiments using phytoplankton pellets (Newell et al. 2002). To better understand the direct and indirect effects of oysters on nitrogen dynamics, I conducted a microcosm experiment

using live oysters (Chapter 3). Results from this experiment found that oyster-mediated denitrification accounted for 16-40% of the inorganic nitrogen flux. The accumulation and remineralization of oyster-produced organic matter, coupled with the oxygen consumption by the oyster created conditions favorable for denitrification. Furthermore, the addition of an oyster to sediment helped to shift the primary nitrogen cycle process from nitrogen fixation to denitrification, probably due to the increase in organic matter from biodeposits and ammonium production from excretion.

Results from the microcosm experiment provided a mechanistic understanding of how oysters affect nitrogen availability. However, oysters do not exist alone but rather build reefs that consist of many oysters. In Chapter 4, I examined the role that the reef has in the formation of a biogeochemical hot spot. I conducted a field experiment to distinguish between the effects of biotic deposition and abiotic accumulation associated with the oyster reef on sediment denitrification. Experimental oyster reefs were constructed with live oysters to represent fully functioning oyster reefs, oyster shells to represent reef structure and mud flats without reefs served as controls. Results indicated that the reef helps to concentrate organic matter, but that collection of biologically derived material had the greatest effect on sediment denitrification. This experiment demonstrated the potential for restored reefs to remove nitrogen and that these effects are achieved quickly--just two weeks after construction.

Results from previous chapters lead me to investigate whether the location of the reef impacted nitrogen removal benefits of restoration. In Chapter 5, I examined denitrification from oyster reefs restored in three different habitat settings under ambient and elevated levels of nutrient loading. I found that oyster reefs restored in a mudflat setting had the greatest

effect on sediment denitrification, likely due to the relative isolation of the mudflat reef. Additionally, the accumulation of high quality organic matter due oyster biodeposits helped to prime the sediments for enhanced denitrification in response to anthropogenic nutrient loading.

Coastal ecosystems are experiencing an array of stressors resulting from human activities. Two of the most concerning alterations have been nutrient enrichment and decrease in the oyster population. My research is among the first to quantify rates of sediment nitrogen removal attributable to oyster reefs and to assess the efficacy of oyster reef restoration in alleviating nutrient pollution. As more resources are devoted to restoring oyster reefs to enhance the fishery, scientists and managers need to ensure that ecological services are also restored, all in the most economically efficient manner.

## **6.2 A Conceptual Model of Oyster Reef Nitrogen Removal**

From the results of this dissertation and information in the literature, I developed a conceptual model examining how oyster mediated benthic-pelagic coupling modifies sediment nitrogen dynamics (Fig 6.1). I hope that this model will provide a framework for determining how to design oyster reef restoration projects to enhance nitrogen removal. If oyster reefs are restored in areas with high sediment organic matter content the capacity for oysters to enhance nitrogen removal will be influenced by the availability of nutrients and the concentration of oxygen in the water column. If the O<sub>2</sub> concentration is low, remineralization of additional organic matter from the oysters will increase ammonium production. If the O<sub>2</sub> concentration is above hypoxic/anoxic levels, the effect will be dependent on the nutrient concentration, where high levels of nitrate (>10μM) will increase direct denitrification, but

