# RESTORATION OF ESTUARINE HABITATS SUPPORTS CHANGES IN NITROGEN CYCLING AND REMOVAL OVER TIME

Kathleen Marie Onorevole

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Approved by:

Michael F. Piehler

Jaye E. Cable

F. Joel Fodrie

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#### ABSTRACT

## Kathleen Marie Onorevole: Restoration of Estuarine Habitats Supports Changes in Nitrogen Cycling and Removal Over Time (Under the direction of Michael F. Piehler)

Salt marshes and oyster reefs can be restored as living shorelines to prevent coastal erosion and provide ecosystem functions, including denitrification. This microbial process transforms N to a non-bioavailable gas, possibly also producing the powerful greenhouse gas N<sub>2</sub>O. This study used a chronosequence space-for-time replacement design spanning 0 to 20 years to evaluate N cycling following restoration. Sediment cores were collected seasonally. Dissolved N<sub>2</sub> and O<sub>2</sub> fluxes in the overlying water were analyzed with a membrane inlet mass spectrometer (MIMS). Denitrification always increased from the 0- to 7-year-old sites; changes in rates between the 7- and 20-year-old sites were not consistent across seasons. Sediment oxygen demand (SOD) was significantly correlated with annual denitrification and may be a viable proxy. These habitats may be a small sink for N<sub>2</sub>O. This research shows that restored salt marshes and oyster reefs can augment denitrification without increasing fluxes of N<sub>2</sub>O.

To my parents, Richard and Marie Onorevole, who have always encouraged my interest in the environment and who continue to articulate the importance of science to our planet's future

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# LIST OF ABBREVIATIONS

Army	Army Marsh		
Carrot	Carrot Island		
CHN	carbon hydrogen nitrogen		
$CO_2$	carbon dioxide		
DNE	denitrification efficiency		
GC	gas chromatograph		
IMS	UNC Institute of Marine Sciences		
LOI	loss on ignition		
MIMS	membrane inlet mass spectrometer		
Ν	nitrogen		
$N_2$	dinitrogen gas		
N <sub>2</sub> O	nitrous oxide		
NF-DNF	nitrification-denitrification		
$\mathrm{NH_4^+}$	ammonium		
NO <sub>3</sub> -	nitrate		
NO <sub>x</sub>	nitrate and nitrite		
NOAA	NOAA Beach at NOAA Beaufort Lab (location of one sampling site;		
	context indicates distinction from NOAA as an institution)		
ON	organic nitrogen		
PO <sub>4</sub> <sup>3-</sup>	phosphate		
SH	shell height		
SOM	sediment organic matter		

SOD sediment oxygen demand

TN total nitrogen

#### **INTRODUCTION**

There is increasing awareness of the deleterious impacts of anthropogenic activity on the marine environment (Lotze *et al.* 2006). Both regional practices and global phenomena can affect marine ecosystems. On a local level, resource exploitation, pollution, nutrient enrichment, habitat destruction, and changes in sediment delivery threaten marine environments and the communities they support. Risks to marine environments associated with climate change include ocean acidification, rising sea levels, and intensified storms. Local stressors will likely be compounded by climate change in ways that are difficult to predict, possibly resulting in a synergistic relationship between the two scales of impact (Crain *et al.* 2008).

Habitat degradation is a broad consequence of local and global stressors. Human wellbeing is dependent upon many ecosystem functions and services provided by marine environments (Costanza *et al.* 1997, Millennium Ecosystem Assessment 2005), making habitat degradation relevant for social and ecological reasons. There are therefore multiple motivations for combating habit degradation. Habitat restoration is increasingly identified as a means of stymieing habitat loss and providing resiliency against future changes (Seavy *et al.* 2009). Holling (1973) initially defined resilience as an ecosystem's ability to withstand disturbance before changing state, although that definition has since been interpreted in many ways (Gunderson 2000). Since climate change will impact all ecosystems in some capacity, designating resiliency as an overall restoration goal can help restoration practitioners maintain a holistic, non-linear perspective (Boesch 2005).

Among marine environments, coastal habitats have been heavily impacted (Halpern *et al.* 2008) and identified as candidates for restoration due to their ecological and cultural importance (Borja *et al.* 2010). Estuarine habitats provide functions such as nursery habitat for juvenile fish and invertebrates (Beck *et al.* 2001), resilience to rising sea level (Morris *et al.* 2002), and nutrient cycling (Jordan *et al.* 2011). Estuaries are often bordered by high human population densities, adding additional pressure to these systems that makes habitat restoration imperative (Callaway & Zedler 2004).

One common goal of estuarine restoration is mitigation of nutrient loading. Anthropogenic nutrient loading to coastal systems has long been recognized as a concern, largely because it increases the probability of eutrophication and poor water quality (Nixon 1995, Pinckney *et al.* 2001). In nutrient-rich systems, eutrophication can lead to a host of deleterious impacts, including harmful algal blooms (Paerl & Otten 2013), hypoxia (Hagy *et al.* 2004), and fish kills (Paerl *et al.* 1998). Nitrogen (N) is a common nutrient of concern in coastal systems, where it enters primarily in the form of NO<sub>3</sub><sup>-</sup> via runoff from residential, commercial, and agricultural sites (McClelland & Valiela 1998, Valiela *et al.* 2000). Restoration of streams and coastal waterways increasingly seeks to manage NO<sub>3</sub><sup>-</sup> concentrations, thus providing a critical ecosystem service (Dodds *et al.* 2008, Craig *et al.* 2008).

Strategies for managing aquatic N loads can include decreasing inputs and increasing removal. The latter can be accomplished through denitrification, a pathway that removes bioavailable N from terrestrial and aquatic environments. Through denitrification, NO<sub>3</sub><sup>-</sup> is reduced to biologically non-available N<sub>2</sub> gas, which is released to the atmosphere (Herbert 1999). The importance of denitrification has been recognized on a global scale (Sutherland *et al.* 2010). Estuarine habitats such as salt marshes and oyster reefs can facilitate denitrification,

making them key moderators of  $NO_3^-$  concentrations (Seitzinger *et al.* 2006, Sousa *et al.* 2012, Beseres Pollack *et al.* 2013).

In estuarine habitats, denitrification is performed by heterotrophic bacteria (Herbert 1999). Fungal denitrification is also possible (Sumathi & Raghukumar 2009). In bacterial denitrification, facultative anaerobes use  $NO_3^-$  as a terminal electron receptor in the absence of oxygen, when the more energetically-favorable reduction of oxygen is not possible (van Rijn *et al.* 2006). Denitrification also depends on available sources of  $NO_3^-$ , organic matter, suitable redox conditions, and a lack of inhibitors such as sulfides (Knowles 1982, Brunet & Garcia-Gill 1996). As a result, denitrification activity is very localized: high rates are found in microsites with appropriate conditions (Parkin 1987). These denitrification hotspots can account for a disproportionate amount of the total denitrification capacity of a given system (Duncan et al. 2013). Intertidal habitats may have more of these hotspots due to their varied microtopography, created by bioturbation, sediment accretion, vegetation, and wave energy, suggesting that they could support substantial rates of denitrification (Wolf *et al.* 2011).

In environments with limited ambient NO<sub>3</sub><sup>-</sup>, denitrification is typically accomplished via coupled nitrification-denitrification (NF-DNF). Since nitrifiers are aerobes, coupled NF-DNF depends upon the existence of proximate oxic and anoxic microsites that can support both processes (Jenkins & Kemp 1984). Intertidal habitats can support conditions conducive to coupled NF-DNF because they experience periodic inundation that alters sediment oxygen concentrations. Tidal inundation leads to oxygen draw-down in sediments, which are reoxygenated during ebb tide. As a result, adjacent oxic and anoxic zones can develop in both time and space (Seitzinger *et al.* 2006), making it possible for NH<sub>4</sub> to be nitrified and subsequently reduced.

When considering N dynamics in a global context, it is necessary to also consider N<sub>2</sub>O gas production. N<sub>2</sub>O is a powerful greenhouse gas. It has a radiative forcing approximately 300x that of CO<sub>2</sub> and contributes to the destruction of the ozone layer (Cicerone 1987). Consequently, production of N<sub>2</sub>O has been termed an ecosystem disservice (Burgin *et al.* 2013). N<sub>2</sub>O is produced as a byproduct of nitrification and an intermediate in denitrification. In estuarine environments, higher emissions are typically associated with denitrification (Dong *et al.* 2002), although some studies have identified nitrification as the dominant source of N<sub>2</sub>O (de Wilde & de Bie 2000). N<sub>2</sub>O is produced via incomplete denitrification, when N<sub>2</sub>O is not reduced to N<sub>2</sub> gas and is instead released to the atmosphere. Lower rates of N<sub>2</sub>O flux have been reported for estuarine environments dominated by coupled NF-DNF (Cartaxana & Lloyd 1999, LaMontagne *et al.* 2002). To balance the value of the ecosystem service of NO<sub>3</sub><sup>-</sup> removal with the disservice of N<sub>2</sub>O production, it is useful to evaluate both denitrification and N<sub>2</sub>O production when studying denitrification.

