Inputs and fluxes of nitrogen in the Virginia coastal bays: Effects of newly-restored seagrasses on the nitrogen cycle

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Abstract

Seagrasses are ecosystem engineers—providing nursery ground and refuge for fish and invertebrates, sediment stabilization, and regulate the nitrogen (N) cycle. Seagrasses worldwide are in decline, bringing about a shift in N dynamics in shallow coastal systems (Waycott *et al*. 2009). The decline of seagrasses is driven largely by the chronic increase in anthropogenic N (Nixon 1995), which triggers a state change where shallow coastal systems become dominated by epiphytes (Bulthuis & Woelkerling 1983) and algae (McGlathery 2001).

This study used a land use-based N loading model to calculate the inputs of N to the Virginia coastal bays—a shallow coastal system characterized by a low human population density, low water nutrient concentrations, and the most successful seagrass (*Zostera marina* [L.]) restoration in the world. In addition, this study measured N fluxes across a restoration chronosequence to quantify the effects of restored seagrass on the N cycle.

Using the N loading model, we determined that agricultural fertilizer was the dominant terrestrial N source, though deposition of rainfall to the surface of the bays was the largest overall source of N. On average, Virginia coastal bays received 7.2×10^4 kg N y^{-1} or 2.11 g N m^{-2} y^{-1}, markedly lower than most other shallow coastal waterways in temperate systems (Boynton *et al*. 1995, McGlathery 2007).

Nitrogen fixation (N_2 fixation) rates were significantly higher in vegetated sediments—compared to bare sediment—and decreased with depth. Furthermore, the older seagrass meadow (8 years old; seeded in 2001) fixed significantly more N_2 than the younger meadow (3 years old; seeded in 2006) and bare sediment. N_2 fixation rates in the older meadow and bare sediment were comparable to other *Z. marina* and bare sediment systems, respectively. Denitrification (N_2 loss), however, was the dominant process. Denitrification rates increased as seagrass meadows aged, and were correlated to increases in sediment organic matter. The N removal capacity of bare sediment was half of the total N loading rate into the bays (1 g N m^{-2} y^{-1}), and vegetated sediments removed 3.9–5.8 g N m^{-2} y^{-1}, supporting the concept of seagrass beds as a “nutrient sponge”.

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Chapter one: An introduction to the dissertation

INTRODUCTION

Background

In many temperate coastal marine systems, nitrogen (N) is often the limiting nutrient for benthic and pelagic primary producers (Taylor *et al.* 1995). Yet throughout the world, N over-enrichment has caused deleterious effects in may coastal ecosystems (Boesch 2002a, Lapointe and Clark 1991, Nixon 1995, McGlathery et al. 2007, Waycott *et al*. 2009). In these ecosystems, increased anthropogenic N sources have often led to eutrophication events, including anoxia, harmful algal blooms, and fisheries losses that occur at a higher frequency and magnitude than in less impacted systems (Boesch 2002b). In the last few decades, coastal scientists have made great efforts to monitor N concentrations and to understand the mechanisms influencing N turnover and the impacts on receiving water bodies.

Shallow bays constitute approximately 13% of the world's coastline and 18% of the coastline of North America (Cromwell 1973, Kjerfve 1989, Kennish & Paerl 2010). Within shallow coastal bays, the photic zone typically penetrates to the sediment surface and benthic primary producers (*e.g.*, seagrasses, algae, cyanobacteria) mediate the nitrogen dynamics within the system (McGlathery *et al*. 2007). Seagrasses in particular are ecosystem engineers—providing nursery ground and refuge for fish and invertebrates, sediment stabilization, and regulation of the nitrogen (N) cycle. Seagrasses worldwide are in decline, the result of which induces a state change from seagrass-dominated benthos to benthos dominated by epiphytes (Bulthuis & Woelkerling 1983) and algae (McGlathery 2001).

Seagrass communities typically sequester more N and carbon (C) than bare sediment alone. The high rates of primary production in seagrass meadows are among the highest in the world, and require large N inputs to sustain growth. Bacteria (Howarth *et al*. 1988, Welsh 2000) and archaea (Dekas et al. 2009) associated with seagrasses "fix" or convert N_2 gas into biologically-usable forms, which can then be used by the plants or other bacterial communities for growth (Fig. 1). N_2-fixing bacteria associated with the temperate seagrass, *Zostera marina* [L.], have been show to provide as much as 5% of the total N demand to the plant (McGlathery *et al*. 1998). In addition, denitrifying bacteria—which convert biologically-usable nitrate (NO_3^-) into N_2 gas—are also found in seagrass sediment and can remove N from the system at rates comparable to, and often higher than, N_2 fixation (Hemminga *et al*. 1991, Welsh *et al*. 2000, McGlathery 2008). The balance of N_2 fixation and denitrification provides a net N_2 flux for the seagrass system (Fig. 1). Seagrasses are referred to as a nutrient "sponge" as

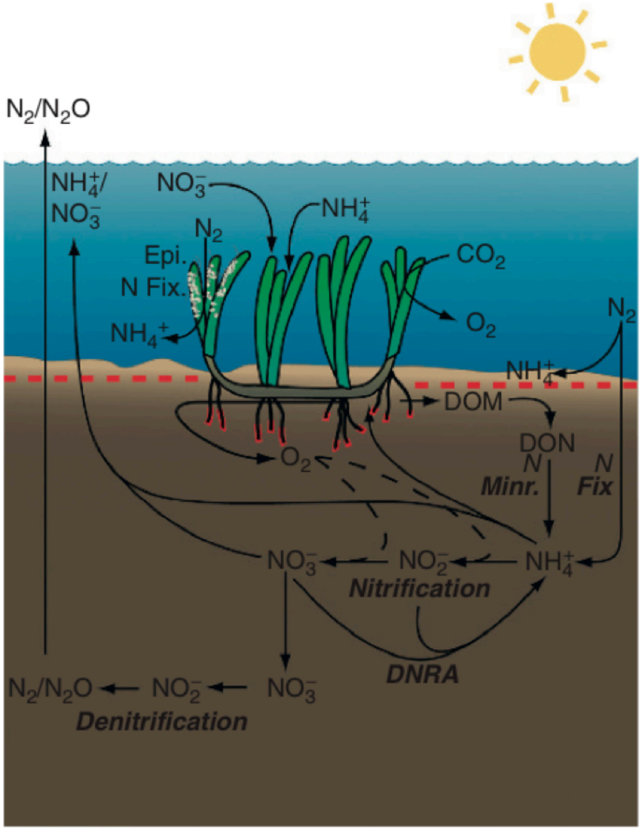

Figure 1. The N cycle in seagrass-vegetated sediments.

N traveling across the land sea margin is intercepted by the plants and used for growth, or, when the seagrasses are net denitrifying (positive net N_2 flux), the system is able to remove the N from the system entirely.

Site description

The Virginia coastal bays are part of a 200 km long stretch of embayments extending from Delaware to the southern tip of Virginia along what is called the Delmarva Peninsula. The entire east coast of Virginia's portion of the Delmarva Peninsula is a series of small bays, separated by mudflats and marshes, and are bound to the east by barrier islands and to the west by the mainland (Fig. 2). A continuous 17-year water quality dataset collected by the Virginia Coast Reserve Long-Term Ecological Research program shows that the water column N (0.5–4 μM NO_2+NO_3, 3.2–3.6 μM NH_4^+) and chl-*a* (1–6.5 μg L^{-1}) were quite low relative to other shallow coastal systems with little inter-annual variability (VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq). Following a 70-year absence in seagrasses, restoration efforts began in the Virginia

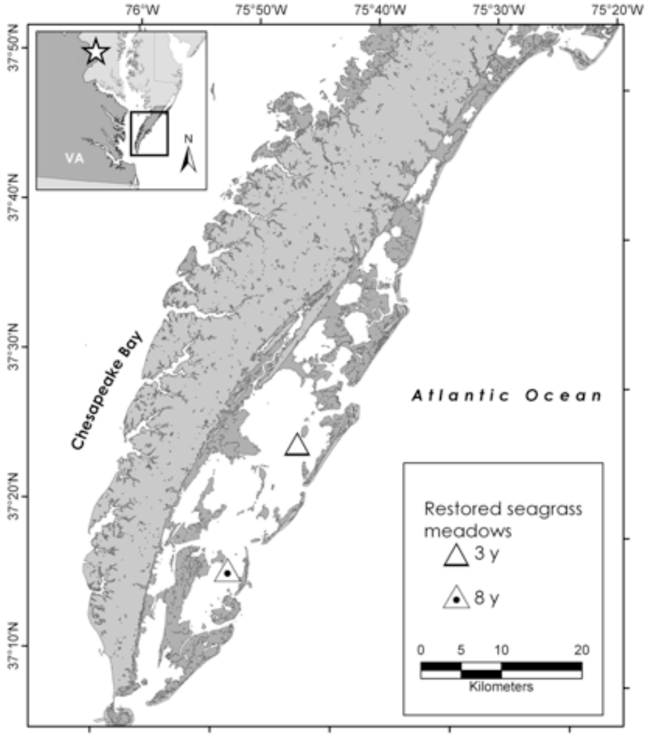

Figure 2. Map of the Virginia coastal bays and locations of seagrass restoration.

coastal bays in 2001, and continued through 2008, resulting in seagrass meadows of different ages (time since seeding). Overall, the seagrass restoration efforts have resulted in the largest areal recovery of seagrasses (*Z. marina*) worldwide (Orth *et al*. 2010).

A guide to the dissertation

This dissertation has been divided into six chapters: an introduction chapter, four chapters written as manuscripts for submission to peer-reviewed publications, and a conclusion chapter. Chapter 1 provides context to this dissertation by reviewing the role of nitrogen in shallow coastal systems, as well as the role of seagrasses in these systems and impact that seagrasses have on the nitrogen cycle. Chapter 2 describes the application of a nitrogen loading model to the watersheds of the Virginia coastal bays and identifies the dominant sources of N. The ratio of watershed to bay area is identified as a major predictor of N loading processes. Chapter 3 has been submitted to *Marine Ecology Progress Series* and presents rates of sediment and epiphytic N_2 fixation across the seagrass restoration chronosequence, and identifies mechanisms driving the rates of N_2 fixation and the impact of N_2 fixation on the associated seagrass community. Chapter 4 details the rates of seagrass-associated N_2 fixation along a depth gradient that approaches the depth limit of seagrasses in the Virginia coastal bays. Chapter 5 for the first time reports net N_2 flux rates across a seagrass restoration chronosequence using the N_2:Ar technique. Chapter 6—the conclusion chapter—summarizes the key findings of this novel research, and identifies where future research is needed.

Chapter Two: Application of a nitrogen loading model to estimate nitrogen inputs to minimally-impacted mid-Atlantic bays

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ABSTRACT

Many coastal embayments are subject to high and increasing nitrogen (N) loads from the land and atmosphere. The watersheds draining to the Virginia coastal bays present a unique ecosystem with low human population, mixed agricultural and forested land use, and as a result, low N inputs to their receiving waters. Using the N loads from a stream survey, we verified the Waquoit Bay Nitrogen Loading Model (NLM) for 6 small watersheds in the Virginia portion of the Delmarva Peninsula, then applied the model to 9 larger surrounding watersheds. Fertilizer from agricultural land use was the dominant terrestrial source of N to the coastal bays (avg. 2.8×10^4 kg N y^{-1} or 1.17 g N m^{-2} y^{-1}, 77% of groundwater N, 39% total N). Direct deposition to the surface of the coastal bays was the largest source of N (avg. 4.0×10^4 kg N^{-1} or 0.76 g N m^{-2} y^{-1}, 54% total N). The average N loading rate was 7.2×10^4 kg N y^{-1} or 2.11 g N m^{-2} y^{-1}. Compared to other watersheds along the east coast of the United States, the N loads from the relatively undeveloped coastal watersheds to the Virginia coastal bays were among the lowest

reported, and these systems provide an “end member” for comparisons of N loading to other, more impacted systems.

INTRODUCTION

Throughout the world, nitrogen over-enrichment has caused deleterious effects in many coastal ecosystems (Boesch 2002a, Lapointe and Clark 1991, Nixon 1995, McGlathery et al. 2007). In these ecosystems, increased anthropogenic nitrogen (N) sources have often led to eutrophication events, including anoxia, harmful algal blooms, and fisheries losses that occur at a higher frequency and magnitude than in less impacted systems (Boesch 2002b). In the last few decades, coastal scientists have made great efforts to monitor N concentrations and to understand the mechanisms influencing N turnover and the impacts on receiving systems, and in turn, models using these data have been developed to predict future changes in marine ecosystems (Valiela *et al.* 1997, Kellogg *et al.* 1996, Caraco & Cole 1999). These models—simple or complex—provide a powerful tool for assessing and predicting the impact of N in the marine environment.

Predictive N loading models range from simple equations with few constants to intensive computer models requiring detailed non-linear equations. Valiela *et al.* (2002) found that while increasing the number of variables in most models provides more accurate predictions of N inputs, simpler models (*e.g.*, Valiela *et al.* 1997) often perform well and are typically easier to use. Simpler models often provide “order of magnitude” predictions—a clear limitation, whilst intermediate models, like the Waquoit Bay Nitrogen Loading Model (Valiela *et al.* 1997)—which was used in this study—operate using multiple iterative inputs and attenuation constants and provide modest accuracy (WB NLM: SE ± 12–14%). One potential drawback of the NLM is that it has not been tested or verified in rural systems with little human disturbance, so its accuracy for those conditions was uncertain.