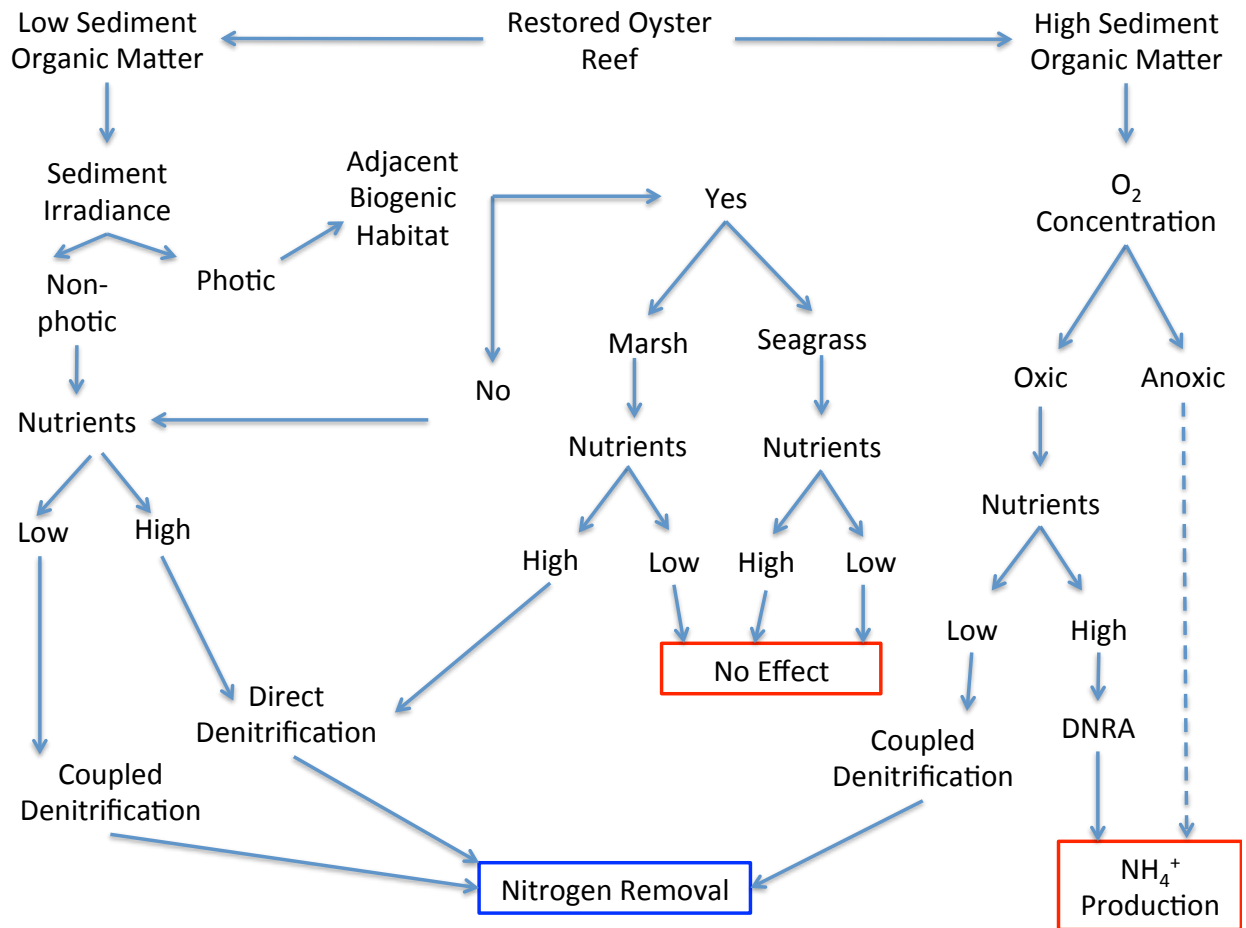
dissimilatory nitrate reduction to ammonium (DNRA) will probably be the dominate process and result in production of ammonium. However, low levels of nutrients ( $<10\mu\text{M}$ ) will lead to increased coupled nitrification-denitrification and nitrogen removal. In systems where sediment organic matter content is low, oyster mediated biodeposition can help to shift the system from net nitrogen fixation to net denitrifying, under both high and low levels of nutrients. When the location is in the photic zone, the habitat setting will determine the success of oyster reef restoration at removing nitrogen. If the location for restoration is not adjacent to other biogenic habitats (i.e. on a mudflat), oyster reefs will enhance denitrification under both high and low levels of water column nutrients. If the adjacent habitat is a salt marsh, oyster reefs will be most effective under high levels of nitrogen loading, because of priming associated with organic matter deposition from the oysters. If oyster restoration occurs adjacent to seagrass beds, the oyster reef will have be functionally redundant and have no effect on the amount of nitrogen that is removed.