As previously described, there are many factors that determine whether denitrification is possible. The relative rate of denitrification is influenced by temperature (Kemp *et al.* 1990, Bachand & Horne 2000), and seasonal variability has been observed in estuarine environments (Thompson *et al.* 1995, Nowicki *et al.* 1997, Cabrita & Brotas 2000). Consequently, single measurements of denitrification could fail to adequately describe trends throughout time, making it important to evaluate denitrification with a high degree of temporal resolution.

Routine measurement of denitrification in estuarine habitats can complement long-term studies of restored habitats. Estuarine habitat restoration in North Carolina commonly includes the restoration of salt marshes with fringing oyster reefs. Salt marshes have long been identified as important habitats for denitrification (George & Antoine 1982, DeLaune *et al.* 1983), and

recent work has shown that oyster reefs also facilitate high rates of denitrification (Carmichael *et al.* 2012, Piehler & Smyth 2011). Oyster biodeposits are posited to provide substrate for denitrifying bacteria (Kellogg *et al.* 2013), enabling augmented rates of denitrification in the sediment surrounding the reef. Restoration of salt marshes and oyster reefs may therefore be a powerful way to increase denitrification in estuarine systems.

Organisms are considered ecosystem engineers when they provide physical structure that influences surrounding biology (Jones *et al.* 1994). Oysters are a prominent example of an ecosystem engineer in coastal North Carolina, as they facilitate physical-biological coupling (Lenihan 1999). Their key role in the aquatic environment has led to oysters' inclusion in living shoreline designs. Living shorelines are a type of nature-based infrastructure, a type of design that is an alternative to traditional gray infrastructure. Living shorelines are intended to minimize coastal erosion. They consist of marsh vegetation with an optional fringing hard substrate whose composition ranges from built materials, such as rocks or cement, to natural hard structures like oyster reefs (NOAA Living Shorelines Workgroup 2015). Living shorelines can reestablish some ecosystem functions lost through habitat degradation. For processes such as nutrient removal, these functions are considered ecosystem services that increase the value of the living shoreline complex beyond its role in shoreline stabilization (Grabowski *et al.* 2012).

Habitat restoration is an expensive and time-intensive effort. As such, it is desirable for restoration practitioners to predict, to the extent possible, the development of desired ecosystem services following restoration. Restoration trajectories are a theoretical framework that visualizes changes in an environmental parameter over time (Kentula *et al.* 1992). Trajectories are constructed by graphing the relative magnitude of a parameter against time since restoration. Using a restoration trajectory, managers can compare field measurements from a known

timepoint to expected values. The comparison can suggest whether the site is developing as expected or whether additional intervention may be advisable. The general shape of a restoration trajectory can also help managers anticipate benchmarks and craft monitoring plans. Monitoring could be timed to measure a parameter during a period of expected change, for example (see La Peyre *et al.* 2014 for an example of an oyster reef restoration trajectory). Although restoration trajectories can be useful for planning purposes, there is speculation regarding their efficacy (Zedler & Callaway 1999). Habitats are influenced by a range of biotic and abiotic factors that could complicate efforts to identify a common restoration trajectory for a given parameter. Although restoration trajectories can help guide planning, they should be employed pragmatically, with an awareness of potential limitations.

The goal of this study was to identify changes in nitrogen cycling, particularly denitrification and N<sub>2</sub>O production, among estuarine habitats restored in the living shoreline framework at known points in the past. It was hypothesized that denitrification rates would increase following restoration and eventually reach a plateau. It was further expected that salt marshes would exhibit higher denitrification rates than the oyster reefs and sandflats. The greater elevation range in salt marshes was expected to facilitate the oxic/anoxic conditions necessary for denitrification. This study also measured relevant biotic and abiotic parameters to examine their influence on the development of denitrification rates. Overall, this study aimed to elucidate the impact of living shoreline restoration on N removal and identify whether other site parameters could be proxies for the development of N cycling.

#### **METHODS**

#### Study Sites

This study employed a chronosequence space-for-time replacement design. Chronosequence studies have been widely used to illustrate the development of restored sites over long periods of time. Restoration projects have been criticized for lack of monitoring, which is often time- and resource-intensive (Hobbs & Norton 1996). Well-designed chronosequence studies can provide the benefits of long-term monitoring without the expense. To be suitable for inclusion in a chronosequence, study sites should have comparable geographic proximity, habitat types, and environmental conditions. It has also been advised that chronosequences include temporal ranges of up to 100 years and focus on environments with lower biodiversity and few disturbances (Walker *et al.* 2010). When using a chronosequence design, it is important to be conservative in attributing change in parameters to the passage of time due to the potential influence of site-specific variables.

Sampling was conducted in Bogue Sound, located near Cape Lookout in the southern Outer Banks, North Carolina (Fig. 1). Study sites were salt marshes dominated by *Spartina alterniflora* with fringing oyster reefs (*Crassostrea virginica*). All sites were restored at known timepoints for ecosystem restoration or mitigation purposes using a living shoreline restoration design (Sutton-Grier *et al.* 2015) (Table 1). Sites spanned an age range from 0 to 20 years.



**Figure 1.** Location of the sampling sites included in this study. Sites were located within a 13 km radius in Bogue Sound near Morehead City, NC. Years reference the amount of time since restoration.

**Table 1.** Details of living shoreline restoration sites chosen for this study. All sites were restored for the overall goal of limiting coastal erosion, with additional restoration impetuses noted below.

Site	Abbreviation	Restored age (years)	<b>Restoration Impetus</b>
Institute of Marine	IMS	0	Ecosystem restoration
Sciences			
Carrot Island	Carrot	2	Ecosystem restoration
NOAA Beach at	NOAA	7	Ecosystem restoration
NOAA Beaufort			
Lab			
Army Marsh	Army	20	Mitigation

The four sites chosen for this study are located within a 13 km radius and are exposed to similar environmental conditions, such as rainfall and temperature. Wave energy varies between sites. Carrot (2-year-old site) is exposed to direct wave energy, and IMS (0-year-old site) is subject to boat wakes from traffic on the Atlantic Intracoastal Waterway. NOAA (7-year-old site) is in a more protected no-wake zone. Army (20-year-old site) has a bowl-shaped morphology that reduces wave energy and may influence tidal exchange. This collection of sites is similar to those chosen for other chronosequence studies (Salmo *et al.* 2013), and are located within a tighter radius than other published studies (e.g. Ballantine & Schneider 2009).

Sampling was conducted during each season from summer 2014 through spring 2015 along transects of five elevations at each site: seaward and landward sides of the oyster reef and three elevations in the salt marsh (Fig. 2). Fieldwork was conducted at approximate low tide to maximize access to lower elevations. Adjacent tidal sandflats within 15 m were sampled at an elevation matching the oyster reef/marsh border to evaluate the impact of restoration on surrounding sediment.



**Figure 2.** Diagram of the sampling scheme. Oyster habitat cores were collected from the landward and seaward sides of the oyster reef; marsh cores were collected from low, mid, and high marsh elevations; and sandflat cores were collected in adjacent sandflats. Triplicate cores were collected at each sampling point. The same sampling scheme was used for each site. This photo illustrates the NOAA location.

## Sediment Core Collection

Triplicate sediment cores were collected by hand using plastic polycarbonate tubes (6.4 cm diameter x 30 cm). Cores were inserted into the sediment to 17 cm, topped with site water, and capped with rubber stoppers. Care was taken to exclude marsh grass and megafauna such as oysters, snails, and crabs from the cores. Oyster habitat cores were collected from sediment

adjacent to the oyster reef. Site water was also collected for core incubation. All cores were immediately stored in a cooler and transported to an environmental chamber at UNC Institute of Marine Sciences (IMS). Cores were incubated in site water in the dark at average *in situ* temperature. Following an overnight equilibration, cores were capped and connected to a flow-through system with a pump rate of 1 mL min<sup>-1</sup> (see Piehler & Smyth 2011).

#### N<sub>2</sub> and O<sub>2</sub> Measurements

Water samples (5 mL) were collected from the outflow of sediment cores and from an inflow line for all cores following initial incubation of at least 16 hours to achieve steady state. Samples were also collected from an inflow line to assess background concentrations of dissolved gas and to isolate any influence of the plastic tubing. Sampling was repeated several times at 5 hour intervals following approximate turnover of overlying water to assess the duration of steady state.