The east coast of the United States has undergone intense human population growth. In coastal counties from Maine to Virginia, the average population has more than doubled in the last two decades, increasing from 131 people km^{-2} in 1988 (Boynton *et al.* 1993) to 369 people km^{-2} in 2000 (U.S. Census GIS Data, ESRI 2010). Wastewater, fertilizers, and anthropogenic atmospheric deposition all increase with an increase in human population, and the added N load from these anthropogenic sources has directly and indirectly affected estuaries by altering primary production processes and energy pathways throughout the water column and benthos (Nixon 1997, Boesch 2002b, Smith *et al.* 2003, Greene *et al.* 2004). Despite this surge in human population along the east coast of the US, some coastal watersheds have seen virtually no major increase in population over the last few decades (*e.g.*, the Virginia coastal bays, http://www.ecotrends.info). When human influences like sewage, agriculture, golf courses, high-density housing, impervious services, and physical shoreline protection (*e.g.*, rip rap) are a minor proportion of the watershed, fewer variables are needed in the predictive model, suggesting the appropriateness and applicability of a simpler nitrogen loading model. These minimally impacted watersheds provide a unique opportunity for using the NLM, as their landscape is appropriate for using the model, but are less altered than the more developed and populated watersheds in Waquoit Bay, MA which were used to verify the model (Heberlig *et al*. 1997, Valiela *et al*. 2000).

Along the coastal plain, particularly in the Delmarva Peninsula (Fig. 1), the primary means of water transport and nutrient delivery from the watersheds to the coastal bays is groundwater (Dillow and Greene 1999, Giblin & Gaines 1999). In the watersheds of the Delmarva Peninsula, surface runoff is minimal or even absent because of the highly permeable sands and absence of impermeable surfaces, in costrast to more suburban watersheds (Dillow & Greene 1999, Dillow *et al*. 2002). The groundwater is partitioned between direct discharge to the bays (11%) and baseflow to streams (88%) (Jordan *et al.* 1997, Bohlke and Denver 1995). In low- relief coastal plains, the flow paths of groundwater are concordant with surface elevation. Immediately north of the Virginia coastal bays in the Chincoteague Bay watershed, groundwater flows from northwest to southeast (Dillow and Greene 1999, Dillow *et al.* 2002). Throughout Virginia coastal bays watersheds, the water table ranges from 0–3 m below sea level indicating geographical, geological, and hydrologic similarities (Galavotti 2004). This shallow water table, combined with the short distance from the landward side of the watershed to the bay edge, suggests that groundwater constituents like N in baseflow and direct groundwater discharge likely undergo similar attenuation processes along the flow path to the bay.

The Virginia coastal bay watersheds have a small watershed to bay area ratio, potentially limiting the impact of terrestrial N relative to bay size. In addition, groundwater N inputs into streams are reduced or completely removed by denitrification activity in the stream beds (Gu *et al*. 2007, Flewelling 2009) and further downstream, extensive tidal marshes that act as a nutrient “sponge”. By the time the water parcel reaches the marine system, it is possible that N has been largely attenuated and may have very little impact on the water quality in the bays.

The relatively undisturbed watersheds of the Virginia coastal bays provide a unique opportunity to evaluate the impacts of low-level human disturbance. Additionally, by testing the accuracy of the nitrogen loading model with stream data from smaller rural watersheds within the peninsula, we will be able to further verify our N loading estimates to the bays.

METHODS

Site description

The Virginia coastal bays are part of a 200 km long stretch of embayments extending from Delaware to the southern tip of Virginia along what is called the Delmarva Peninsula. A continuous 17-year water quality dataset collected by the Virginia Coast Reserve Long-Term Ecological Research program shows that the water column N (0.5–4 µM NO_2+NO_3, 3.2–3.6 µM NH_4^+) and chl-a (1–6.5 µg L^{-1}) were quite low relative to other shallow coastal systems with little inter-annual variability (VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq). For this study, we used groundwater and aquifer characteristics from the Chincoteague Bay, MD/VA watershed as a reference for the Virginia systems because of their inherent similarities. The entire east coast of Virginia's portion of the Delmarva Peninsula is a series of small bays, separated by mudflats and marshes, and are bounded to the east by barrier islands and to the west by the mainland (Fig. 1). These bays are shallow, have sandy or muddy sediments, and have low watershed to bay area ratios (land:water). Sandy, unconsolidated sediments underlie these coastal watersheds; the surficial aquifer to the north of the Virginia coastal bays is saturated to a depth of 12–37 m below the ground surface (Owens and Denny 1978), suggesting a shallow aquifer basement in the Virginia portion of the Delmarva Peninsula. The depth of the aquifer is correlated with the elevation of topographic features; that is, the higher in elevation the feature, the deeper the aquifer. Human population in the Virginia watersheds is low and is considered rural (<30 people km^{-2}). Forest and agriculture are the primary terrestrial land use types in most watersheds (38% agriculture, 32% forest, 27% wetlands, 2% developed; Stanhope et al. 2009). Cultivation agriculture is primarily corn and soybean, but tomato plasticulture and bean crops are present in the southern Virginia watersheds (USDA NASS 2009). There are only 2 poultry farms in the Virginia watersheds (Assawoman), unlike Chincoteague Bay to the north (USDA NASS 2009, Giordano et al. 2011).

Nitrogen Loading Model and associated data sources

The Waquoit Bay Nitrogen Loading Model developed by Valiela *et al.* (1997, 2000) is a useful model for predicting terrestrial groundwater N loads to the marine system. The Waquoit Bay NLM (hereafter called NLM) predicts the total terrestrial N load to an estuary, provided the watersheds are suburban and underlain by a sandy substrate. The model's N sources are atmospheric deposition, fertilizer, and human wastewater (Fig. 2). Attenuation constants are applied to different land use types and include volatilization and uptake at the soil surface, in the unconsolidated aquifer (vadose zone), in the saturated aquifer, and for wastewater, in the septic system (Valiela *et al.* 1997).

A method for calculating total atmospheric N deposition is described in Valiela *et al.* (1997). This method uses available wet deposition data and literature constants to calculate total wet and dry (including organics) N. In this study, these data came from the NADP (National Atmospheric Deposition Program, http://nadp.sws.uiuc.edu/). In addition, the EPA Models-3 Community Multiscale Air Quality (CMAQ) Modeling System (Byun & Ching 2009) was run to compare to the total N deposition calculation presented in Valiela *et al.* (1997). Similarity of the two atmospheric deposition datasets was tested with a two-tailed type 1 t-test, and was not statistically different.

Land cover data used for the sub-watersheds gauged by Stanhope *et al.* (2009) were generated from the 2001 National Land Cover Database (NLCD, 1:24,000 scale). These land use data were used in the NLM and verified with the Stanhope *et al.* (2009) stream N data. For the whole watershed analysis, USGS NLCD land use rasters were analyzed with GIS software (ArcGIS, ESRI, Redlands, CA) to calculate the land cover area of forested (*i.e.*, evergreen and deciduous trees), agricultural (*i.e.*, crow crops), and impervious surfaces (*i.e.*, roofs, driveways, roads, parking lots).

Initially, fertilizer application rates were run with both published and modified values from Valiela *et al.* (1997). The mean fertilization rate suggested by Valiela *et al.* (1997) was 136 kg N ha^{-1} y^{-1}; this was the average of a variety of crops grown in the Cape Cod, MA watersheds for which the model was developed. For this study, crop-specific fertilization and N_2 fixation rates were used for corn and soybeans, respectively. Corn was fertilized at a rate of 119 kg N ha^{-1} y^{-1} as reported by farmer interviews

(Giordano *et al*. 2011). Soybean N was determined to have leached into the groundwater post-harvest as chaff (32 kg N ha^{-1} y^{-1}, Cole 2005). The annual per capita release rate of 4.8 kg N used by Valiela *et al.* (1997) was applied to the human population living in the watersheds of the coastal bays for the most recent census year, 2000 (US Census Bureau, http://www.census.gov/), knowing that the majority of the population uses individual septic disposal systems (ISDS) rather than municipal sewage. Retention rates for septic tanks and fields were applied as described in Valiela *et al.* (1997).

Verification and sensitivity analysis

Baseflow stream nutrient monitoring conducted in the creeks of 14 sub-watersheds in Virginia from 2001–2002 (Stanhope *et al.* 2009) were used to verify the nitrogen loading model. All samples in the Stanhope *et al.* (2009) study were analyzed using standard methods for NO_3^-, NO_2^-, and NH_4^+. Once verified, we ran the nitrogen loading model for each of the larger watersheds that drain into the Virginia coastal bays (Fig. 1).

A regression was made comparing the measured N load for 6 small gauged streams from different watersheds (Fig. 1, Stanhope *et al.* 2009) and the N load was predicted by the NLM (Fig. 3a). The areal ratio of the dominant crops: corn and soy, in Northhampton County, VA in 2002 was 23:77, so this ratio was applied to the land use area marked as “agricultural” in each watershed. County-level agricultural census data were the finest resolution data available for 2002 (the year of both the agricultural census and of the Stanhope *et al.* (2009) study). Since each individual stream watershed is only a small portion of the county area, we cannot say with confidence that the 23:77 corn:soy ratio was accurate, as many of these small, gauged watersheds contained only one or two single-crop farms. NLM estimates were low relative to estimates from monitoring data, indicating that soybean crops were being fertilized. Adjusted fertilizer application rates which included soy fertilization, equaled 136 kg N ha^{-1} y^{-1}, which is also the rate suggested by Valiela *et al.* (1997). This correction brought the N load predicted by the NLM for the gauged watersheds to within the 12–14% SE suggested by Valiela *et al.* (1997).

RESULTS

Watershed characteristics for model verification

The six watersheds used to verify the modified Nitrogen Loading Model (Valiela *et al.* 1997, Cole 2005) were Assawoman, Nickawampus, Partings, Greens, Mill, and Holt (Fig. 1). These watersheds ranged in area from 117–238 ha. Forested area ranged from 24–67%, developed: 0.6–13.5%, and agricultural: 29–69%. NLM loads (in kg N y^{-1}) were statistically similar to the N loads measured by Stanhope *et al.* (2009) (Fig. 3a; slope: 0.94, r^2: 0.69, F: 7.47, p: 0.07). The largest possible source of error was that a county-level crop area ratio was used while the crops grown at each specific watershed were not known. Agricultural inputs accounted for 70–90% of the total N draining from these 6 stream watersheds.

Whole watershed N inputs

Agriculturally-derived groundwater N was the largest terrestrial source of N delivered to the Virginia coastal bays (Fig. 4). One exception, however, was Fisherman

Island, a small, uninhabited National Wildlife Refuge at the southern tip of the Delmarva Peninsula. Direct deposition to the surface of the coastal bays was also a major input, particularly for watersheds with a small land to water ratio (*e.g.*, Machipongo, Quinby, Fisherman Island, and Sand Shoal; range: 16–98% total N; Table 1, Fig. 4). The Virginia coastal bays had a lower N input compared to Chincoteague Bay (*i.e.*, Chincoteague, Sinepuxent, and Newport bays; 4.4 x 10^5 kg N y^{-1}; Cole and Nixon *submitted*), and when normalized by bay area, the areal loading rate for all systems fell within a range of 0.7 and 5 g N m^{-2} y^{-1} (Table 2, Fig. 5a). A latitudinal trend was seen in the N inputs normalized to bay area for the Virginia bays. N input rates decreased from north (3.47–4.80 g N m^{-2} y^{-1}) to south (0.77–1.65 g N m^{-2} y^{-1}), driven primarily by a general increase in bay area relative to land area, and to a lesser extent, population density (Table 2, Fig. 4). The agricultural N input was similar across the Virginia watersheds, but on the basis of bay area, the loading rate from agricultural sources to the northern bays was higher than the southern (2.62–3.57 g N m^{-2} y^{-1} and 0–0.77 g N m^{-2} y^{-1}, respectively; Table 2).

The total N load (kg N y^{-1}) for the Virginia coastal bays was regressed with bay area, watershed area, agricultural area, and forested area (Fig. 6). Agricultural area was the best predictor of total N load for these systems (r^2: 0.99, $p < 0.001$), followed by watershed area (r^2: 0.93, $p < 0.001$), forested area (r^2: 0.75, $p = 0.003$), and bay area (r^2: 0.20, $p = 0.23$). Agricultural area increased linearly with watershed area (r^2:0.911, Fig. 6), emphasizing the role of agricultural N inputs in this system.

The percentage of the total N coming from direct deposition of N to the water surface of the coastal bays was similar for all the watershed–bay systems (0.76 g N m^{-2} y^{-1}, with direct deposition providing >50% of total N to 5 watersheds, and <50% to four.

All watersheds received 75% or less of total N from agriculture, and <25% at only two watersheds (Quinby and Fisherman Is.) (Fig. 5b). All 9 watersheds received <25% of their total N from both non-agricultural (forested, turf, impervious surfaces) and wastewater sources.