### **6.3 Importance of Scale**

This dissertation has investigated oyster-mediated changes in nitrogen dynamics over at the scale of the individual organism, the oyster reef, the habitat setting and the ecosystem. This holistic approach allowed for me to better understand the complex interactions between oysters and the nitrogen cycle. For example, if I only examined the individual oyster I would grossly over-estimate the amount of denitrification associated with the reef ecosystem. Similarly, examining the interactions between the reef and other habitats in a given area provided insight into how material and energy flow throughout the ecosystem. Enhanced denitrification is often considered a benefit of oyster reef restoration; however, until now, we



have lacked the information necessary to include oysters in nutrient management plans. As interest in oyster restoration, oyster aquaculture and nutrient trading programs increases, such measurements will be necessary to ensure high levels of water quality are maintained. This dissertation can serve to help shift the view of oysters as an exploitable commodity to a valued habitat.



**Figure 6.1** Conceptual model showing how the locations of oyster reef restoration projects can affect the removal and regeneration of nitrogen. This model is designed to provide a framework for managers in designing oyster reef restoration plans in intertidal systems. Dotted lines indicate processes that were not directly measured in this study.

## APPENDIX A: Methods for Measuring Sediment Denitrification

### A.1 Measuring Denitrification

Denitrification is an important ecological process, which permanently removes fixed nitrogen from ecosystems. Direct measurements of rate of denitrification are challenging due to the high concentrations of N<sub>2</sub> in the atmosphere and the relatively small changes in concentration resulting from denitrification (Cornwell et al. 1999, Groffman et al. 2006). Numerous methods have been used to measure denitrification including acetylene block technique, stoichiometric and mass balance approaches, isotope pairing techniques and the N<sub>2</sub>:Ar method using membrane inlet mass spectrometry (MIMS). Each method has its own limitations and assumptions (Cornwell et al. 1999, Groffman et al. 2006). The difficulty associated with measuring denitrification and complexities of coastal ecosystems have made it challenging to evaluate denitrification on larger spatial scales. Therefore, rates of denitrification determined from two independent methods were compared.

### A.2 Flux Calculations and Analytical Methods

Flux calculations were based on the assumption of steady-state conditions and a well-mixed water column in each microcosm (Miller-Way and Twilley 1996). The system was assumed to be at steady state when the slope of concentration vs. time for each microcosm was not different from zero. Benthic fluxes were calculated using the following equation:

$$J = ([i_{\text{outflow}}] - [i_{\text{inflow}}]) * \frac{F}{A}$$

where  $J$  is the flux in  $\mu\text{mol m}^{-2} \text{hr}^{-1}$ ,  $[i_{\text{outflow}}]$  and  $[i_{\text{inflow}}]$  is the concentration ( $\text{mmol m}^{-3}$ ) of any dissolved constituent leaving and entering the core, respectively,  $F$  is the peristaltic pump flow rate ( $\text{m}^3 \text{hr}^{-1}$ ), and  $A$  is the surface area of the core ( $\text{m}^2$ ).  $[i_{\text{outflow}}]$  is the average of three measurements of concentrations leaving each the microcosm collected over a 48 hour period;

[ $i_{\text{inflow}}$ ] is the average of three measurements of concentrations entering the microcosm measured from a bypass, that flowed directly into the sample vial and was collected at the same time as the outflow sample. For nitrogen species, a positive flux indicates an exchange from the sediment to the water column, and a negative flux indicates an exchange from the water column to the sediment. For  $\text{O}_2$ , a positive flux indicates an exchange from the water column to the sediment and is denoted as sediment oxygen demand (SOD). Treatment specific fluxes were calculated as the mean of microcosm specific values from replicates ( $n=3$ ). Errors presented here are the standard error of the means ( $n=3$ ).

Samples were analyzed for concentrations of  $\text{N}_2$ ,  $\text{O}_2$  and Ar gases dissolved in water using a Balzers Prisma QME 200 quadruple mass spectrometer (MIMS; Pfeiffer Vacuum, Nashua, NH, USA; Kana et al. 1994). Concentrations of  $\text{O}_2$  and  $\text{N}_2$  were determined using the ratio with Ar (Kana et al. 1994; Ensign et al. 2008). MIMS has a rapid analysis time, requires a small sample volume, little sample preparation and has good precision (Kana et al. 1994). Coefficients of variation (CV) for  $\text{N}_2/\text{Ar}$  and  $\text{O}_2/\text{Ar}$  were calculated from 25 replicate samples of deionized water maintained at  $16^\circ\text{C}$  and at 0 salinity. The maximum observed CV for  $\text{N}_2/\text{Ar}$  was 0.05% and was 0.04% for  $\text{O}_2/\text{Ar}$ . The MIMS method determines the net flux (production-demand) across the sediment-water interface such that a positive  $\text{N}_2$  flux indicates denitrification dominates the net  $\text{N}_2$  flux and a negative  $\text{N}_2$  flux indicates nitrogen fixation dominates the net  $\text{N}_2$  flux (An et al. 2001, Fulweiler et al. 2007). The MIMS method does not discern between  $\text{N}_2$  production from denitrification, anammox or any other  $\text{N}_2$  producing process.