Dissolved N<sub>2</sub> and O<sub>2</sub> were measured using a Balzers Prima QME 200 quadropole mass spectrometer (MIMS; Pfeiffer Vacuum, Nashua, NH, USA; Kana *et al.* 1994). The MIMS measures dissolved N<sub>2</sub> and O<sub>2</sub> concentrations in relation to inert Ar gas, making it sensitive to small changes in concentration. It is therefore able to measure net N<sub>2</sub> flux very precisely. Without the use of radioactive tracers, the MIMS cannot distinguish between N<sub>2</sub> produced through denitrification or anammox or lost through nitrogen fixation, which has been shown to occur in restored salt marshes (Piehler *et al.* 1998). However, previous research has indicated that anammox is negligible in habitats similar to those studied here (Koop-Jakobsen & Giblin 2009), indicating that positive net N<sub>2</sub> flux can be interpreted as denitrification.

Use of the MIMS requires that cores be maintained in a dark environment to prevent the formation of oxygen through benthic microalgae photosynthesis. Since water constantly overlies

the sediment, incubation mimics high tide conditions. Cores experience a gradual draw-down of oxygen over the course of the incubation that was quantified as sediment oxygen demand (SOD). Use of the MIMS avoids limitations associated with older methods of measuring denitrification, such as acetylene block, which blocks nitrification and therefore is not applicable when measuring coupled NF-DNF (Groffman *et al.* 2006).

All fluxes ( $\mu$ mol m<sup>-2</sup> h<sup>-1</sup>) were calculated per the following equation:

#### [Equation 1.]

$$flux = ([x]_{outflow} - average [x]_{inflow}) * \frac{pump \ rate}{core \ area}$$

where  $[x]_{outflow}$  is concentration in the sediment core outflow tube ( $\mu$ M), average  $[x]_{inflow}$  is average concentration in the inflow tubes ( $\mu$ M), pump rate is the incubation flow-through rate (L h<sup>-1</sup>), and core area is the surface area of the sediment sample in the core (m<sup>2</sup>).

Positive and negative dissolved gas fluxes were interpreted as flux out of and into the sediment, respectively. Denitrification was calculated as net positive N<sub>2</sub> gas flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>). Sediment oxygen demand (SOD) was calculated as the flux of O<sub>2</sub> into the sediment ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>).

Denitrification efficiency (DNE) was calculated per the following equation (Eyre & Ferguson 2002):

#### [Equation 2.]

$$DNE (\%) = \frac{N_2 flux}{N_2 flux + DIN flux} * 100$$

where DIN flux included NH<sub>4</sub> and NO<sub>x</sub> ( $\mu$ mol m<sup>-2</sup> h<sup>-1</sup>).

#### N<sub>2</sub>O Concentration and Flux Measurements

Water samples (100 mL) were collected in vented N<sub>2</sub>-sparged glass serum bottles (260 mL) to prevent inclusion of ambient N<sub>2</sub>O. Vials were shaken vigorously to equilibrate gases, and headspace gas was transferred to an evacuated glass vial (13 mL). A syringe was used to transfer a 5 mL air sample to a Shimadzu GC-2014 (Shimadzu Corporation, Kyoto, Japan) for detection of headspace N<sub>2</sub>O gas.

 $N_2O$  concentrations were calculated based on the assumptions of Henry's Law.  $N_2O$  concentration ( $\mu$ M) in each water sample was calculated using the Bunsen solubility coefficient ( $\beta$ ), which was in turn calculated from the Henry's Law solubility constant ( $K_o$ ). Equations from Weiss & Price 1980 were used to calculate  $K_o$  based on published constants and *in situ* temperature and salinity. Concentration was converted to flux ( $\mu$ mol  $N_2O$  m<sup>-2</sup> h<sup>-1</sup>) per Eqn. 1.

## Nutrient Concentration and Flux Measurements

Water samples (40 mL) were collected from inflow and outflow tubing following achievement of steady state. Samples were processed on a Lachat Quick-Chem 8000 (Lachat Instruments, Milwaukee, WI, USA) to measure concentrations of NO<sub>x</sub>, NH<sub>4</sub>, PO<sub>4</sub><sup>3</sup>-, total nitrogen (TN), and organic nitrogen (ON) ( $\mu$ M). Detection limits were 0.05  $\mu$ M N-NO<sub>x</sub>, 0.24  $\mu$ M N- NH<sub>4</sub>, 0.02  $\mu$ M P-PO<sub>4</sub><sup>3-</sup>, and 0.75  $\mu$ M N-TN. Nutrient flux was calculated per Eqn. 1.

#### Sediment Characteristics

Surface sediment (0-3 cm) was collected from each core at the end of the incubation and analyzed for sediment organic matter (SOM) via loss on ignition (LOI) (Byers *et al.* 1978). Known volumes of surface sediment from spring 2015 samples were baked and combusted to determine bulk density (g cm<sup>-3</sup>). Sediment samples from spring 2015 were pulverized and analyzed with a Costech ECS 4010 CHNS-O Elemental Analyzer (Costech Analytical

Technologies Inc., Valencia, CA, USA) to determine bulk percent nitrogen, percent carbon, and C:N ratios. Bulk density and CHN data were assumed to represent sediment conditions during the study period.

#### **Oyster Filtration and Marsh Grass Density**

Oyster density was measured in summer 2015 to describe conditions during the study period. A total of four  $1/16 \text{ m}^2$  quadrats were randomly tossed onto the oyster reef. Two quadrats were located on the reef crest and two were located on the landward side of the reef. Live oysters were excavated and transported to IMS for processing. The number of mature oysters and their shell heights (SH) were recorded. Spat < 25 mm SH were excluded (zu Ermgassen *et al.* 2013).

Oyster filtration provides a more comprehensive representation of oyster populations than count data. Oyster filtration was calculated for each season per the following equation (zu Ermgassen *et al.* 2013):

#### [Equation 3.]

filtration rate 
$$(L m^{-2} h^{-1}) = N(8.02W^{0.58} e^{[-0.015*((T-27)^2)]})$$

where N is oyster density (number of mature oyster m<sup>-2</sup>), W is dry tissue weight (g), and T is temperature (° C). Dry tissue weight was calculated from SH using an equation developed for South Carolina (Grizzle *et al.*2008; zu Ermgassen *et al.* 2016). SH to biomass conversion varies regionally, making it most appropriate to use an equation developed for the nearest geographical location. The process used to calculate oyster filtration was recommended for assessing restored oyster populations by the Nature Conservancy (zu Ermgassen *et al.* 2016).

*S. alterniflora* density was measured in fall 2015 prior to senescence to describe conditions during the previous growing season. Three marsh elevation zones (low, mid, and

high) were sampled at each site. Three  $\frac{1}{4}$  m<sup>2</sup> quadrats were distributed horizontally in each zone in accordance with the site's sediment core sampling scheme. Quadrats were tossed in a manner favoring vegetated areas within each elevation. The number of live *S. alterniflora* culms in each quadrat were recorded. *S. alterniflora* densities were adjusted by estimated percent cover to calculate overall density for each elevation zone.

#### Inundation Calculation

HOBO water level loggers (Model: U20-001-01, Onset Corporation, Bourne, MA, USA) were deployed at approximately the same elevation as the seaward oyster cores at each site. Water level data were logged at 15-minute intervals for at least one month. Data were corrected using barometric pressure recorded at a NOAA monitoring station in Beaufort, NC, and incorporating a brackish salinity correction factor built into the HOBOware software (Onset Corporation, Bourne, MA, USA). HOBO data were standardized to local tide records measured at the NOAA monitoring station, as described below. The relationship was used to hindcast tide patterns at each study site during sampling seasons. NOAA records were hindcast without standardization at Army, where HOBO data were not successfully recorded. Elevation data and field records indicated that conditions were similar enough at Army and the NOAA monitoring station for direct comparison.

Seasonal percent inundation was calculated using inverse cumulative percent histograms that modeled the hindcasted water level at the elevation of each sampling zone. Elevations were obtained using an automatic laser level (Model SAL24N, CST/Berger, Watseka, IL, USA).

There is some error inherent in hindcasting tidal predictions. The HOBO data first underwent a phase shift to account for the slight temporal difference in tidal extremes between the study sites and the NOAA tide gauge. Units of one hour were used for the phase shift, which

could have resulted in some loss of precision. Linear equations were then fit to the HOBO and NOAA records, and the difference in y-intercepts was added to or subtracted from the HOBO data. The pair of linear equations had extremely similar slopes at all sites, indicating that the HOBO logger and NOAA gauge were measuring the same tidal patterns. In some instances, the heights of maximum low and high tide following the vertical offset were slightly different between the HOBO and NOAA data, introducing a small source of error. When the corrected HOBO data were regressed against the NOAA data,  $R^2 \ge 0.92$  at all sites. This indicates strong correlation between the two data sets and confirms the appropriateness of the correction process. However, since  $R^2 \neq 1$ , there was some error introduced by the correction. HOBO data were not available for Army, so it is possible that there was error created by using the NOAA gauge data as a proxy for that site.