DISCUSSION

The N loads to the Virginia coastal bays were driven primarily by direct deposition of N to the bays' surface and agriculturally-derived sources (Fig. 4). The N load predicted by the nitrogen loading model (NLM; Valiela *et al.* 1997) was statistically similar (Fig. 3a, t-test: $p = 0.8$) to the stream measurements made by Stanhope *et al.* (2009) and were positively and significantly correlated (linear regression: $p = 0.04$). The verification watersheds were of comparable size to those measured for the development of the NLM in Waquoit Bay, MA (Valiela *et al.* 1997, 2000) and like the Cape Cod watersheds, the Virginia watersheds had mixed agriculture and groundwater as the primary means of water transport. The Virginia human population density, however, was lower.

The predicted N load from the NLM run on the larger watersheds was regressed with the equation presented in Fig. 3a, resulting in an over-prediction of only 7%, which is within the range reported with the original NLM (Valiela *et al.* 1997). As the watershed size increases, so too does the likelihood of multiple crop types being farmed, and the multiple crop fertilization rate suggested by Valiela *et al.* (1997) is more applicable, thus reducing the potential error. It is important to note that the results of the

NLM only represent terrestrial N inputs and do not include direct deposition to the bay surface (Fig. 3b).

Source contributions

The nitrogen loading model provides both a total N input from the watershed and a breakdown of the source inputs and attenuation. Attenuation occurs at all stages of the model, including volatilization at the watershed surface, the unsaturated aquifer (vadose zone), the saturated aquifer, and within septic tanks. While the NLM does not account for specific attenuation processes, absorption, denitrification, and burial (among others) are assumed to be occurring within the groundwater pathway (Valiela *et al.* 1997). Consistent with the findings of Giordano *et al*. (2011) in their analysis of the northern VA coastal bays, agriculture was the major land use type, and despite an assumed 39% volatilization of fertilizer N, 69% attenuation in the vadose zone, and 35% attenuation in the aquifer, agricultural N was the major terrestrial source of N delivered to the bays, with the exception for Fisherman Island, which had no agricultural land. In 7 out of 9 watersheds, agricultural N was >25% of the total and averaged to be 50% of total N (Fig. 5b).

The ratio of watershed land area to bay surface area has direct implications for the delivery of N to the marine system. N in the NLM is attenuated throughout the groundwater transport process, but in contrast, N deposition to the bay surface is a direct input to the system. This implies that with a smaller watershed:bay, the addition of direct deposition goes up at a much higher rate than does the same loading to the watershed surface. The 6 southern watersheds in this study have markedly lower watershed area:bay areas (mean: 0.3) than the northern 3 (mean ratio: 2.6; Table 1). This same trend was seen in the referenced Chincoteague Bay complex (Cole and Nixon *submitted*) to the north where the watershed:bay area was 1.3, and direct deposition accounted for 63% of the total whilst agriculture contributed 29%.

Comparison of bay N inputs

In the last few decades, nutrient loading rates have been assembled in order to demonstrate the role of N in anthropogenic eutrophication (Nixon *et al.* 1986, Boynton *et al.* 1996, McGlathery *et al.* 2007). The coastal bays of the mid-Atlantic (particularly in Virginia) have some of the lowest N input rates on the east coast of the United States (Table 1, McGlathery *et al*. 2007). This is potentially due to the low watershed:bay areas, the low population density, and the absence of high concentration poultry and livestock farming which is prevalent in the Maryland, Delaware, and North Carolina systems (Cole and Nixon *submitted*, USDA NASS 2009). In Virginia, the north to south decreasing N input rate (g N m^{-2} y^{-1}) is likely a combination of the low land:water area and the overall low population density, particularly at the southern tip of the Delmarva Peninsula.

The contribution of direct atmospheric N inputs to the total N load to large water bodies with relatively small watersheds are often high in both large scales (N. Pacific Ocean: 40–70%, Atlantic Ocean: 35–60%, Mediterranean Sea: 10–60%) and smaller scales (Rhode River, MD: 40%, New York Bight: 38%) (Paerl 1997). Of the fifteen water bodies with N loading rates under 10 g N m^{-2} y^{-1} presented in Table 1, ten are shallow bay systems with narrow, ribbon-like watersheds with a land:water area under 5. It is likely that terrestrial N sources in these systems are area limited, meaning that the small size of the watershed limits the amount of N that can be produced to then travel to the receiving waters, and N inputs would likely be dominated by direct N deposition. Yet, while bay watersheds represent a specific morphology typified by a low land:water, their N loading rate increases concomitantly with other more typical funnel-shaped watersheds (Fig. 7). The watersheds with a land:water of <5—which include all of the Virginia coastal bays—have a slope that is 7% higher than the slope that includes all 25 watersheds in Table 1.

The impacts of N inputs to marine systems do not necessarily parallel the associated loading rates. Coastline morphology, residence time, benthic N cycling, depth, and climate can all potentially influence the impact N has on these shallow marine systems (Boynton *et al.* 1995, Herbert 1999, Cloern 2001). The low N inputs to the Virginia coastal bays are consistent with the low water column N concentrations (1992–2008 DIN: 1.08–6.84 µM) and chlorophyll (chl-*a*: 1.38–65.69 µg L^{-1}; Virginia Coast Reserve LTER *http://www1.vcrlter.virginia.edu/home1/?q=data_wq*). With fewer than 30 people km^{-2}, nearly all of whom use septic tanks for wastewater, the direct impact of human waste was a minor constituent to the N load. In 1960, the average coastal county population density was 72 km^{-2}, by 1994: 105 km^{-2}, and is projected to be 126 km^{-2} by 2015 (US Census 2010, ESRI 2010). Without the typical urban/wastewater inputs to the Virginia coastal bays, coupled with the low land:water, these bays are among the least impacted along the mid-Atlantic coast.

Efficacy of an N loading model

In this study, we have shown the efficacy of the NLM presented (Valiela *et al.* 1997) and verified (Valiela *et al.* 2000) in Cape Cod, MA for the streams gauged in Virginia in 2001–2002 (Stanhope *et al.* 2009). The NLM has been used successfully in various other New England locations (Latimer and Charpentier 2010, Latimer and Rego 2010), Maryland (Cole and Nixon *submitted*), and Virginia (Giordano *et al*. 2011). The methods and results presented in this study using the NLM do not account for residence time, transformations, loss processes within the bay (*e.g.*, N_2 fixation, burial, denitrification, among others). The area and morphology (*e.g.*, residence time, aquifer–sediment structure) of the receiving waters will likely have a strong impact on the dispersion and consumption of land- and atmospherically-derived N, whose pathways require further study. Data from the NLM can be used for restoration planning, build-out scenarios, assessing past conditions, and coastline protection policy. Giordano *et al*. (2011) estimated N loads to three Virginia lagoons for possible residential and agricultural expansion using the NLM providing a directly applicable planning tool for the local governments. The applications are many, and given the variety of locations tested and the simplicity of the inputs, this model can be used worldwide, where, if only satellite images or crude land use data are available, N inputs to even remote coastal locations can be assessed.

Conclusion

The watersheds of the Virginia Coast Reserve represent some of the few coastal locations remaining in the eastern United States that are minimally impacted by human activities, in part due to the very low population densities (www.ecotrends.info). Virginia's shallow, well-mixed bays receive low N inputs from watersheds that are primarily agriculture and forest, and terrestrial sources provide on average 31827 kg N y^{-1} (29–64664 kg N y^{-1}) or 1.3 g N m^{-2} y^{-1} (0.009–4.0 g N m^{-2} y^{-1}) which is 13% of the total

N loads for all of the watersheds (avg. 7.2×10^4 kg N y^{-1} or 2.11 g N m^{-2} y^{-1}). We have shown that the NLM can be verified using past literature values for stream measurements, and then applied to larger watersheds, highlighting the usefulness of the NLM for predicting N loads to coastal systems. There is little doubt that the data resulting from long-term stream gauging and nutrient sampling provide very useful and factual watershed nutrient loading rate information. However, when a large-scale, low-budget, or fast nutrient loading rate is needed, a model can provide a worthwhile first order estimate.

Table 1. Watershed characteristics and areal loading rates of nitrogen to various coastal systems.

Location	Land Area (km^2)	Water Area (km^2)	Land:Water	Total N Loading Rate g N m^{-2} y^{-1}
Patapsco River, MD[1,*]	3415	261	13	49.0
St. Martin River, MD[‡]	96	8	11	39.7 (2.0)
Potomac River,VA/MD/WV/DC[1,*]	97938	3313	30	29.3
Narragansett Bay, RI/MA[3,*]	12194	850	14	27.6
San Francisco Bay, CA[1,*]	119178	1325	90	22.6
Chesapeake Bay, PA/DE/DC/MD/VA[1,*]	464732	25690	18	20.5
Delaware Bay, DE/PA/NJ/MD[2,*]	90222	5151	18	18.2
Mobile Bay, AL[1,*]	114415	1022	112	17.9
Patuxent River, MD[1,*]	2271	116	20	12.7
Pamlico River, NC[1,*]	26840	5588	5	12.0
Apalachicola Bay, FL[1,*]	52214	593	88	7.8
Albemarle Sound, VA/NC[1,*]	45036	2497	3.9	7.1
Isle of Wight Bay, MD[‡]	17.5	15.8	1.1	6.5 (0.7)
Assawoman Bay, VA	*36*	*7*	*4.9*	*4.8*
Choptank River, MD[1,*]	1886	411	5	4.3
Assawoman Bay, MD[‡]	24.7	22.5	1.1	4.1 (1.0)
Gargathy Bay, VA	*19*	*12*	*1.6*	*4.0*
Metompkin, VA	*67*	*49*	*1.4*	*3.5*
Wachapreague, VA	*56*	*84*	*0.7*	*1.6*
Chincoteague[1,†]	488	364	1.3	1.2 (0.9)
Machipongo, VA	*100*	*180*	*0.6*	*1.2*
Sand Shoal, VA	*60*	*169*	*0.4*	*1.2*
Smith, VA	*18*	*47*	*0.4*	*1.1*
Quinby, VA	*5*	*148*	*0.04*	*0.8*
Fisherman Is., VA	*0.25*	*5*	*0.1*	*0.8*

Watershed and Lagoon Characteristics
1. DOC/NOAA 2010
2. DOC 1985
3. Nixon *et al*. 1995
4. Valiela *et al.* 1997
5. Cole and Nixon *submitted*
italicized: this study

N Loading Rate Sources
* Boynton *et al*. 1995
† Cole and Nixon *submitted*
‡ parenthetical terms calculated from concentrations in Wazniak et al. 2004 and lagoon characteristics from Boynton et al. 1995

Table 2. Nitrogen loads to the Virginia coastal lagoons expressed in both cumulative and lagoon-area normalize loading rates. The groundwater column includes values from the agricultural, non-agricultural, and wastewater components.

	Watershed Area	Bay Area	kg N y^{-1} (g N m^{-2} y^{-1})					
Watershed	(ha)	(ha)	Groundwater	Ag. Component	Non-Ag.	Wastewater	Direct Dep	Total
Assawoman	3624	732	28520 (3.39)	26119 (3.57)	2401 (0.33)	1094 (0.15)	16571 (0.76)	35158 (4.80)
Gargathy	1875	1158	15316 (3.10)	12923 (2.62)	2393 (0.48)	565 (0.11)	8766 (0.76)	19644 (3.97)
Metompkin	6655	4877	61692 (2.62)	56306 (2.40)	5386 (0.23)	2009 (0.09)	36919 (0.76)	81495 (3.47)
Wachapreague	5619	8404	39619 (0.85)	35639 (0.77)	3980 (0.09)	1696 (0.04)	63618 (0.76)	76460 (1.65)
Machipongo	9987	18041	61649 (0.47)	54679 (0.41)	6970 (0.05)	3016 (0.02)	136570 (0.76)	164466 (1.25)
Quinby	528	14804	4333 (0.04)	3962 (0.04)	371 (0.00)	159 (0.00)	112066 (0.76)	77744 (0.80)
Sand Shoal	6037	16946	50520 (0.40)	46121 (0.37)	4399 (0.03)	1822 (0.01)	128281 (0.76)	147535 (1.17)
Smith	1841	4713	13849 (0.38)	12552 (0.34)	1297 (0.04)	556 (0.02)	35677 (0.76)	42320 (1.15)
Fisherman Is.	25	475	29 (0.01)	0 (0.00)	29 (0.00)	0 (0.00)	2357 (0.76)	2376 (0.77)