Water samples (50ml) were collected for nutrient analysis from the bypass line and the outflow port of each microcosm after steady state had been established with respect to

dissolved gasses (typically 24-hours after the incubation began). Water was filtered through Whatman GF/F filters (25 mm diameter, 0.7  $\mu\text{m}$  nominal pore size), and the filtrate was analyzed with a Lachat Quick-Chem 8000 (Lachat Instruments, Milwaukee, WI, USA) automated ion analyzer for nitrate plus nitrite (reported as  $\text{NO}_x^-$ ) and ammonium ( $\text{NH}_4^+$ ) (detection limits: 0.04  $\mu\text{M}$  and 0.18  $\mu\text{M}$ , respectively). The precision of  $\text{NO}_x^-$  and  $\text{NH}_4^+$  were calculated from five replicated samples. The maximum observed CV was 0.9% for  $\text{NO}_x^-$  and 2.6% for  $\text{NH}_4^+$ .

The standard deviation of the flux measurement ( $\sigma_J$ ) is calculated by error propagation as:

$$\sigma_J = ([\sigma_{i_{\text{outflow}}}]^2 + [\sigma_{i_{\text{inflow}}}]^2)^{0.5} * F/A$$

where  $\sigma_i$  is the standard deviation of  $i_{\text{outflow}}$  and  $i_{\text{inflow}}$ ,  $F$  is the peristaltic pump flow rate (0.60  $\text{l hr}^{-1}$ ),  $A$  is the surface area of the microcosm ( $3.22 \times 10^{-3} \text{ m}^2$ ). The limit of detection (LD) of an experimental measurement is often defined as two standard deviations (Miller and Miller 1993). Assuming  $\sigma_{i_{\text{outflow}}} \approx \sigma_{i_{\text{inflow}}}$ ,  $F = 6.0 \times 10^{-5} \text{ m}^3 \text{ hr}^{-1}$ ,  $A = 3.22 \times 10^{-3} \text{ m}^2$ ,

$$\text{LD} = 2 * \sqrt{2} * 0.0186 (\text{m hr}^{-1}) * \sigma_i (\text{mmol m}^{-3}).$$

Given CV = 0.05% for  $\text{N}_2$  and CV=0.04%  $\text{O}_2$ ,  $[\text{N-N}_2] \approx 580 \text{ mmol m}^{-3}$ ,  $[\text{O}_2] \approx 170 \text{ mmol m}^{-3}$ , the detection limit for the  $\text{N-N}_2$  and  $\text{O}_2$  fluxes are 30.5 and 38.8  $\mu\text{mol m}^{-2} \text{ hr}^{-1}$ , respectively. For dissolved nitrogen fluxes, given  $[\text{NO}_x^-] \approx 2 \text{ mmol m}^{-3}$  and CV=0.9% for  $\text{NO}_x^-$ ,  $[\text{NH}_4^+] \approx 25 \text{ mmol m}^{-3}$  and CV=2.6% for  $\text{NH}_4^+$ , the detection limit for nitrate and ammonium fluxes are: 0.95  $\mu\text{mol m}^{-2} \text{ hr}^{-1}$  and 34  $\mu\text{mol m}^{-2} \text{ hr}^{-1}$ , respectively.

### A.3 Mass Balance Approach For Denitrification

The use of MIMS with continuous flow benthic microcosms reduces environmental dependency of the sample and achieves steady state fluxes (Miller-Way and Twilley 1996).