#### Statistical Analysis

N<sub>2</sub> flux data were analyzed for normality and heteroscedasticity. A constant was added to convert all N<sub>2</sub> flux data to positive values. Data were transformed using the Box-Cox transformation with a lambda value that maximized a log-likelihood function (Box 1964). Transformation achieved heteroscedasticity and improved normality. Several factors remained non-normal following transformation. ANOVA testing can tolerate non-normality (Underwood 1997). Non-parametric methods were also used to analyze the data, as described below.

A three-way ANOVA was used to identify significant differences ( $\alpha = 0.05$ ) in denitrification rates, with site, season, and habitat as interactive fixed factors. The effect of habitat was not significant (p = 0.17), so a two-way ANOVA was run with site and season as interactive fixed factors. A Tukey post-hoc test was also conducted. Regression was used to assess the relationship between restored age and site parameters. Regression was modeled using second-order polynomial equations. Separate regression analyses were conducted for annual and seasonal groupings of parameters (SOD, SOM, bulk density, and N<sub>2</sub> flux). Annual regression was conducted using data collected during the entire year; data were not averaged. Regressions for specific seasons used data collected during that season. Correlations were conducted to identify collinearity among site parameters and between LOI and CHN data. Regression and correlation results are reported with the p-value and the Pearson correlation coefficient.

Regression trees were used to explore the relative impact of site parameters (SOD, O<sub>2</sub> concentration, SOM, percent inundation, NH<sub>4</sub> flux, and NO<sub>x</sub> flux) on N<sub>2</sub> fluxes. A second regression tree was constructed with the addition of site age, season, and habitat. Separate regression trees were also constructed for each season to eliminate the potentially confounding influence of temperature, which is a known driver of denitrification (Seitzinger 1988, Bachand & Horne 2000). Each seasonal regression tree included all parameters except habitat. Data were not transformed because regression trees do not rely on assumptions regarding data distribution or homoscedasticity. Regression trees were constructed using the ANOVA version of recursive partitioning, and pruned with a complexity parameter corresponding to the smallest tree with a cross-validation error within one standard deviation of the minimum (De'Ath & Fabricius 2000). If this method did not sufficiently prune the tree, the next smallest complexity parameter was applied.

All analyses were conducted using R Version 3.3.1 (R Core Team 2016). Regression trees were constructed using the rpart package (Therneau *et al.* 2015).

#### RESULTS

#### **Overall Trends**

N<sub>2</sub> flux rates (µmol N m<sup>-2</sup> h<sup>-1</sup>) were generally positive, indicating net denitrification (Fig. 3). Subsequent discussion of statistical differences in N<sub>2</sub> flux refers to Box-Cox transformed values. Rates were not significantly different across habitats (p = 0.18). Site and season each had a significant impact on transformed denitrification rates ( $p = 2 \times 10^{-16} \& 1.9 \times 10^{-13}$ , respectively). The interaction of site and season was also significant ( $p = 9.8 \times 10^{-13}$ ), indicating that transformed denitrification rates did not respond to seasonal variation in the same way across sites. Post-hoc testing indicated that all seasons were significantly different from one another except for winter and fall (p = 0.99), and that NOAA was the only site that was significantly different from all other sites (p = 0.00). Denitrification was highest in the summer for most sites and habitats, and was consistently high at NOAA compared to other sites.



**Figure 3.** Seasonal average net  $N_2$  flux (µmol N m<sup>-2</sup> h<sup>-1</sup>) divided by site and grouped by habitat. Fluxes were generally positive, indicating net denitrification. Denitrification was generally highest during the summer and at the 7-year-old site. Error bars represent standard error.

Some seasonal grouping was apparent when N<sub>2</sub> flux was presented as a function of O<sub>2</sub> concentrations (mg L<sup>-1</sup>) (Fig. 4). O<sub>2</sub> concentrations were highest in the winter (> 7.5 mg L<sup>-1</sup>) and lowest in the summer (< 5.0 mg L<sup>-1</sup>). The highest N<sub>2</sub> fluxes were associated with lower O<sub>2</sub> concentrations.



**Figure 4.** N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>) as a function of average O<sub>2</sub> concentrations (mg L<sup>-1</sup>) demonstrates some seasonal grouping, particularly for samples collected in the summer and winter.

Annual N<sub>2</sub> flux was positively related to SOD ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>) (Fig. 5). The highest N<sub>2</sub>

flux and SOD were observed in the summer and spring; the lowest were observed in the winter.



**Figure 5.** Annual N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>) is positively related to sediment oxygen demand (SOD) ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>). General seasonal trends indicated higher N<sub>2</sub> flux and SOD in the summer and spring, and lower values in the winter.

Denitrification efficiency was always greater than 50%, indicating that N<sub>2</sub> flux was greater than DIN flux and that net N removal was occurring (Fig. 6). DNE was typically greater than 75%. DNE not follow a discernable seasonal pattern, although the lowest rates were generally recorded in the winter (data not shown). DNE remained relatively stable across restored age, and was not notably different between habitats. Statistical testing was not conducted on denitrification rates because the term is a proportion.



**Figure 6.** Average annual denitrification efficiency (DNE) for each site, grouped by habitat. DNE was always greater than 50%, indicating net N removal. DNE was generally stable across restored site age and did not exhibit strong differences between habitats. Error bars represent standard error.

DNE generally increased with SOD, especially between 0-1000  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> (Fig. 7). DNE plateaued above 1000  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. NH<sub>4</sub> flux was not positively correlated with SOD or SOM (data not shown).



**Figure 7.** DNE generally increased with increasing sediment oxygen demand (SOD) ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), especially between 0 and 1000  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. There was not a clear difference in the relationship among habitats.

Positive N<sub>2</sub>O fluxes ( $\mu$ mol N<sub>2</sub>O m<sup>-2</sup> h<sup>-1</sup>) were less than 0.2  $\mu$ mol m<sup>-2</sup> h<sup>-1</sup> (Fig. 8). Fluxes were often negative, particularly at the youngest site. The most extreme positive fluxes were recorded in the summer and winter, whereas the most extreme negative fluxes were recorded in the summer and fall.



**Figure 8.** Seasonal N<sub>2</sub>O flux ( $\mu$ mol N<sub>2</sub>O m<sup>-2</sup> h<sup>-1</sup>) divided by season. Within each season, fluxes are reported for each site, identified by its restored age, and divided by habitat. Fluxes were generally low and did not follow a clear pattern. Error bars represent standard error.

Average annual N<sub>2</sub>O fluxes were <  $0.5 \ \mu$ mol N<sub>2</sub>O m<sup>-2</sup> h<sup>-1</sup>. There were two positive average fluxes: the oyster habitat at the 2-year-old site and the marsh habitat at the 20-year-old site (Fig. 9). All other fluxes were negative or had a range of error that included 0. The 0-yearold site exhibited the largest negative fluxes. Annual averages did not obscure any trends apparent in the seasonal data.


**Figure 9.** Annual average N<sub>2</sub>O flux ( $\mu$ mol N<sub>2</sub>O m<sup>-2</sup> h<sup>-1</sup>) by restored age, divided by habitat. Annual fluxes were less than 0.5  $\mu$ mol N<sub>2</sub>O m<sup>-2</sup> h<sup>-1</sup>. The 0- and 7-year-old sites exhibited negative fluxes across all habitats. Error bars represent standard error.

When data from the entire year are included, N<sub>2</sub>O flux was significantly correlated with N<sub>2</sub> flux at the two oldest sites (p = 0.009 & 0.0027,  $R^2 = 0.27 \& 0.34$ , respectively; Fig. 10). The relationship was positive at the 7-year-old site and negative at the 20-year-old site.



**Figure 10.** Relationships between N<sub>2</sub>O flux ( $\mu$ mol N<sub>2</sub>O m<sup>-2</sup> h<sup>-1</sup>) and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>) for each site. Data from the entire year are included. N<sub>2</sub> flux demonstrated a positive relationship with N<sub>2</sub>O at the 7-year-old site and a negative relationship at the 20-year-old site. P-values and the Pearson correlation coefficient (R<sup>2</sup>) are reported for each site.