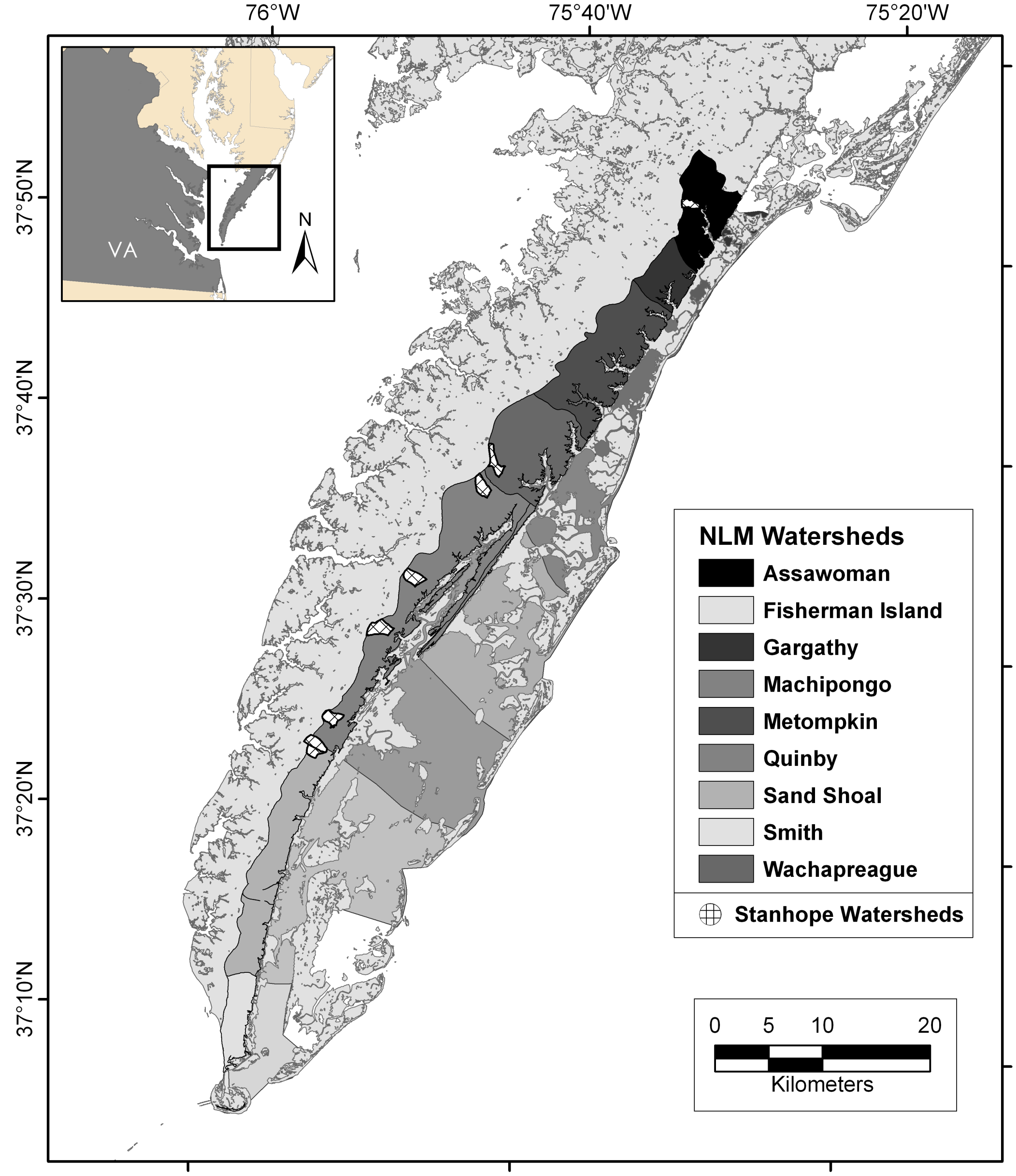

Figure 1. Basemap of the Virginia coastal bays. The bays are shown in a slightly lighter shade than their corresponding watershed. The Stanhope watersheds were used in the verification of the Nitrogen Loading Model.

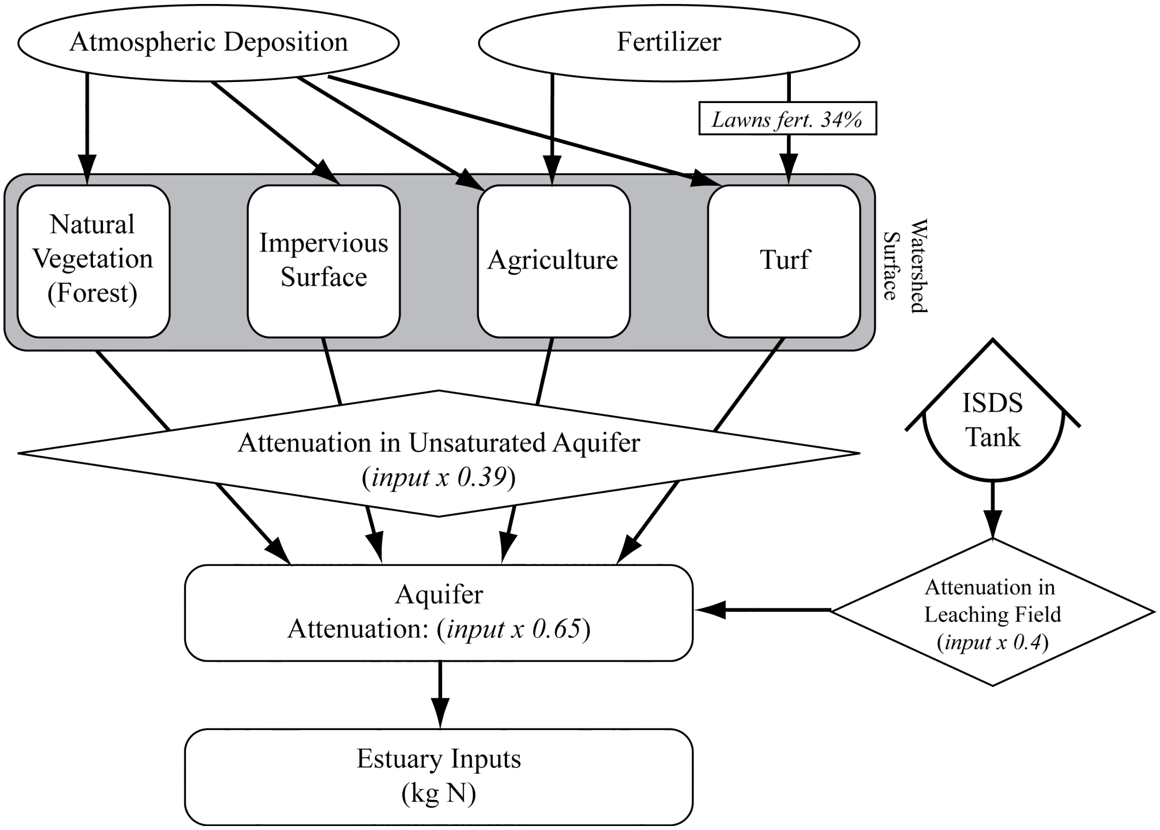

Figure 2. Schematic of the Waquoit Bay Nitrogen Loading Model (NLM; Valiela *et al.* 1997).

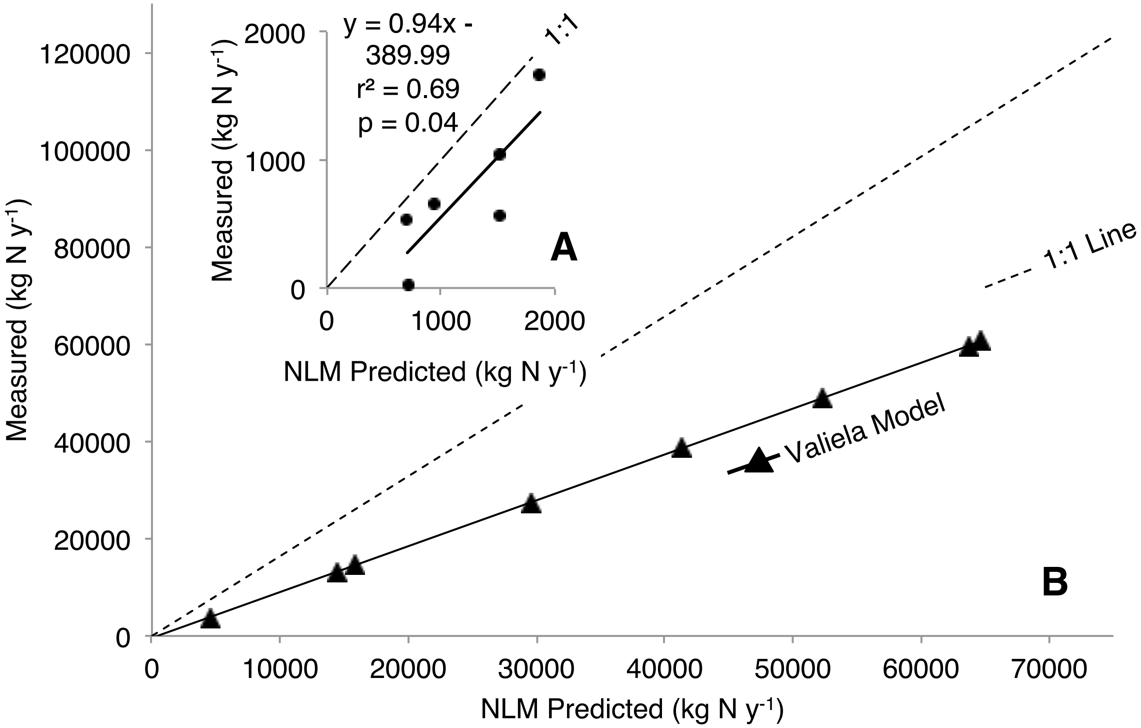

Figure 3. Regressions showing N inputs from (A) 6 subwatersheds using the Valiela NLM and stream measurements from Stanhope *et al*. (2009); and (B) 9 watersheds, adjusted using the equation presented in (A) against the predicted values from the original NLM (Valiela *et al*. 1997).

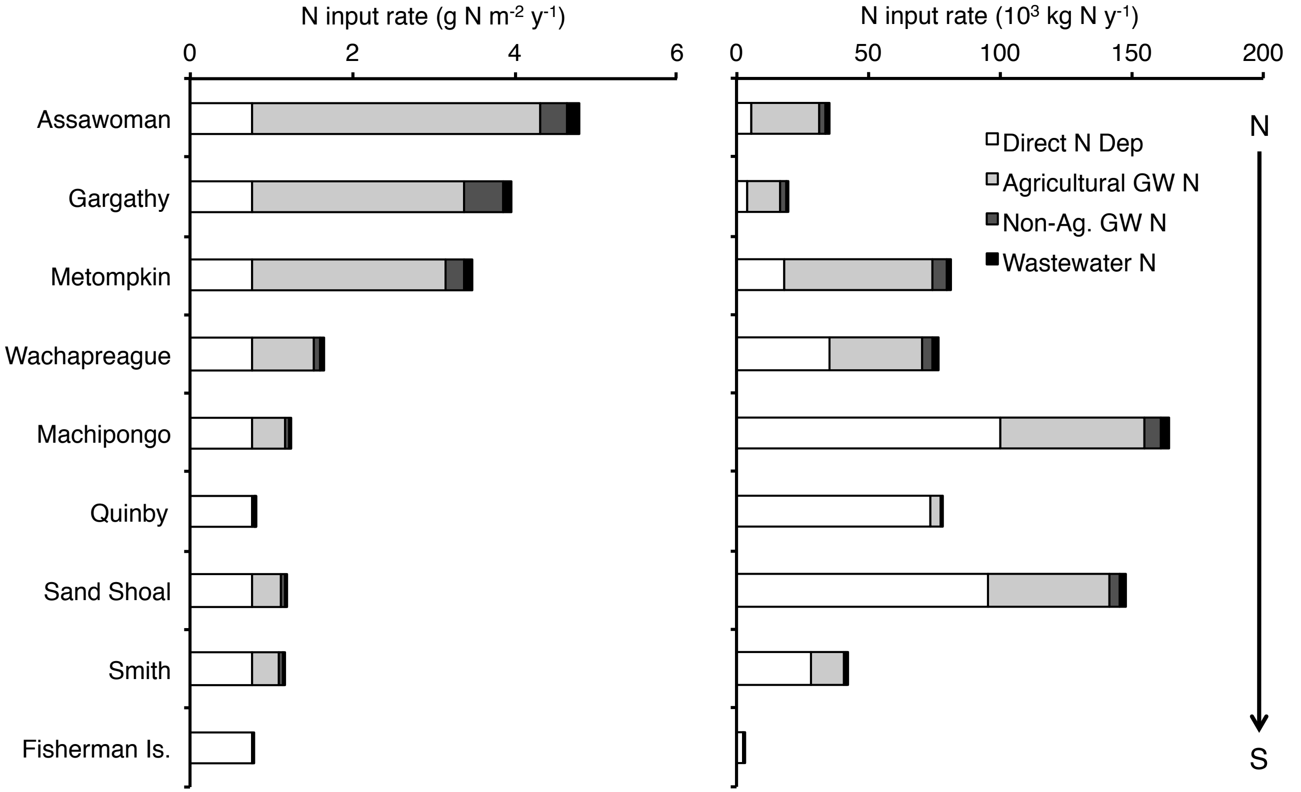

Figure 4. Total N loads to the Virginia coastal bays. Watersheds are arranged N to S. Left panel: bay area-weighted input rates. Right panel: total N loads.