Steady state fluxes, where concentrations did not change overtime, were measured for  $N_2$ ,  $O_2$ ,  $NH_4^+$ ,  $NO_x^-$ . The assumption of steady state conditions and types of fluxes that were measured allowed denitrification to be calculated using mass balance equations, in addition to direct measurements with MIMS (Miller-Way and Twilley 1996, Groffman et al. 2006, Fennel et al. 2009). The mass balance approach assumes that organic matter with Redfield ratios of C:N is decomposed with  $O_2$ , and the end products are defined by stoichiometry (Groffman et al. 2006). Additional assumptions include: denitrification is the major nitrogen removal pathway and dominant source of  $N_2$ , there is minimal nitrogen fixation and assimilation, minimal DNRA and remineralization and nitrification are dominant  $O_2$  consuming processes. An ammonium absorption coefficient of 1 (Rosenfeld 1979, Klump and Martens 1989) and respiratory coefficient of 1 (Hopkinson 1985, Giblin et al. 1997) were used for calculations.

The relationship between  $N_2$  flux and sediment oxygen demand (SOD) contains information about the source of nitrate used for denitrification. Previous studies suggest that a strong positive correlation between  $N_2$  flux and SOD results from a coupling between nitrification and denitrification (Seitzinger and Giblin 1996, Piehler and Smyth 2011). SOD is primarily a function of mineralization and nitrification and any other  $O_2$  consuming process. Thus, a relationship between  $N_2$  flux and SOD, especially when ambient nitrate concentration is less than  $10 \mu M$  suggests nitrate used for denitrification is generated through nitrification (Seitzinger 1994, Piehler and Smyth 2011, Fennel et al. 2009).

#### **A.4 Comparison of Mass Balance Rates to MIMS Measured Rates**

We measured denitrification for each individual cores measured from Piehler and Smyth 2011 and Chapter 2 using two independent methods: mass balance and MIMS ( $N_2:Ar$ ) technique. After removing outliers from the mass balance model and excluding MIMS

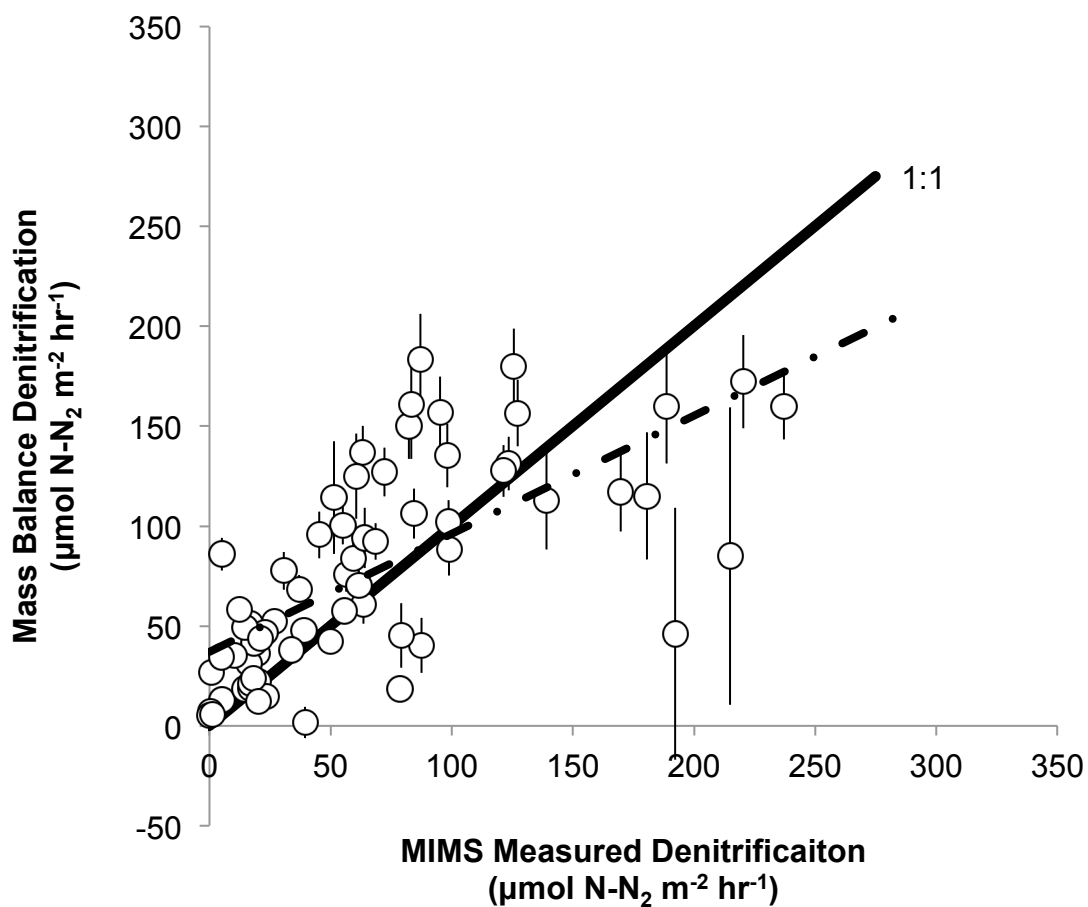
measurements that were negative, 67 of the 120 microcosms were used in the analysis. A paired t-test was used to determine if denitrification rates measured by the two methods were different. Results were considered statistically significant at an alpha level of 0.05. Statistical analyses were performed using R 2.13.1 (The R Foundation for Statistical Computing 2011). Rates of denitrification determined by the MIMS method ( $N_2:Ar$ ) were compared to rates modeled using a mass balance approach (Figure A.1). Error bars represent the random error associated with each measurement. Error for the mass balance approach was calculated by error propagation for each flux measurement.