# Regression

Regression of all data collected during the study year indicated that  $N_2$  flux and SOD were significantly associated with age, although the relationships did not explain a large amount of the variability (p < 0.01; Fig. 11, Table 2). Both parameters increased to the 7-year-old site, then decreased to the 20-year-old site. SOM and bulk density were also significantly associated with age (p < 0.01; Fig. 11, Table 2). Their regression curves were mirror images: SOM increased to the 7-year-old site then plateaued, whereas bulk density decreased to the 7-year-old site and remained constant to the 20-year-old site. This is not surprising, as SOM and bulk density are usually inversely related.



**Figure 11**. Regression for sediment oxygen demand (SOD) ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), sediment organic matter (SOM) (%), bulk density (g cm<sup>-3</sup>), and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>) as a function of restored age (years). All data collected during the study year are included. Regressions are fitted with a second-order polynomial equation. Pearson's correlation coefficient (R<sup>2</sup>) and p-values are reported in Table 2.

When separate equations were fitted for each habitat, all habitats exhibited a significant relationship between N<sub>2</sub> flux and age (p < 0.01 for oysters and marsh, p < 0.05 for sandflat; Fig. 12, Table 2). N<sub>2</sub> flux in all habitats increased to the 7-year-old site, then gradually decreased to the 20-year-old site. All habitats also exhibited significant relationships between SOM and age (p < 0.01). Oyster and marsh SOM values increased to the 7-year-old site, then plateaued. In comparison, SOM values in the sandflat remained low through the 7-year-old site, but increased to roughly the same SOM value as the oyster and marsh habitats at the 20-year-old site. Each

habitat exhibited the opposite trend for bulk density, although none of the relationships was significant.



**Figure 12.** Regression for sediment oxygen demand (SOD) ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), sediment organic matter (SOM) (%), bulk density (g cm<sup>-3</sup>), and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>) as a function of restored age (years). All data collected during the study year are included. Data from each habitat were fitted with a second-order polynomial equation. Pearson's correlation coefficient (R<sup>2</sup>) and p-values are reported in Table 2.

Seasonal regressions were also conducted.  $N_2$  flux was significantly correlated with age every season except fall (Table 2), although the shape of the regression line was not consistent across seasons (data not shown). The regression line for  $N_2$  flux remained high to the 20-yearold site in the summer (Fig. 13), but decreased from the 7- to the 20-year-old site every other season (data not shown). SOM was the only parameter significantly correlated with age every season (Table 2).



**Figure 13.** Regression for sediment oxygen demand (SOD) ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), sediment organic matter (SOM) (%), and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>) as a function of restored age (years) for data collected during summer 2014. Data were fitted with a second-order polynomial equation. Pearson's correlation coefficient (R<sup>2</sup>) and the p-value are reported in Table 2.

**Table 2.** Results of regressions conducted using annual or seasonal data for various site parameters regressed against restored age. Data were fitted with a second-order polynomial equation. Only significant relationships (p < 0.05) are included in the table. Pearson's correlation coefficients ( $R^2$ ) and p-values are reported.

Season	Parameter	Pearson's	p-value	
		correlation		
		coefficient (R <sup>2</sup> )		
Annual	SOD	0.13	< 0.01	
Annual	SOM	Total: 0.39	9 All: < 0.01	
		Oyster: 0.33		
		Marsh: 0.59		
		Sandflat: 0.72		
Annual	Bulk density	Total: 0.44	< 0.01	
Annual	N <sub>2</sub> flux	Total: 0.3	Total: < 0.01	
		Oyster: 0.27	Oyster: < 0.01	
		Marsh: 0.3	Marsh: < 0.01	
		Sandflat: 0.38	Sandflat: < 0.05	
Summer 2014	SOM	0.69	< 0.01	
Summer 2014	N <sub>2</sub> flux	0.54	< 0.01	
Fall 2014	SOM	0.54	< 0.01	
Fall 2014	SOD	0.45	< 0.01	
Winter 2015	N <sub>2</sub> flux	0.52	< 0.01	
Winter 2015	SOM	0.33	< 0.05	
Winter 2015	SOD	0.54	< 0.01	
Spring 2015	N <sub>2</sub> flux	0.63	< 0.01	
Spring 2015	SOM	0.38	< 0.05	
Spring 2015	SOD	0.42	< 0.01	

# **Correlations**

Correlation analyses were conducted on all data collected during the study year and on data separated by seasons. Significant correlations with  $R^2$  values > 0.20 are reported in Table 3. Based on correlations of all annual data, N<sub>2</sub> flux was significantly correlated with SOD and O<sub>2</sub> concentrations (p < 0.01; Fig. 14, Table 3). There was a stronger correlation with SOD than with O<sub>2</sub> concentrations. SOD was significantly correlated with O<sub>2</sub> concentrations (p < 0.01).



**Figure 14.** Correlation matrix for all annual measurements of O<sub>2</sub> concentrations (mg O<sub>2</sub> L<sup>-1</sup>), sediment oxygen demand (SOD) ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), sediment organic matter (SOM) (%), percent inundation, NO<sub>x</sub> flux ( $\mu$ mol NO<sub>x</sub> m<sup>-2</sup> h<sup>-1</sup>), NH<sub>4</sub> flux ( $\mu$ mol NH<sub>4</sub> m<sup>-2</sup> h<sup>-1</sup>), and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>). Column labels describe the x axes and row labels describe the y axes. Pearson's correlation coefficients (R<sup>2</sup>) and p-values are reported for each correlation.

Correlations were also conducted for data collected during each season. N<sub>2</sub> flux was significantly correlated with SOM only during the summer (p < 0.01; Fig. 15, Table 3). N<sub>2</sub> flux was significantly correlated with SOD every season, and with O<sub>2</sub> concentrations in the fall, winter, and spring (data not shown). Percent inundation did not display a clear pattern of correlation. It was significantly correlated with O<sub>2</sub> concentrations in the spring and winter, with N<sub>2</sub> flux in the fall, and with SOD in the spring (data not shown).



**Figure 15.** Correlation matrix for data collected during summer 2014. Parameters include  $O_2$  concentrations (mg  $O_2 L^{-1}$ ), sediment oxygen demand (SOD) (µmol  $O_2 m^{-2} h^{-1}$ ), sediment organic matter (SOM) (%), percent inundation, NO<sub>x</sub> flux (µmol NO<sub>x</sub> m<sup>-2</sup> h<sup>-1</sup>), NH<sub>4</sub> flux (µmol NH<sub>4</sub> m<sup>-2</sup> h<sup>-1</sup>), and N<sub>2</sub> flux (µmol N m<sup>-2</sup> h<sup>-1</sup>). Column labels describe the x axes and row labels describe the y axes. Pearson's correlation coefficients (R<sup>2</sup>) and p-values are reported for each correlation.

Time	Parameter 1	Parameter 2	Pearson's correlation	p-value
Frame			coefficient (R <sup>2</sup> )	
Annual	O <sub>2</sub> concentration	SOD	0.67	< 0.01
Annual	O <sub>2</sub> concentration	N <sub>2</sub> flux	0.30	< 0.01
Annual	SOD	N <sub>2</sub> flux	0.54	< 0.01
Summer	O <sub>2</sub> concentration	SOD	0.92	< 0.01
Summer	O <sub>2</sub> concentration	SOM	0.31	< 0.01
Summer	SOD	SOM	0.37	< 0.01
Summer	SOD	N <sub>2</sub> flux	0.48	< 0.01
Summer	SOM	N <sub>2</sub> flux	0.49	< 0.01
Fall	O <sub>2</sub> concentration	SOD	0.94	< 0.01
Fall	O <sub>2</sub> concentration	N <sub>2</sub> flux	0.81	< 0.01
Fall	O <sub>2</sub> concentration	% inundation	0.21	< 0.05
Fall	SOD	N <sub>2</sub> flux	0.79	< 0.01
Fall	% inundation	N <sub>2</sub> flux	0.31	< 0.01
Winter	O <sub>2</sub> concentration	SOD	0.82	< 0.01
Winter	O <sub>2</sub> concentration	SOM	0.21	< 0.05
Winter	O <sub>2</sub> concentration	% inundation	0.35	< 0.01
Winter	O <sub>2</sub> concentration	N <sub>2</sub> flux	0.59	< 0.01
Winter	SOD	SOM	0.26	< 0.05
Winter	SOD	N <sub>2</sub> flux	0.74	< 0.01
Spring	O <sub>2</sub> concentration	SOD	0.97	< 0.01
Spring	O <sub>2</sub> concentration	% inundation	0.31	< 0.01
Spring	O <sub>2</sub> concentration	N <sub>2</sub> flux	0.32	< 0.01
Spring	SOD	% inundation	0.29	< 0.01
Spring	SOD	N <sub>2</sub> flux	0.41	< 0.01

**Table 3.** Results of correlations conducted using annual or seasonal data for various site parameters. Only significant relationships (p < 0.05) with  $R^2$  values > 0.20 are included in the table. Pearson's correlation coefficients ( $R^2$ ) and p-values are reported.