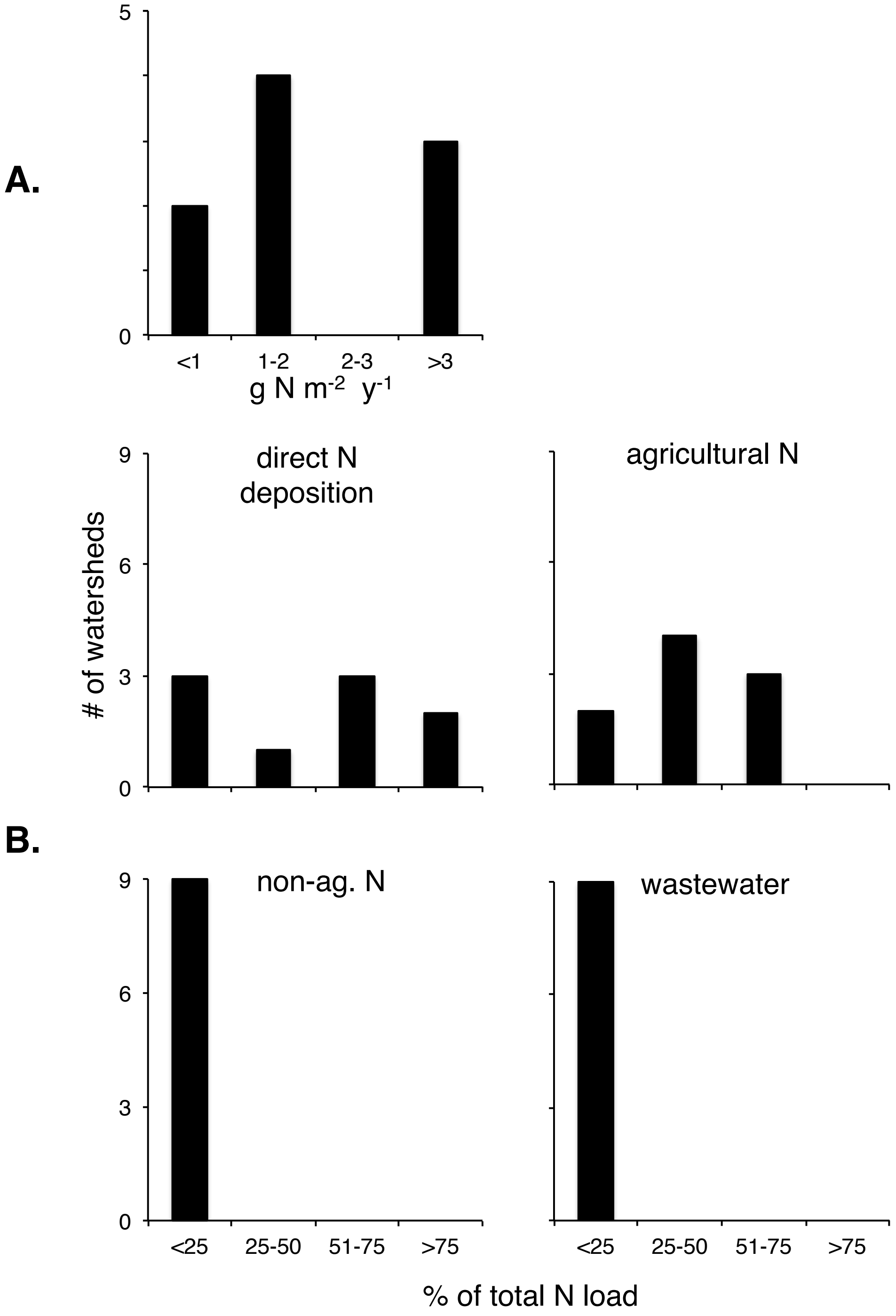

Figure 5. Frequency distribution of (A) N loading rates and (B) categories of N sources. For all panels, the x-axis shows the number of watersheds corresponding to each distribution.

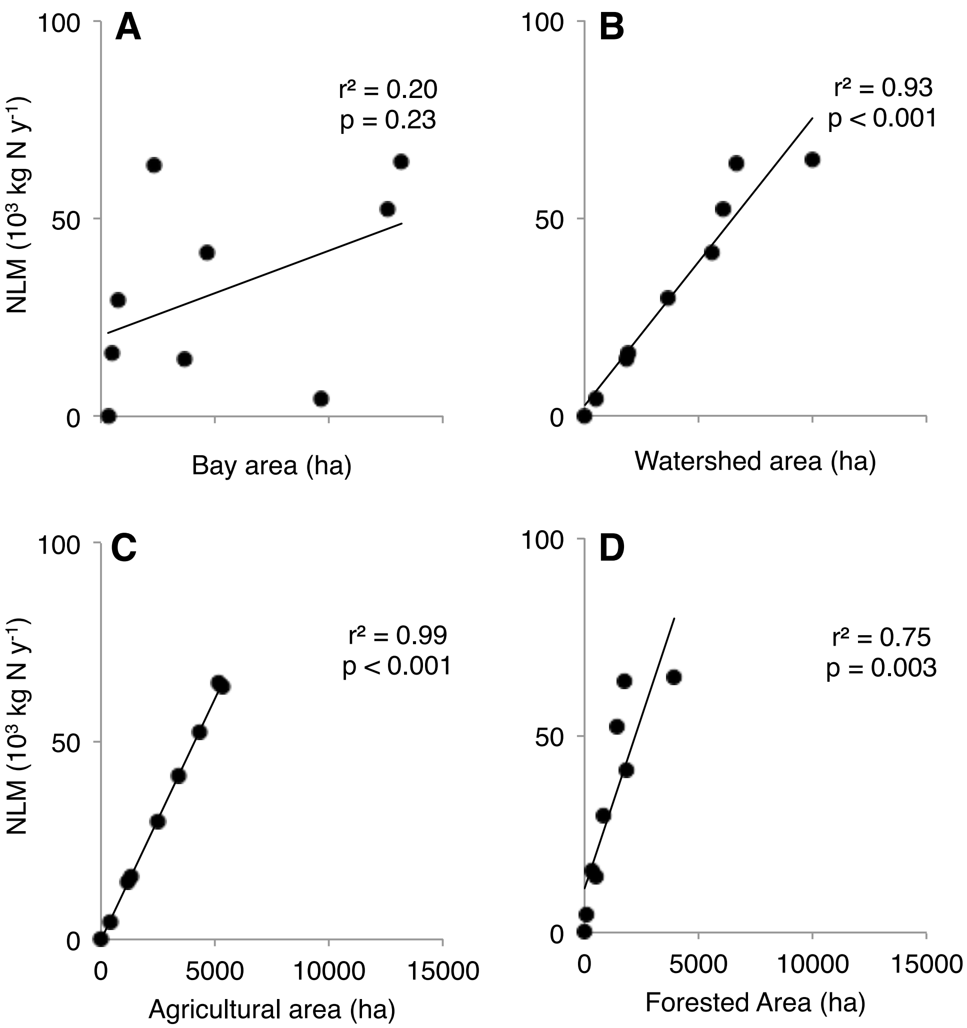

Figure 6. Regressions showing best predictors for the NLM in the Virginia coastal bays. Agricultural fertilizer was often the largest source of terrestrial N, which likely accounts for the high r^2 and low p values.

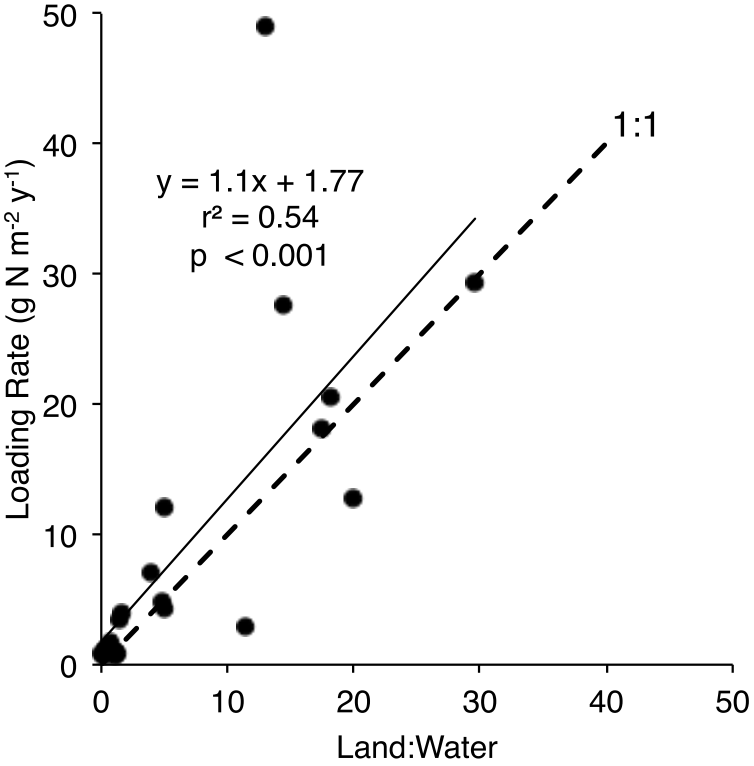

Figure 7. The N loading rates to shallow coastal waterbodies along the east coast of the US as predicted by the associated land:water. All points come from locations in Table 1, excluding those with land:water > 30 (Mobile Bay, AL; San Francisco Bay, CA; Apalachicola Bay, FL).

Chapter Three: Nitrogen fixation in restored seagrass (*Zostera marina* L.) meadows

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ABSTRACT

Biological nitrogen (N_2) fixation is the primary input of new nitrogen to marine systems, and is important in meeting the nitrogen (N) demands of primary producers. In shallow marine systems, seagrass-vegetated sediments often have higher rates of N_2 fixation than bare sediments, although there can be considerable overlap in rates. In this study, we determined if seagrass (*Zostera marina*) restoration in a shallow coastal bay system facilitated increasing rates of N_2 fixation as the meadows aged. Rates of N_2 fixation were measured in a system that had been devoid of seagrass following local extinction in the 1930s until restoration by seeding began in 2001. Restored *Z. marina* meadows of different ages (time since seeding) were compared with nearby bare sediment sites during times of summer peak metabolism over two years. Nutrient sequestration by N_2 fixation was enhanced as the meadows aged. Rates of N_2 fixation in the older (7–8 year old) meadows were 2.7 times more than the younger (2–3 year old) meadows (average 390 µmol N m^{-2} d^{-1} and 146 µmol N m^{-2} d^{-1}, respectively), and 28 times more than bare sediments (average 14 µmol N m^{-2} d^{-1}). The rates of N_2 fixation in the older seagrass meadows were within the range reported for established seagrass meadows in the United States and Europe, indicating that by 7–8 years after initial seeding this function had been restored. This was largely due to the high rates of N_2 fixation by heterotrophic epiphytes on the seagrass leaves, which accounted for ~90% of the total N_2 fixation in *Z. marina* meadows of both age classes. Both sediment and epiphyte N_2 fixation were strongly related to *Z. marina* density, and also to areal productivity and sediment organic content, suggesting that as shoot density increases the positive feedback of plant presence on N_2 fixation through the release of organic carbon exudates into the rhizosphere and phyllosphere and the build up of sediment organic matter also increases. Inhibition experiments showed that sulfate-reducing bacteria were responsible for 12–20% of the N_2 fixation in the sediments. The N provided though fixation represented a large fraction (20.5–30%) of the total N demand to support seagrass aboveground growth during this period of peak summertime production. Our study indicates that continued spread of *Z. marina* would facilitate a positive feedback of N sequestration that supplements the plant and bacterial communities, potentially supporting continued expansion of the seagrass meadow into uncolonized, bare sediment.

INTRODUCTION

In many temperate coastal marine systems, nitrogen (N) is often the limiting nutrient for benthic and pelagic primary producers (Taylor *et al.* 1995). Although dinitrogen (N_2) gas constitutes 62.6% of the gases dissolved in seawater when at equilibrium with air (Pilson 1998), N_2 is biologically unavailable except to specialized prokaryotic N_2-fixing bacteria (Howarth *et al*. 1988, Welsh 2000) and archaea (Dekas *et al*. 2009). To meet the N demand required for growth, primary producers rely on external N sources as well as internal recycling of N. In the open ocean, pelagic N_2-fixing bacteria provide a large source of N on an areal basis compared to sediments, though deep-sea benthic N_2 fixation has only been minimally assessed (Carpenter & Capone 2008, Dekas *et al*. 2009). In estuarine and lagoonal systems, pelagic N_2 fixation is rarely considered to be an important process, and N inputs from benthic N_2 fixation can vary, with higher rates typically measured in tropical systems (Howarth & Marino 2006). Although rates of benthic N_2 fixation have often been assumed to be low in temperate coastal systems (Howarth & Marino 2006), recent studies have shown high rates of benthic N_2 fixation, both in bare sediments (Gardner *et al*. 2006, Fulweiler 2007) and in macrophyte-dominated sediments (Capone & Budin 1982, Howarth *et al*. 1988, McGlathery *et al.* 1998, McGlathery 2008). Thus N_2 fixation can be a major source of N to benthic macrophytes (*e.g.*, McRoy *et al.* 1973, O'Donohue *et al*. 1991, Moriarty & O'Donohue 1993, Welsh *et al*. 1996a, among many others), particularly in systems where pools of inorganic N in the porewater are depleted (O'Donohue *et al.* 1991). Nutrient sequestration is one of the many ecosystem services provided by seagrass habitats in shallow coastal systems. Given the significant loss in seagrass habitats globally (Waycott *et al*. 2009), and concurrent large-scale restoration efforts (Orth *et al*. 2010), it is important to understand the influence of seagrass habitats on N inputs via fixation.