Comparisons between the two methods indicated that rates were not statistically different ( $t_{55}=-0.96$ ,  $p=0.34$ ,  $r^2=0.46$ ). Predicted mass balance rates were generally higher than MIMS measured rates. The MIMS method results in a net  $N_2$  flux ( $N_2$  production- $N_2$  consumption); thus, if nitrogen fixation was high, the method may underestimate denitrification. Recent studies have indicated that denitrification and nitrogen fixation can co-occur in estuarine sediment (Fulweiler et al. 2013); thus, the positive  $N_2$  flux measured from MIMS methods may be less than the actual rate of denitrification. Under such a scenario mass balance may be a better predictor of the actual rate of denitrification. Results did not indicate a clear pattern between seasons or habitats. There is a cluster of measured rates around  $200 \mu\text{mol m}^{-2} \text{hr}^{-1}$ , that are higher than values predicted by mass balance. Measured rates may be higher than mass balance rates because the mass balance model assumes a minimal contribution of  $N_2$  production from anammox. If anammox is contributing to the  $N_2$  production mass balance rates will underestimate denitrification. These data points were from oyster reef sediments in the summer and SAV sediments in the spring. It is likely that the organic matter at these sites during these seasons deviating from Redfield ratio organic

matter. A closer relationship between the measured and modeled values for these samples was achieved by adjusting the C:N lower. This suggests that the quality of organic matter was higher (more nitrogen rich) than Redfield for these sites. Oyster reef sediments were likely more nitrogen rich during the summer because of the production and accumulation of biodeposits is higher as oysters feed more during this time and there is more particulate organic nitrogen (phytoplankton) in the water column. The spring is the growing seasons for *Halodule wrightii* in this system, which has high nitrogen content.

Mass balance rates rely on the assumption of Redfield organic matter (i.e. C:N=6.625) and that nitrification and remineralization are the dominant oxygen consuming processes. If sediment samples have organic matter with elemental ratios different from Redfield ratios or processes other than nitrification and remineralization consumes large amounts of oxygen (i.e. iron oxidation, sulfate oxidation), mass balance calculations may not be as accurate (Jørgensen 1977, Groffman et al. 2006). Unfortunately, we do not have measures of C:N from these site; however, C:N may be estimated by assuming the MIMS N<sub>2</sub> flux is accurate and adjusting the C:N ratio as well as the oxidation state of the carbon until rates calculated from the two different approaches agree. However, measurements of net N<sub>2</sub> fluxes may underestimate denitrification if nitrogen fixation rates are high. Each technique has assumptions and limitations and the researcher should select a method that is most appropriate for the research question. The MIMS method will be most valuable when determining net sources and sinks, while other methods are beneficial for quantifying rates of specific reactions.





**Figure A.1** Comparison of denitrification determined by mass balance and the N<sub>2</sub>:Ar techniques (n=67). Error bars represented the propagated error for each method. Dotted line is best fit (p=0.34, R<sup>2</sup>=0.46), solid if a 1:1 line is plotted for reference.

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