A separate set of correlations was conducted on sediment parameters quantified by CHN (%C, %N, and C:N) and loss on ignition (LOI) (SOM (%)) (Fig. 16). This was to determine whether LOI results were supported by CHN analysis. Percent C was significantly correlated with percent N and SOM (p < 0.01).



**Figure 16.** Correlation matrix for spring CHN data and average annual SOM (%) data. Parameters included % C, % N, C:N, and SOM. Column labels describe the x axes and row labels describe the y axes. Pearson's correlation coefficients ( $\mathbb{R}^2$ ) and p-values are reported for each correlation.

### **Regression Trees**

A regression tree for denitrification rates was first constructed using all annual data for parameters measured every season: percent inundation, SOM, SOD, O<sub>2</sub> concentration, NH<sub>4</sub> flux, and NO<sub>x</sub> flux (Fig. 17). SOD explained the most variation for the first two levels of the tree, and SOM explained the most variation for the third level ( $R^2 = 0.67$ , full tree).

The first node split the data based on SOD. Samples with SOD < 530.7  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 17.11  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. The second bifurcation in this subgroup was also based on SOD. Cores with SOD < 232.1  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 9.481  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>, and cores with SOD > 232.1  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 24.05  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. Samples with SOD > 530.7  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 49.17  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. From that subgroup, samples with SOD > 1916  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 77.8  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. For samples with SOD between 530.7 and 1916  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>, denitrification rates were bifurcated by SOM. Samples with SOM < 94.2% had an average denitrification rate of 31.59  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>, whereas samples with SOM > 94.2% had an average denitrification rate of 49.88  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>.



**Figure 17.** Results of pruned regression tree for denitrification rates using all annual data for parameters measured every season: percent inundation, SOM (%), SOD ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), O<sub>2</sub> concentration (mg O<sub>2</sub> L<sup>-1</sup>), NH<sub>4</sub> flux ( $\mu$ mol NH<sub>4</sub> m<sup>-2</sup> h<sup>-1</sup>), and NO<sub>x</sub> flux ( $\mu$ mol NO<sub>x</sub> m<sup>-2</sup> h<sup>-1</sup>). SOD explained the most variation in denitrification rates for the first two levels of the tree, and SOM explained the most variation on the third level.

A second regression tree was constructed by adding season, habitat, and site age to the parameters included in the first tree (Fig. 18). SOD explained the most variation for the first level of the tree. SOD and age explained the most variation on the second level, and SOD explained the most variation on the third level ( $R^2 = 0.72$ , full tree).

The first bifurcation was identical to the previous regression tree. Among samples with SOD < 530.7  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>, those with SOD < 232.1  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 0.481  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. Samples with SOD > 232.1  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 24.05  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>.

Samples with SOD > 530.7  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> were subsequently split by age. Samples with age < 4.5 years had an average denitrification rate of 32.46  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>. Samples with age > 4.5 years had an average denitrification rate of 59.91  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>, and those samples were further bifurcated by SOD. Samples with SOD < 1393  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 45.43  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>, and those with SOD > 1393  $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup> had an average denitrification rate of 72.4  $\mu$ mol N<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>.



**Figure 18.** Result of pruned regression tree results when site, age, and habitat were added to parameters measured every season: percent inundation, SOM (%), SOD ( $\mu$ mol O<sub>2</sub> m<sup>-2</sup> h<sup>-1</sup>), O<sub>2</sub> concentration (mg O<sub>2</sub> L<sup>-1</sup>), NH<sub>4</sub> flux ( $\mu$ mol NH<sub>4</sub> m<sup>-2</sup> h<sup>-1</sup>), and NO<sub>x</sub> flux ( $\mu$ mol NO<sub>x</sub> m<sup>-2</sup> h<sup>-1</sup>). SOD explained the most variation on the first level, but SOD and age explained the most variation on the second level. SOD explained the most variation on the third level.

Separate regression trees were constructed for each season to eliminate the potentially confounding influence of temperature, which is a known driver of denitrification. Sediment cores from all sites were incubated at the same temperature during each season. Constructing separate seasonal trees therefore controls for temperature and potentially identifies variables whose influence on denitrification rates may have been obscured. None of the seasonal regression trees was notably different than the annual tree (data not shown). This indicates that controlling for season, and by proxy temperature, in the construction of the regression tree did not alter the results, suggesting that it was not problematic to include temperature. It is therefore sufficient to consider trees that include all annual data.

# **Physical Site Features**

Oyster filtration (L m<sup>-2</sup> h<sup>-1</sup>) was highest at the 0-year-old site, followed by the 7-year-old site (Fig. 19). The 20-year-old site exhibited the lowest filtration rates. Filtration rates at all sites were highest in the summer and negligible in the winter. There was no clear relationship between oyster filtration and N<sub>2</sub> flux (Fig. 20), nor with other site parameters such as SOM, NH<sub>4</sub> flux, or SOD (data not shown).



**Figure 19.** Seasonal oyster filtration rates (L m<sup>-2</sup> h<sup>-1</sup>) at each site. Rates were highest at the 0year-old site and lowest at the 20-year-old site. Filtration was highest in the summer and negligible in the winter. Filtration rates were calculated based on shell height and temperature using an equation from zu Ermgassen *et al.* 2016.



**Figure 20.** There was no apparent relationship between seasonal averages of oyster filtration rates (L m<sup>-2</sup> h<sup>-1</sup>) and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>). Data were collected in summer 2015 to describe conditions during the study period.

Adjusted *S. alterniflora* stem density (number of stems m<sup>-2</sup>) roughly corresponded to restored age (Fig. 21). There was no clear relationship between marsh grass density and N<sub>2</sub> flux (Fig. 22). Stem density was positively correlated with annual average SOM (p = 0.0502,  $R^2 = 0.9$ ; Fig. 23). Older sites expressed higher SOM and marsh grass density.



**Figure 21.** *S. alterniflora* stem density (number of stems m<sup>-2</sup>) for all study sites. Data were collected in fall 2015 to describe conditions during the 2014-2015 growing season.



**Figure 22.** There was no clear relationship between *S. alterniflora* stem density (number of stems m<sup>-2</sup>) and N<sub>2</sub> flux ( $\mu$ mol N m<sup>-2</sup> h<sup>-1</sup>). Stem density were collected in fall 2015 to describe conditions during the 2014-2015 growing season.



**Figure 23.** There was a positive relationship between *S. alterniflora* stem density (number of stems m<sup>-2</sup>) and average SOM (% organic matter). Stem density data were collected in fall 2015 to describe conditions during the 2014-2015 growing season.

#### DISCUSSION

One goal of this research was to identify distinctions in N cycling among restored estuarine habitats. The three habitats sampled- oyster reefs, salt marshes, and sandflats- were expected to express different N cycling attributes, particularly denitrification rates, because of different sediment organic and redox conditions and possible inundation patterns (Sousa *et al.* 2012). However, denitrification rates in the three habitats were not statistically different from one another. All seasons except fall and winter had a significantly different effect on denitrification across sites, suggesting that season and therefore primarily temperature could not predict denitrification rates at these sites. It was surprising that denitrification was not significantly different during the summer, as previous studies have found rates to be higher in warmer temperatures (Nowicki *et al.* 1997, Barnes & Owens 1999, Cabrita & Brotas 2000, Kellogg *et al.* 2013, Kuschk *et al.* 2003).

A related goal of this study was to determine whether denitrification could be predicted by restored site age. Linear regression of annual averages indicated that denitrification rates increased from the 0- to 7-year-old sites, then decreased slightly to the 20-year-old site. This pattern was similar for all habitats, emphasizing the similarity between habitats on an annual basis. The annual regression indicates that there was a relationship between denitrification and site age, although changes in rates after the 7-year-old site were less predictable. The relationship was not linear, likely due to the influence of many other site parameters. Sediment characteristics and physical features of a restored site change over time, making it difficult to identify a relationship between a single process and time without accounting for simultaneous changes in other parameters. This study did attempt to capture variability in other parameters, as discussed below.

Trends in regression lines were not the same for each season, which complicates interpretation of the annual results. During the summer, denitrification rates increased from the 0- to 7-year-old site and remained high to the 20-year-old site. In subsequent seasons, however, denitrification rates decreased markedly from the 7- to the 20-year-old site. The seasonal regressions suggest that it would be misleading to identify a single relationship between denitrification and age. Other studies have also found it challenging to identify a consistent relationship between site age and denitrification. A chronosequence study of denitrification in forests converted to pastures in Costa Rica also found that denitrification did not correspond to site age, although other measurements of N cycling did (Veldkamp *et al.* 1999). Evaluation of genes related to denitrification along a glacial retreat chronosequence suggested that denitrifying communities develop at different rates (Kandeler *et al.* 2006). Analysis of genetic diversity was beyond the scope of this research, but it could have contributed to some differences observed between sites.