Seagrasses-vegetated sediments often have higher rates of N_2 fixation than bare sediments, although there can be considerable overlap in rates. Studies in temperate estuarine and shallow bay systems report rates from 7–520 μmol N m^{-2} d^{-1} (McGlathery

2008 and sources therein), while in bare sediments free of macrophytes or cyanobacterial mats, reported rates range from 0–300 µmol N m^{-2} d^{-1} in (Howarth *et al.* 1988 and sources therein, Nixon *et al.* 1996). In shallow, vegetated (sub)tropical systems. Much higher N_2 fixation rates have been measured in the rhizosphere of *Zostera capricorni* (1785–2800 µmol N m^{-2} d^{-1}; O'Donohue *et al.* 1991) and in *Thalassia testudinum* (360–10000 µmol N m^{-2} d^{-1}; McGlathery 2008 and sources therein). In vegetated sediments of some temperate systems, N_2-fixing bacteria have been shown to provide 5% to 12% of the annual N requirement to the plant (Welsh *et al*. 1996a, McGlathery *et al.* 1998).

Heterotrophic N_2 fixers are typically the dominant functional group in seagrass-vegetated sediments (McGlathery *et al*. 1998), although autotrophic N_2 fixers are common on illuminated surface sediments (Stal *et al*. 1985, Paerl *et al*. 1996). Heterotrophic N_2-fixing bacteria require organic carbon to fuel N_2 fixation (Penhale & Smith 1977, Kirchman *et al*. 1984, Welsh *et al*. 1996a), and the supply of organic carbon is enhanced by the presence of seagrasses through biological and physical mechanisms. Seagrass photosynthesis results in passive diffusion of photosynthate (dissolved organic carbon) into the rhizosphere, contributing a carbon source for heterotrophic N_2-fixing bacteria (McRoy & Goering 1974, Penhale & Smith, 1977, Oremland & Taylor 1977, Moriarty & Pollard 1982). Seagrasses also apply drag to the overlying water column, slowing water velocities and causing organic particles to fall out of solution and accumulate in seagrass sediments (Fonseca 1982, Marbá *et al.* 2006). This increase in sediment organic matter potentially provides a carbon source for sediment N_2 fixers.

The relative importance of epiphytic N_2 fixation in seagrass meadows has been less studied than sediment N_2 fixation, and most studies have focused on tropical systems

(Goering & Parker 1972, Patriquin & Knowles 1972, McRoy *et al*. 1973, Capone & Taylor 1977, Capone *et al*. 1979, O'Donohue 1991). Epiphytic N_2 fixers can be either autotrophic (Capone & Taylor 1977, Welsh *et al*. 2000) or heterotrophic (Goering & Parker 1972, Capone & Taylor 1977). Reported ratios of autotrophic to heterotrophic epiphyte N_2 fixation have ranged from one-third to two-thirds (Goering & Parker 1972, Capone & Taylor 1977), though most studies have been performed in the light and thus have been limited to gross autotrophic N_2 fixation. Heterotrophic epiphytes require an external carbon source, primarily photosynthate excreted from leaves (McRoy *et al*. 1973, McRoy & Goering 1974, Capone & Taylor 1977, Wetzel & Penhale 1979). It has been shown that the seagrass, *Zostera marina* excretes photosynthate in proportion to the rate of primary production (Penhale & Smith 1977), suggesting that plants with high rates of primary productivity may in turn support high rates of epiphytic N_2 fixation.

The chronic, global loss of seagrass has resulted in a reduction in the capacity of shallow coastal systems to act as carbon and nutrient sinks (Duarte *et al.* 2010). Given the positive feedback between seagrass and N_2 fixation, restoring seagrass in a system devoid of rooted macrophytes can potentially increase the quantity of N sequestered by fixation in these ecosystems. The Virginia coastal bays became a benthic algae-dominated system following the loss of *Zostera marina* due to disease and massive coastal storms in 1933. Following a nearly 70–year absence of *Z. marina*, a large-scale restoration program by seeding began in 2001 that has resulted in approximately 4500 acres of seagrass habitat from 200 acres initially seeded (Orth *et al.* 2006, Orth *et al*. 2010). A restoration program of this scale provides the opportunity to quantify the effects of a state change from an algae-dominated to seagrass-dominated system on N cycling processes by comparing replicate plots of different ages (*i.e.*, time since seeding). Here we report the effects of this state change on N_2 fixation, including those occurring in the sediments and by epiphytes in the seagrass canopy.

METHODS

Site Description

Over 200 km of shallow coastal bays extend from Delaware to Virginia, bounded to the east by barrier islands and to the west by the Delmarva Peninsula. Our study sites are within the Virginia coastal bay system, which is part of the Virginia Coast Reserve Long Term Ecological Research (VCR LTER) site. A large-scale experiment to mimic a state change from an algae- to a seagrass-dominated system was initiated in 2001 by broadcasting seeds into 1-acre plots at 10^5 seeds acre^{-1} (Orth *et al.* 2006, Orth *et al.* 2010), four of which were monitored in this study. In 2006, a similar area was seeded at 1-acre plots with 10^5 seeds acre^{-1}, four of which were monitored in this study. Four bare sites were identified adjacent to each seagrass-restoration site and were sampled concurrently. Here we compare the bare sites with the 2–3 year old (seeded 2006) and 7–8 year old (seeded 2001) seagrass-vegetated sites.

The study sites were located within 20 km of each other (37º24’47” N, 75º43’36” W and 37º15’54” N, 75º48’50” W) and were separated only by mudflats and a tidal channel. The sites are similar with respect to bathymetry, hydrodynamics, and water column and sediment characteristics. Both sites were shallow (<1 m at low tide) and had light penetration capable of supporting the seagrass, *Zostera marina* to -1.6 m MSL

(Lawson *et al*. 2007, Carr *et al*. 2010). The study sites were located between 1.3 and 1.6 m depth at MSL. The tidal amplitude was similar at both sites (1.3 vs. 1.2 m) (McGlathery *et al*. *this issue*). Bare sediment characteristics at the sites, including organic matter, N and C content, exchangeable NH_4^+, and grain size, were also not significantly different (McGlathery *et al*. *this issue*). The largest input of N to the Virginia coastal bay system is direct atmospheric deposition, and the total N load from all sources was calculated to be approximately 2.1 g N m^{-2} y^{-1} (Cole & McGlathery *submitted*), which is extremely low compared to similar shallow coastal bay systems (McGlathery *et al*. 2007).

N_2 fixation

In June and July of 2008 and 2009, sediment and epiphyte N_2 fixation rates were quantified using the acetylene-reduction technique. Data from June and July were selected to represent peak rates of N_2 fixation based on seasonal and monthly data from 2008 and 2009, respectively. Two sediment cores were collected in each of the four replicate plots of each seagrass age class and the eight bare sites. The two sediment cores were taken by hand in both vegetated and bare sediment using a 1” ID clear acrylic corer at random locations within each replicate seagrass plot, making sure the aboveground seagrass biomass was not included. Cores were closed on either end with rubber stoppers, held at ambient temperature in site water, and taken into the laboratory where the top stoppers were removed and the cores were bubbled with an aquarium motor equipped with a diffuser until processing (not more than 2 h). For the incubation, a sub-core was taken from each large core using a 5 cc syringe corer down to 5 cm. The sub-core was split lengthwise to provide adequate exposure to the acetylene and to give a depthintegrated estimate of N_2 fixation rates. Each half was placed in a 40 cc incubation vial with a glass bead and 0.7 mL of ammonium-free artificial seawater, and capped with a screw-top collar holding a septum in place. Samples were sparged with Ar for 2 min, after which time 10 mL of acetylene (C_2H_2) gas was injected into the vials, and the vials were then shaken for 10 seconds and vented to atmospheric pressure. Vials were kept in the dark at field temperature and incubated for 6-8 h. The incubation was ended by transferring the gas samples into a pre-evacuated vacutainer. Gas samples were run on a Varian Star 3400 Cx gas chromatograph equipped with a flame ionization detector and a 6’ x 1/8” x 0.098” Teflon-coated column filled with Porapak N 80/100. For scaling purposes, the halved sub-core samples were summed and extrapolated to area units using the aperture of the core. Rates of sediment acetylene reduction were scaled to fixed N_2 (as NH_4^+) using a 3 : 1 molar conversion (Seitzinger & Garber 1987). An initial analysis to determine the potential for autotrophic sediment N_2 fixation in vegetated sediments showed no differences within a 2 x 2 design for oxic vs. anoxic and light vs. dark incubation ($F = 1.76$, $p = 0.2$), with all rates were comparable to N_2 fixation rates in dark anoxic sediment. Based on this finding and to increase the number of replicates, we used anoxic and dark conditions in subsequent incubations. Autotrophic N_2 fixation was calculated as the difference between incubations in the light (reflecting activity of both autotrophs and heterotrophs) and dark (heterotrophic activity only).

A profile of sediment N_2 fixation with depth was done at 1 cm intervals down to 5 cm in both bare and vegetated plots in May 2009. Samples were inoculated with C_2H_2 and incubated in the same manner as described above.

Shoots of *Zostera marina* were collected at random locations in each of the four replicate plots in the 2–3 and 7–8 year-old meadows, placed into clear, zip-top bags filled with site water, and kept at field temperature. Shoots were taken directly to the laboratory (within 2 h of collection) for analysis. Eight shoots per bay were analyzed for N_2 fixation, chosen randomly from the 5 shoots collected in each replicate plot (2 per replicate plot). For each shoot, the length and width of each leaf was recorded to calculate leaf area, and the top 15 cm of leaves 2 and 4 were placed into 120 mL serum vials filled with 90 mL of filtered site water, and then 10 mL of acetylene-saturated, filtered site water was added. The vials were corked with a septum, crimped with an aluminum collar, shaken for 30 seconds, and incubated at field temperatures. For the epiphytes, an initial test comparing light and dark incubations similar to that done for the sediments indicated that 99% of the epiphyte N_2 fixation was heterotrophic (dark incubations), and so subsequent incubations were done in the dark. Incubations lasted for 4–6 h, and were ended by shaking the bottle vigorously for 30 s, waiting 10 s to allow bubbles trapping gases to dissolve, and then the headspace was drawn into a pre-evacuated vacutainer using a double-ended needle for storage until it was run on the gas chromatograph. As for the sediments, rates of epiphyte acetylene reduction were scaled to N_2 fixed (as NH_4^+) using a 3 : 1 molar conversion (Seitzinger & Garber 1987). The N_2 fixed was representative of the leaf area of the top 15 cm of the leaves incubated. To scale to an areal rate of N_2 fixation, the average of the two leaves incubated was scaled up per shoot, using the area of the top 15 cm of the leaves on the shoot. We incubated the top 15 cm of the leaf knowing that N_2 fixation is less at the base of the leaf since epiphyte colonization is a function of leaf age, and we observed little epiphyte biomass below the top 15 cm of the leaves. However, since our calculated values do not include the entire leaf surface of the plant, our method may give a conservative estimate of epiphyte N_2 fixation. This value was multiplied by shoot density to calculate an areal N_2 fixation rate in μmol N m^{-2} d^{-1}.

Site Characterization

Seagrass density and plant productivity were measured concurrently with the N_2 fixation measurements in both 2008 and 2009. At each of the four replicate plots for each age category, ten 0.25 m^2 PVC quadrats were thrown and the shoots were counted. Plant productivity was measured on all shoots (n = 5 - 20) within two 20 cm x 10 cm anchored grids using the leaf marking method with the top of the sheath bundle as the reference point, puncturing the blades with a 22-gauge needle (Zieman 1974). Shoots were collected 10-15 d after marking. Leaf area and dry weight of the older growth (above the scar) and the new growth (below the scar, but excluding the sheath bundle and including new shoots) were measured separately.

Sediment organic and C:N contents were determined in July in both 2008 and 2009 at the 4 replicate plots in both the 2–3 year old and 7–8 year old seagrass meadows and the eight adjacent bare sites. Five cores were collected equidistant along a 50 m transect in the middle of each plot using a 60 cc syringe and placed immediately on ice. Sediment organic content was calculated as loss on ignition from sediment dry weight after combustion in a 500 ºC muffle furnace for 8 h. Carbon and nitrogen contents were measured on dried sediment (60 ºC for at least 48 h) using a Carlo Erba Elemental Analyzer with a 1020 ºC combustion tube and 650 ºC reduction tube and helium as a carrier gas.

Statistics

For sediment characteristics, replicate samples from each replicate plot were averaged to provide values for each of the bare sites and for the vegetated sites (n=8 for bare, n=4 for each seagrass age class). An initial test showed that the sediment characteristics of the bare sites at the two locations were not significantly different, so all bare sites were pooled for further comparisons with the seagrass sites in each age class. For the seagrass characteristics, replicate measurements of densities and productivities within each plot also were averaged, and then all replicate plots for each age class were then averaged. Differences in pooled sediment and seagrass parameters were tested using ANOVA; all parameters conformed to ANOVA assumptions (heterscedasticity and normality). Epiphyte N_2 fixation rates were first scaled to the whole shoot, and then rates were averaged within each replicate plot based on average shoot densities for that plot; these values were then averaged to obtain mean and variance for the two seagrass age classes. Analyses of epiphyte and sediment N_2 fixation, seagrass characteristics, and sediment profiles as a function of *Zostera marina* meadow age (time since seeding) were done using a two-way ANOVA, and pairwise differences were tested using ad-hoc tukey tests (SAS version 9.2, SAS Institute Inc., Cary, NC). Linear regressions were used to analyze relationships between N_2 fixation rates and plant characteristics or chemical variables using statistical software (Minitab). For all comparisons, p values ≤ 0.05 were considered significant.

RESULTS

Sediment N_2 Fixation

Heterotrophs were the dominant functional group fixing N_2 in the sediments, as indicated by the similar rates between light and dark incubations. Sulfate-reducing N_2-fixing bacteria were responsible for a relatively small percentage of the sediment nitrogen fixation, contributing 16% of the sediment N_2 fixation in both bare and vegetated sites. Sediment N_2 fixation rates were significantly higher in 2008 than in 2009, likely due to low temperatures in 2009 (Fig. 1). In 2008, peak sediment N_2 fixation rates (June–July) in the 7–8 year old seagrass meadows (32.5 µmol N m^{-2} d^{-1}, SE ± 1.2) were significantly higher than in the 2–3 year old meadows (21 µmol N m^{-2} d^{-1}, SE ± 2.0) and bare sediments (18 µmol N m^{-2} d^{-1}, SE ± 1.5, Fig. 1). In 2009, however, the only significant differences were between the 7–8 year old meadows (18 µmol N m^{-2} d^{-1}, SE ± 1.0) and bare sediments (9.7 µmol N m^{-2} d^{-1}, SE ± 3.0, Fig. 1). There were no significant differences between the bare sediments and 2–3 year old meadows in either year. Depth profiles of sediment N_2 fixation showed that rates increased with depth up to 5 cm in the seagrass-vegetated sediments and decreased with depth in the bare sediments (Fig. 2).

Shoot density, areal rates of primary productivity, and sediment organic matter were identified as environmental variables that could influence sediment N_2 fixation rates in seagrass-vegetated sediments of different ages (time since seeding) (Table 1). The increase in *Zostera marina* shoot density was positively correlated with sediment N_2 fixation in both 2008 and 2009 (Fig. 3). Additionally, the higher rates of areal productivity were positively correlated with higher rates of sediment N_2 fixation, though only significantly in 2008 (Fig. 3). Lastly, higher sediment organic matter was positively correlated with sediment N_2 fixation, though again only significantly in 2008 (Fig. 3). Despite having an $r^2 = 0.93$, the low number of samples in 2009 yielded results that were not statistically significantly different.

Epiphyte N_2 Fixation

Rates of epiphyte N_2 fixation were higher in the 7–8 year old *Zostera marina* meadows than in the 2–3 year old meadows during both years (Fig. 4). Heterotrophs were the dominant functional group of the epiphytic N_2 fixers in both the 7–8 and 2–3 year old meadows; our initial test indicated that autotrophs fixed less than 1% of the total N_2 fixed. Rates of epiphyte N_2 fixation in 2009 were significantly higher than in 2008 (Fig. 4), an opposite trend than what was measured for sediment N_2 fixation (Fig. 1).

Areal rates of epiphyte N_2 fixation were positively correlated with *Zostera marina* shoot density, though not significantly in either year (Fig. 5). Epiphyte N_2 fixation rates were significantly and positively correlated with areal productivity rates in both 2008 and 2009 (Fig. 5). The slopes of the regressions for epiphyte N_2 fixation versus density and productivity were considerably steeper in 2008 than in 2009 (Fig. 5).

To determine what variable was affecting the difference in rates of N_2 fixation and plant productivity, we analyzed temperature records during the sampling periods. We found a significant difference in air temperature for June–July in 2008 and 2009, with a higher mean temperature in June–July 2008 of 0.81 ºC (VCR LTER, *http://www.vcrlter.virginia.edu/data/metdata/index.html.*).

Seagrass and sediment parameters

Seagrass and sediment parameters for the bare sediments and different-aged seagrass meadows are summarized in Table 1. The 7–8 year old *Zostera marina* meadows had significantly higher shoot densities than the 2–3 year old meadows in both 2008 and 2009 (Table 2). The rates of productivity per shoot were not different between the 2–3 and 7–8 year old meadows in 2008 or 2009. However, productivity rates per shoot were higher at both seagrass sites in 2008 than in 2009, presumably due to the higher mean water temperature in 2009 (Table 2). The average N content of *Z. marina* tissue was significantly higher in the 2–3 year old meadows (2.3% vs. 1.2% in 7–8 year old). Average C:N indicated that the younger meadows were significantly less N limited than the older meadows (C:N 20.6 v. 37.3). The 7–8 year old meadows had a significantly higher sediment organic matter than the younger meadows and the bare sediments, however, there were no differences in sediment organic matter between the 2–3 year old meadows and bare sediments ($p = 0.32$).

DISCUSSION

Total rates of N_2 fixation

Our data show that nutrient sequestration by N_2 fixation in restored seagrass meadows was enhanced as the meadows aged. The markedly higher rates of N_2 fixation in the 7–8 year old *Zostera marina*-vegetated system compared to the unvegetated sediments illustrate the effects of the state change from a bare to a macrophyte-dominated benthos. N_2-fixing bacteria in the older (7–8 year old) meadows fixed on average 390 µmol N m^{-2} d^{-1}, 2.7 times more than the younger, 2–3 year old meadows (146 µmol N m^{-2} d^{-1}), and 28 times more than bare sediment sites (14 µmol N m^{-2} d^{-1}), and the majority of the N_2 fixed was by heterotrophs (Fig. 6). The higher variance at the vegetated sites was a result of the high and variable rates of epiphyte N_2 fixation. Our findings are in agreement with previous studies showing a positive feedback between seagrass presence and rates of N_2 fixation (Capone 1988, Welsh *et al*. 2000, Carpenter & Capone 2008), but show for the first time that this feedback is based on seagrass density. N_2 fixation rates in the younger meadows where shoot densities were on average <100 shoots m^{-2} were not different than nearby unvegetated sediments, indicating there this is a lag time after initial seeding before this feedback is apparent at the m^{-2} scale. Although we do not know the exact threshold density where vegetated sediments had higher N_2 fixation rates that bare sediments, our data show that by 7–8 years since seeding when densities were >400 shoots m^{-2}, rates were significantly greater.

The rates of N_2 fixation in the older meadows of the Virginia coastal bays are within the range reported for seagrass meadows in other temperate regions, indicating that by 7–8 years since seeding this function had been restored relative to established meadows. In a study of *Zostera marina* in the Chesapeake Bay, Capone (1982) found rates of N_2 fixation (279–464 µmol N m^{-2} d^{-1}) comparable to our 7–8 year old site. The results of two European studies of *Z. marina* and *Z. noltii* also are consistent with our findings, with rates of N_2 fixation ranging from 300–429 µmol N m^{-2} d^{-1} (McGlathery *et al*. 1998) and 143–521 µmol N m^{-2} d^{-1} (Welsh *et al*. 1996a), respectively. It is important to note that the total N_2 fixation rates were equivalent primarily because our epiphyte N_2 fixation rates were very high. Our rates of total N_2 fixation only include heterotrophic N_2

fixation based on our findings that autotrophic N_2 fixation contributed a minor amount of N_2 fixed, and thus may be a slightly conservative estimate of total N_2 fixation rates.

Contributions of epiphyte and sediment N_2 fixation

The majority of total N_2 fixation (epiphyte + sediment N_2 fixation) in the seagrass meadows of both age classes was attributed to epiphytes on the seagrass blades, which accounted for 89% and 93% of the total N_2 fixed in the younger and older meadows, respectively (Fig. 6). The epiphytes on the *Zostera marina* shoots in the older meadows fixed significantly more N_2 than in the younger meadows on an areal basis in both years. Because shoot-specific rates of N_2 fixation and primary productivity were not different between meadows in the different age classes, we attribute the higher epiphyte N_2 fixation in the older meadows to differences in shoot densities that were a function of seagrass meadow age (Fig. 5).

The dominance of N_2 fixation by epiphytes compared to sediments has not been shown previously in other temperate seagrass meadows. We know of only one study in a tropical system, the Gulf of Carpentaria, Australia, where N_2 fixation was shown to be dominated by epiphytes for the seagrass *Enhalus acoroides* (300 μmol N m^{-2} d^{-1} compared to rinsed roots (21 μmol N m^{-2} d^{-1} and rhizomes (7 μmol N m^{-2} d^{-1})) during summer months (Moriarty & O'Donohue 1993). In our study, summertime epiphyte N_2 fixation rates ranged from 2–256 μmol N m^{-2} d^{-1} (avg. 129 μmol N m^{-2} d^{-1}) in the younger meadows, and rates were significantly higher (123–604 μmol N m^{-2} d^{-1}; avg. 364 μmol N m^{-2} d^{-1}) in the older seagrass meadows (Fig. 4). For temperate seagrass meadows, previous studies have shown that *Zostera marina* leaves collected in North Carolina had “extremely low or undetectable” rates of epiphyte N_2 fixation (McRoy *et al.* 1973), and *Ruppia maritima* collected from brackish water in Chesapeake Bay had rates of 5 μmol N m^{-2} d^{-1} (Lipschultz *et al.* 1979). A comparison with tropical systems shows some similar rates to those we measured, where N_2 fixation associated with *Thalassia testudinum* leaves ranged from 82–228 μmol N m^{-2} d^{-1} (Capone *et al*. 1979) to 285–357 μmol N m^{-2} d^{-1} (Capone & Taylor 1977). The high rates of epiphyte N_2 fixation in the Virginia coastal bays may be in part related to the low nutrient status and high water quality of these coastal bays. External nutrient loading rates to the Virginia coastal bays are very low compared to other shallow coastal bay systems (Cole & McGlathery *submitted*, Giordano *et al*. 2011, McGlathery *et al*. 2007), and the 16-year data base from the VCR LTER shows that water column chlorophyll and total suspended solids are very low and have shown no trends of decreasing water quality during this period (www1.vcrlter.virginia.edu/home1/?q=data_wq).

Sediment N_2 fixation measured in the rhizosphere of *Zostera marina* only comprised on average 11% of the total N_2 fixed in the younger meadows and 7% in the older meadows. Rates of N_2 fixation in the 7–8 year old *Z. marina* meadows were significantly higher than the younger meadow in 2008—but not in 2009—though the trend of increasing sediment N_2 fixation with seagrass meadow age was evident that year. We attribute this difference to the lower temperatures in 2009 compared to 2008, which translated into lower seagrass productivity rates. We found rates of sediment N_2 fixation in the rhizosphere of the restored *Z. marina* meadows during the summer months were lower than previously reported values for vegetated sediments in coastal Virginia (16–25 μmol N m^{-2} d^{-1} this study, 279–464 μmol N m^{-2} d^{-1} Capone 1982). Peak sediment N_2

fixation rates reported from temperate European seagrass meadows were also consistently higher than those found in this study (143–521 µmol N m^{-2} d^{-1}, Welsh *et al.* 1996a; 300–429 µmol N m^{-2} d^{-1}, McGlathery *et al.* 1998). We suspect the rates of sediment N_2 fixation in this study were much lower than the older, more established seagrass meadows reported in the literature because the shoot densities in our study location are comparatively low (Table 1; Neckles *et al.* 1993, Moore & Wetzel 2000), and this influences the positive feedback between seagrass presence and N_2 fixation.

Drivers of N_2 Fixation

The mechanisms behind the positive relationship between *Zostera marina* shoot density and areal sediment and epiphyte N_2 fixation rates that were apparent in the different aged meadows during both years are likely the stimulation of bacterial activity in the rhizosphere by the excretion of organic carbon from seagrass roots and also by accumulation of organic matter in the sediments. The majority of the N_2 fixation (both sediment and epiphytic) in the Virginia coastal bays was heterotrophic, and it is well known that heterotrophs in seagrass-vegetated sediments can be stimulated by carbon inputs from both plant metabolism (McRoy & Goering 1974, Penhale & Smith 1977, Kirchman *et al.* 1984, Moriarty *et al.* 1986) and by accumulation of organic carbon (Moriarty *et al.* 1986, Welsh *et al.* 1996b, McGlathery *et al.* 1998). The depth profiles of N_2 fixation in the sediment highlighted the influence of seagrass on N_2 fixation, showing peak rates in the rhizosphere of vegetated sediments where maximum root biomass occurred (4–5 cm below surface), while the peak in bare sediment cores was at the surface (0–1 cm) (Fig. 2). This is consistent with other studies showing depth-related differences in bacterial processes in seagrass-vegetated sediments (O'Donohue *et al*. 1991, Moriarty & O'Donohue 1993, McGlathery *et al*. 1998). The sediments in the older, more dense seagrass meadows, where the sediment organic content was significantly higher, also had higher sediment N_2 fixation than bare sediments (Fig. 1). But unlike many other temperate systems with more mature seagrass meadows, the role of N_2-fixing sulfate-reducing bacteria was relatively low; sulfate reducers fixed only 12–20% of the total N_2 compared to > 80% (Capone 1982, Welsh *et al*. 1996b), though similar rates of N_2 fixation by sulfate reducing bacteria have been observed in some meadows (25% of total N_2 fixed, McGlathery *et al*. 1998). The low concentrations of organic matter in the rhizosphere (1–1.8%) may limit sulfate reduction in these sandy sediments.