The concept of restoration trajectories is another way to consider change over time. This study asked whether it is possible to identify a restoration trajectory for denitrification in reefmarsh restoration projects. Although significant for annual and seasonal data, the relationship between denitrification and restored age was not consistent across seasons. The summer results do warrant special consideration, however. Denitrification is a particularly important ecosystem service in the summer, when it can limit eutrophication during high rates of biological productivity. It is therefore promising to observe sustained rates of denitrification in older restored sites during the summer. In areas prone to eutrophication during warmer months,

restoration efforts may be motivated by the prospecting of boosting denitrification during the summer. Other studies have identified seasonal peaks in denitrification: from summer to fall in Japanese estuaries (Senga *et al.* 2010), during the fall in North Carolina salt marshes (Thompson *et al.* 1995), and from winter to spring in intertidal environments in the Netherlands (Kieskamp *et al.* 1991).

Due to seasonal disparity, it is likely ill-advised to ascribe a single restoration trajectory to these systems. Other studies have failed to identify restoration trajectories associated with denitrification (Ahn & Peralta 2012), and some authors have questioned whether restoration trajectories are a useful construct (Zedler & Callaway 1999). Consequently, there is a general sense of caution against their application in management efforts. The differences between seasonal restoration trajectories reinforces the importance of seasonal sampling, and suggests that it may be more feasible to construct accurate restoration trajectories for individual seasons. It has been suggested that chronosequences may be best suited for studying soil development (Walker et al. 2010), which could explain why SOM was significantly correlated with age every season. La Peyre et al. (2009) and Osland et al. (2012) successfully identified restoration trajectories for the development of sediment characteristics in chronosequences of brackish marshes and mangroves, respectively. In their salt marsh chronosequence study, Craft et al. suggested that it might be easier to identify sediment characteristics in a chronosequence spanning fewer than 30 years, but that nutrient dynamics might require more time to develop (1988). These studies suggest that ours is not the first to have difficulty identifying a single restoration trajectory for a biogeochemical process.

The study design could have contributed to difficulty identifying a consistent trajectory, especially beyond 7 years. Since there was only one site older than 7 years, the later 2/3 of the

trajectory was driven by data from the 20-year-old site. Site-specific characteristics, rather than age, could have influenced the parameters measured at that site. The restoration trajectories were interpreted with the awareness that site features, rather than age, could have driven the observed trendline, but it is worth noting that the oldest site could have had a disproportionate influence as the endpoint of the trajectory.

Denitrification may not be reliably predicted by age because of the influence of other site features. As restored sites age, their physical characteristics and biogeochemical cycles also change, and these parameters in turn influence denitrification. For example, increasing SOM is typically a priority in wetland restoration, and SOM is commonly identified as a driver of denitrification (He *et al.* 2016). In this study, although SOM was not correlated with denitrification when all annual data were considered, regression analysis indicated that all habitats exhibited a significant increase in SOM over time. The increase in SOM in the sandflats was particularly striking. Although SOM in the sandflats remained low through the 7-year-old site, it increased in the 20-year-old site to approximately the same level observed in the adjacent oyster reef and salt marsh. This result suggests that the impacts of restoration could have spread beyond the two restored habitats and was affecting surrounding areas. Influences of restored habitats beyond the restoration itself has been termed "outwelling," and has been previously identified for mangroves (Lee 1995), tidal marshes (Odum 2000), and oyster reefs and seagrass (Sharma *et al.* 2016).

It is worth noting that SOM was a reliable predictor of sediment organic matter content in this study. Some studies have challenged that LOI does not adequately reflect organic matter pools (Leong & Tanner 1999), but our results agreed with other studies that LOI provided comparable results to CHN analysis (Byers *et al.* 1978, Kristensen & Andersen 1987, Craft *et al.* 

1991). SOM was highly correlated with %C and C:N, indicating that LOI accurately measured particulate carbon. SOM was therefore used in lieu of C:N data to describe sediment characteristics.

This study found that SOM was significantly correlated with marsh grass stem density. Greater structural complexity is frequently cited as a means of increasing SOM in restored habitats. As *S. alterniflora* develop more complex root systems over time, they can more effectively trap and retain SOM. Similar mechanisms are proposed for oyster reefs (see Carlsson *et al.* 2012), although this study found no correlation between oyster filtration and SOM. Both oyster reefs and salt marshes also directly contribute organic matter to the sediment. Oysters produce biodeposits during filtration, and the annual senescence of marsh grass adds decaying plant matter. Marsh grass stem density was roughly associated with age. Other studies in restored salt marshes have also found a link between restored age, stem density, and SOM (Craft *et al.* 2003). If augmenting SOM is a key goal, it may be useful to increase initial plant density and/or replant following initial restoration.

Since coupled NF-DNF relies on oxic-anoxic microsites, it is expected that inundation would be an important driver of denitrification in systems with low ambient  $NO_x$ . Inundation frequency is expected to interact with sediment particle characteristics, such as bulk density, to alter oxygen concentrations in sediment porewater. Some studies and technical reports even recommend incorporating microtopography in restored wetlands to enhance oxygen gradients and therefore boost denitrification (Wolf *et al.* 2011, Wisconsin Natural Resources Conservation Service 2002). Conceptual models have been developed for inundation time and denitrification in estuarine sediments, and were designed to predict the timing and duration of sediment redox conditions favorable for denitrification (Ensign *et al.* 2008).

This study's sampling design measured denitrification across a range of elevations. Denitrification was not significantly different among habitats, implying that it was not significantly different among the different elevations encompassed by those habitats. This was particularly surprising given that coupled NF-DNF is presumed to constitute the majority of denitrification at these sites. Ambient NO<sub>3</sub><sup>-</sup> concentrations were extremely low, indicating that the NO<sub>3</sub><sup>-</sup> source must have been nitrification. Percent inundation was also not significantly correlated with denitrification on an annual basis. There was a significant correlation in the fall, which may have been driven by larger tidal excursions during that season. This research suggests that maximum denitrification rates may not be correlated with differences in inundation associated with typical tidal patterns.

Some considerations of the study sites and design may be relevant to interpreting the inundation results. Incubation conditions mimicked high tide, which could have obscured the impact of inundation. Core incubation integrates sediment processes, but it is possible that the impact of inundation is only perceptible when diurnal fluctuations are actively occurring. Additionally, wave energy was not equivalent at each site. The 2-year-old site experienced direct wave energy, whereas the 20-year-old site was very sheltered. It is possible that wave energy interrupted predictable patterns between inundation and denitrification. Additionally, inundation patterns should not be interpreted as indications of the importance of topographic variation. Topographic heterogeneity has been identified as an important factor in meeting restoration goals (see review by Larkin *et al.* 2006). If N removal is a stated restoration goal, however, this research indicates that there is not a strong relationship between inundation patterns and denitrification. It is possible that inundation differences are more relevant for N removal in non-tidal or freshwater tidal wetlands, where other studies have found significant

correlations between denitrification and microtopography (Moser *et al.* 2007, Courtwright *et al.* 2011, Duncan *et al.* 2013).

This study measured many site parameters with a high degree of temporal resolution. Regression trees were used to combine all data and identify factors that were best correlated with denitrification on an annual basis. SOD explained the most variation in denitrification, which agreed with other denitrification research in similar environments (Piehler & Smyth 2011). SOD reflects the cumulative influence of all microbial processes. Its position in the regression tree suggests that denitrification rates are best explained by evaluating oxygen-utilizing processes at a site, rather than considering a single descriptive factor such as percent inundation or age. When site age, season, and habitat were added to the regression tree, age explained some variation in denitrification rates. This further suggests that denitrification rates at the older sites were distinct, especially beyond 4.5 years. However, because age covaried with other site parameters, it is difficult to unequivocally equate age to time since restoration in the context of a regression tree.

It is useful to consider the relationship between SOD and SOM. SOM was significantly correlated with denitrification only in the summer, which suggests that although denitrification is typically correlated with process-based parameters, it may be limited by physical parameters, specifically SOM, when overall microbial activity is high. During the summer, microbial activity is elevated but depends on SOM as a carbon source. SOD therefore may be limited by availability of SOM, which in turn could restrict denitrification (Eyre *et al.* 2013). Other studies have also made the link between SOM and microbial activity, even without directly measuring the latter (Groffman & Tiedje 1989). This observation reinforces the importance of seasonal sampling to capture the nuances of biogeochemical processes.

Although denitrification is a valuable ecosystem service, there are concerns that its benefits could be tempered by production of N<sub>2</sub>O. Since N<sub>2</sub>O is a powerful greenhouse gas, some researchers have raised concerns that high N<sub>2</sub>O production associated with incomplete denitrification could negate the benefits of increase N removal (Burgin *et al.* 2013). This study identified only two instances of positive N<sub>2</sub>O flux when fluxes were averaged and represented annually by site and habitat. There was no indication of a predictable pattern in N<sub>2</sub>O fluxes based on site age, season, or habitat. Increased denitrification was not found to increase N<sub>2</sub>O flux. Our findings suggest that reef-marsh restoration is not associated with an increase in N<sub>2</sub>O production within 20 years of restoration, and that N<sub>2</sub>O production is not significantly correlated with denitrification rates in these systems.

Both the positive and negative N<sub>2</sub>O fluxes recorded compare favorably with published values for estuarine environments. Foster & Fulweiler (2016) reported negative fluxes of -0.5 to  $-1 \ \mu mol \ N_2O \ m^{-2} \ h^{-1}$  in sediments from a Massachusetts estuary, and a review by Murray *et al.* (2015) cited numerous instances of negative N<sub>2</sub>O fluxes smaller than -5  $\mu mol \ N_2O \ m^{-2} \ h^{-1}$  in salt marshes. These published values suggest that the negative N<sub>2</sub>O fluxes reported in this study are not unusual, and that the habitats studied may represent a small but notable sink for N<sub>2</sub>O. As explained in Foster & Fulweiler, the mechanism for N<sub>2</sub>O uptake is likely provided by microbes with the nitrous oxide reductase enzyme. These microbes can use N<sub>2</sub>O as an electron receptor during respiration, facilitating fluxes of N<sub>2</sub>O into the sediment. Future research should continue to explore the uptake of N<sub>2</sub>O in estuarine environments, as this is a promising ecosystem service.

Increased  $NH_4$  and  $NO_x$  fluxes are a potential ecosystem disservice sometimes associated with restored oyster reefs. If N is converted to  $NH_4$  and  $NO_x$ , it is recycled to a bioavailable form that can be used for growth. High rates of positive  $NH_4$  and  $NO_x$  fluxes have the potential to eclipse N removal through denitrification, resulting in net N input. In this study,  $NO_x$  fluxes were negligible, and  $NH_4$  fluxes did not exceed 200 µmol m<sup>-2</sup> h<sup>-1</sup>. There was no correlation between  $NH_4$  fluxes and oyster filtration, challenging suggestions that oyster reefs increase ammonification.

DNE provides a metric to contextualize N fluxes (Eyre & Ferguson 2009, Piehler & Smyth 2011). DNE did not follow a clear pattern based on season, habitat type, or restored age. DNE was generally above 75%, indicating that these systems generate net N removal. Although results did not suggest a predictable restoration trajectory for DNE, they do reiterate one of the main findings of this study: restoration augments ecosystem function. In this case, reef-marsh restoration provides net N removal. It has been suggested that denitrification could become less efficient as SOD increases, reducing O<sub>2</sub> concentrations and increasing the likelihood of incomplete denitrification (Gardner & McCarthy 2009). Our results indicated that DNE increased or remained stable as SOD increased. These findings agree with a prior study in the same region, which also demonstrated sustained denitrification efficiency with increasing SOD (Kellogg *et al.* 2013).

Restoration practitioners in coastal areas are often limited by time and financial resources, making it useful to employ constructs such as restoration trajectories to anticipate changes in ecosystem services over time to justify and plan restoration projects. The results of this study do not support advancing a single restoration trajectory for denitrification in oyster reef and salt marsh habitats in N-limited coastal systems, because trajectories differed by season. However, denitrification did consistently increase from the 0- to 7-year-old sites. These results suggest that denitrification does follow a consistent pattern during the first 7 years following restoration, and that practitioners can expect an increase in N removal during that time.

The summer restoration trajectory warrants additional consideration. There was a significant relationship between denitrification rates and restored age only during the summer, during which rates increased and plateaued. This suggests that summer denitrification rates adhered to La Peyre *et al.*'s proposed trajectory for biogeochemical factors (2014). Further summer sampling is recommended to confirm this pattern, which may be a useful framework for addressing summer eutrophication. Sustained rates of denitrification during the summer would perform a valuable ecosystem service during a time of elevated biological activity.

Many coastal restoration efforts seek to enhance ecosystem services by constructing oyster reefs and salt marshes. These habitats frequently co-occur naturally in estuaries in North Carolina and elsewhere on the East Coast. In the context of living shorelines, including both habitats helps maximize desired shoreline stabilization benefits. This research indicates that the denitrification benefits of reef-marsh restoration are distributed in both habitats. Other site parameters influenced the development of denitrification as the restored sites aged, but denitrification rates developed similarly in both habitats. This suggests that, once a reef and marsh are restored, they quickly function as a comprehensive system, at least in terms of biogeochemical indicators. This cohesiveness increases the attractiveness of living shorelines as an ecologically-sound coastal management strategy.

In fact, results suggest that the impact of restoration extends beyond the restored habitats themselves into adjacent areas. Denitrification rates were the same in the sandflat as in the reef and marsh, suggesting that N-removal capacity was enhanced beyond the boundaries of the restoration itself. This idea is also supported by the accumulation of SOM in the sandflats over time. SOM in the sandflats was not comparable to SOM in the restored habitats until the 20-year-old site, indicating that sandflats had lower initial SOM but gradually increased. The

ultimate increase in SOM could point to the compounding influence of the reef-marsh restoration on adjacent habitats.

Since this study employed a chronosequence design, it is important to underscore that the 20-year-old site is an independent location rather than a true snapshot in time. As such, it is possible that SOM was higher in the sandflats at that site for reasons unrelated to the restoration. Future research could discern potential outwelling by measuring denitrification rates and SOM in restored habitats and adjacent habitats at multiple sites restored 7 years ago or more.

Our results suggest that maximizing physical parameters of a site is not guaranteed to result in higher levels of biogeochemical function. Neither oyster filtration nor stem density exhibited a clear relationship with  $N_2$  flux. This result addresses the "field of dreams" restoration myth: that restoring physical features of a site will ultimately translate to ecosystem function (Hilderbrand *et al.* 2005). The sites chosen for this study did not display a connection between the physical presence of oysters and marsh grass and an increase in denitrification. Physical parameters did appear to contribute to other habitat features, such as increased SOM, and likely contributed to the outwelling effect on adjacent sandflats. However, it is unlikely that increasing the number of mature oysters and the density of marsh grass would boost denitrification.

Although this study did not find a predictable link between age and denitrification, it did demonstrate that reef-marsh restoration augments ecosystem function without introducing ecosystem disservices (Burgin *et al.* 2013, Lyytimaki & Sipila 2009). Fluxes of NH<sub>4</sub> and N<sub>2</sub>O were low across habitat and season. SOM increased, denitrification rates were appreciable, and the physical presence of oysters and marsh grass likely introduced a host of ecosystem services not measured in this study, including juvenile fish habitat, wave attenuation, and carbon

sequestration (Broome *et al.* 1988, Davis *et al.* 2015). In this study, reef-marsh restoration was found to be a self-sustaining way to increase ecosystem function.

Restoration projects designed to increase N removal benefit from the ability to measure denitrification. However, denitrification can be a costly and difficult process to measure, particularly for managers who lack access to scientific equipment. Based on the results of this study, measuring SOD can reliably predict relative rates of denitrification in restored oyster reefs, salt marshes, and adjacent sandflats. Oyster reef restoration criteria tends to focus on physical parameters of reefs (Baggett *et al.* 2015). Although the structural attributes of the reef are indicative of common restoration targets, we did not find it to be correlated with denitrification. This emphasizes the importance of clearly defining restoration goals and ensuring that monitoring plans can adequately measure parameters associated with those goals (Ehrenfeld 2000, Hobbs & Harris 2001). When planning restoration projects, it is also important to recognize that that some restoration goals may be mutually exclusive. One study of restored wetlands identified a tradeoff between biodiversity and nutrient removal, including denitrification (Jessop *et al.* 2015).

Future research on denitrification in restored estuarine habitats should continue to assess the many factors that impact this process, especially across a seasonal gradient. It is also critical to consider resiliency of reef-marsh restoration, particularly in response to climate change. Sea level rise and coastal squeeze are likely the most pressing consequences of climate change for the habitats included in this study. Restoration can be planned with an awareness of uncertain future conditions (Harris *et al.* 2006, Seastedt *et al.* 2008), and it is likely that nature-based solutions such as living shorelines can help sustain ecosystems and their function.

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