The low levels of sediment organic matter likely contributed to the overall low levels of sediment N_2 fixation in the Virginia coastal bays compared to other sites. We suspect that the consistently low anthropogenic N input rates, water column N and chlorophyll concentrations in the VCR coastal bays (Cole & McGlathery *submitted*, Giordano *et al*. 2011, VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq), limit the input of organic carbon to the benthos (Nixon *et al*. 2009). With low inputs of organic carbon, heterotrophic N_2 fixers cannot produce the ATP required for N_2 fixation (Herbert 1975, Welsh 2000, among others). As seagrass meadows develop over time, both in size and shoot density, the seagrass community will exert more drag on the overlying water column and will increase the deposition of organic particles from the overlying water column and reduce sediment suspension (Fonseca 1982, Moore 2004, Marbá *et al.* 2006, Chen *et al*. 2007, Gruber & Kemp 2010, Hansen and Reidenbach, *this issue*). Our findings suggest the mechanism of drag-induced particle deposition can be affected by meadow age as seagrass density and sediment organic matter increases because both of these variables correlated positively to an increase in sediment N_2 fixation (Fig. 3).

Contribution to *Zostera marina* N demand

Nitrogen demand was calculated from the N content of the seagrass leaves, the shoot-specific primary production rate, and shoot density. During the study period, the N demand for the 2–3 year old seagrass meadow was 488 µmol N m^{-2} d^{-1} and 1903 µmol N m^{-2} d^{-1} for the 7–8 year old meadow. The 16.4 and 25.8 µmol N m^{-2} d^{-1} supplied by sediment N_2 fixation in the younger and older meadow, respectively, had the potential to supply a 3.4% and 1.4% of the total N demand of each seagrass meadow. The larger amount of N_2 fixed by epiphytes had the potential to provide 19.1% to 26.5% to the total N demand in the 7–8 year old and 2–3 year old meadows, respectively. Combining the sediment and epiphyte N_2 fixation rates, we see that N_2 fixation could supply 29.8% of the total *Zostera marina* N demand in the younger meadows and 20.5% in the older meadows, respectively, during this period of high summer production. These contributions are higher than those reported previously for temperate seagrass meadows on an annual basis (5% to 12% of the N requirement, Welsh *et al.* 1996a, McGlathery *et al.* 1998), largely due to the significant contribution of epiphyte N_2 fixation. The CN ratios for the seagrass tissues in the restored meadows (younger: 21, older: 37) and the low N content of the leaves (younger meadow: 2.3%, older meadow: 1.2%) suggest that the plants were N limited (Duarte 1990), and internal recycling of N before tissue senescence may reduce the demand for external sources (Pedersen & Borum 1993).

We expect that as the restored seagrass beds continue to age, remineralized N will provide a greater proportion of the N required to support growth as sediment organic matter and standing stock nutrient concentrations increase (Bronk & Steinberg 2008, McGlathery 2008). Tyler *et al*. (2003) found this pattern in a *Spartina alterniflora*-dominated salt marsh chronosequence of sites in the VCR ranging in age from 7–150 years, where sediment N_2 fixation decreased with stand age presumably as a result of increased porewater NH_4^+ concentrations.

System-wide scaling

After over a 70-year period when the coastal bays of the Virginia Coast Reserve were devoid of seagrasses, restored *Zostera marina* meadows (7–8 year old in this study) fixed N_2 at rates comparable to established mature meadows primarily because of high rates of heterotrophic epiphyte N_2 fixation, and these rates were nearly 30-fold higher than bare sediments. If the rates of N_2 fixation for the older seagrass meadows are scaled to *Z. marina* potential habit in the VCR coastal bays (depths at MSL between 0.6 and1.6 m; Carr *et al.* 2010, Carr *et al*. *this issue*), we see the potential for nearly 3.3×10^5 kg N to be sequestered each year, which equates to 77% of the allochthonous N from the watersheds of the Virginia Coast Reserve (Cole & McGlathery, *submitted*). In comparison, if the same potential habitat was to remain unvegetated, the N_2 fixed would be only 3% of the total allochthonous N delivered to the coastal bays. Our study indicates that continued spread of *Z. marina* would facilitate a positive feedback of N sequestration that supplements the plant and bacterial communities, potentially supporting continued expansion of the seagrass meadow into uncolonized, bare sediment.

Table 1. Seagrass and sediment parameters of restored *Zostera marina* meadows and associated bare sediment. Standard error represented by values following (±).

		Bare	2–3 y		7–8 y	
	unit		2008	2009	2008	2009
Density	m^{-2}		33 ± 5	88 ± 51	412 ± 21	515 ± 22
Shoot productivity	$cm^2\ d^{-1}$		0.75 ± 0.05	0.18 ± 0.09	0.87 ± 0.16	0.27 ± 0.12
C:N (sed.)		9.0 ± 1.8	18.7 ± 6.7	5.9 ± 2.2	5.2 ± 0.7	8.1 ± 1.0
Organic matter (sed.)	%	1.48 ± 0.1	1.3 ± 0.3		1.74 ± 0.6	

Table 2. ANOVA table of p values (α = 0.05) for biological and physical seagrass characteristics between meadow ages and over the course of this study.

block	comparison	Density	Productivity	foliar N	foliar C	CN
2–3 y	2008 v. 2009	0.26	0.005	—	—	—
7–8 y	2008 v. 2009	0.01	0.04	—	—	—
2008	2–3 v. 7–8 y	< 0.001	0.54	—	—	—
2009	2–3 v. 7–8 y	< 0.001	0.56	—	—	—
all	2–3 v. 7–8 y	< 0.001	< 0.001	< 0.001	0.33	< 0.01

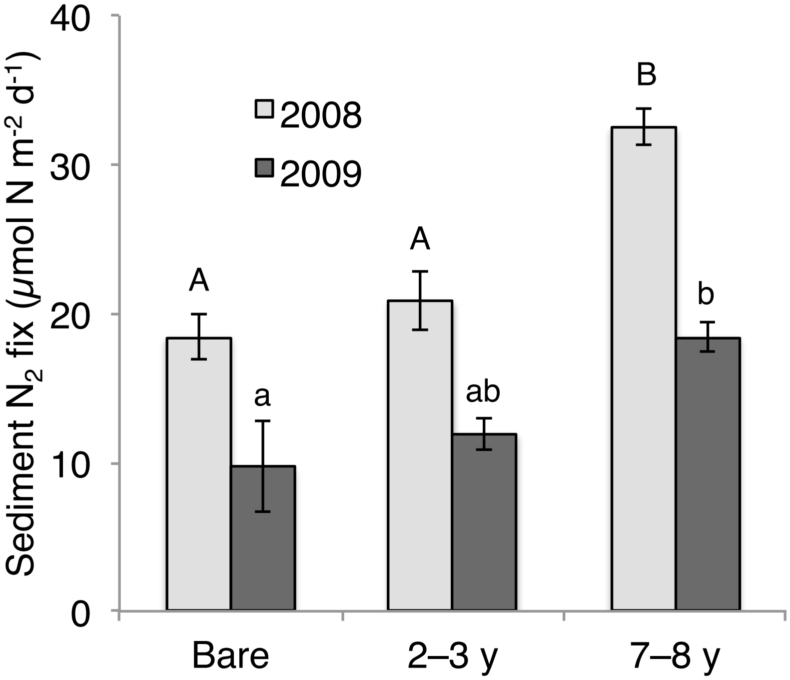

Figure 1. Sediment N_2 fixation in bare sediment (bare) and in the rhizosphere of restored *Zostera marina* meadows. The vegetated sediments are 2–3 and 7–8 years old. Significant differences represented by difference in letter coding (ANOVA, post-hoc Tukey test); case is relative to year. Error bars represent ±1 SE.

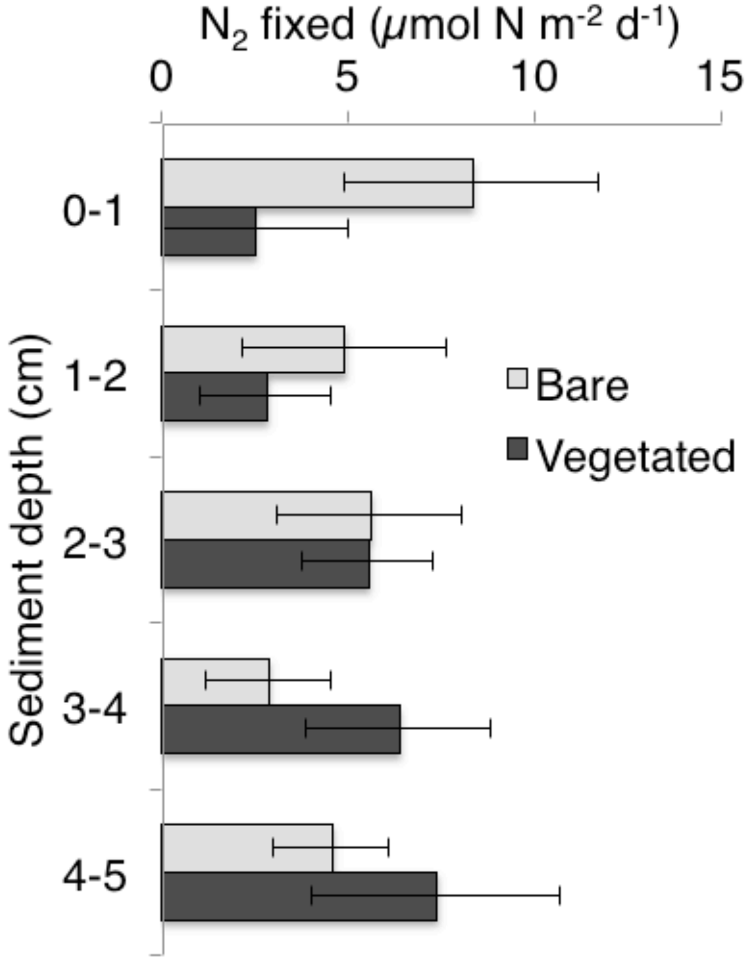

Figure 2. Depth profile of sediment N_2 fixation in bare and *Zostera marina* vegetated sediment. No significant differences across depth or sediment type, though peak rates and depth relationships are different in presence v. absence of seagrass. Error bars represent ±1 SE.

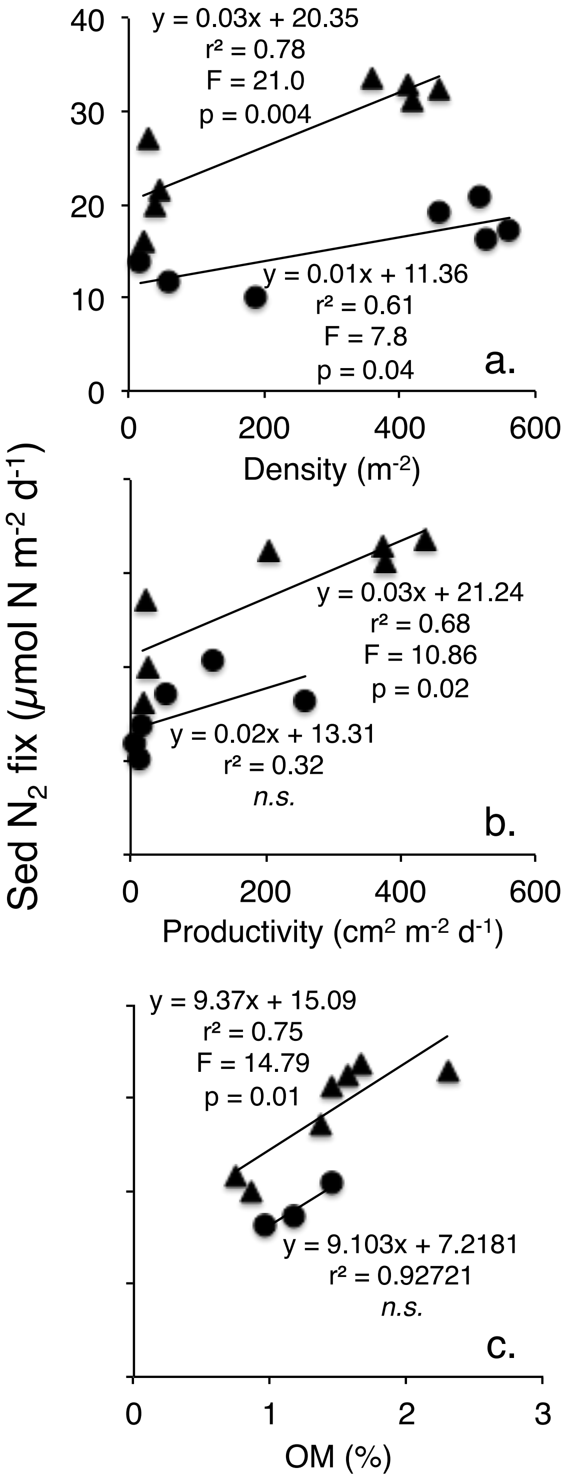

Figure 3. Sediment N_2 fixation regressed with (a) shoot density, (b) areal productivity, and (c) sediment organic matter. Triangles represent 2008 and circles represent 2009.

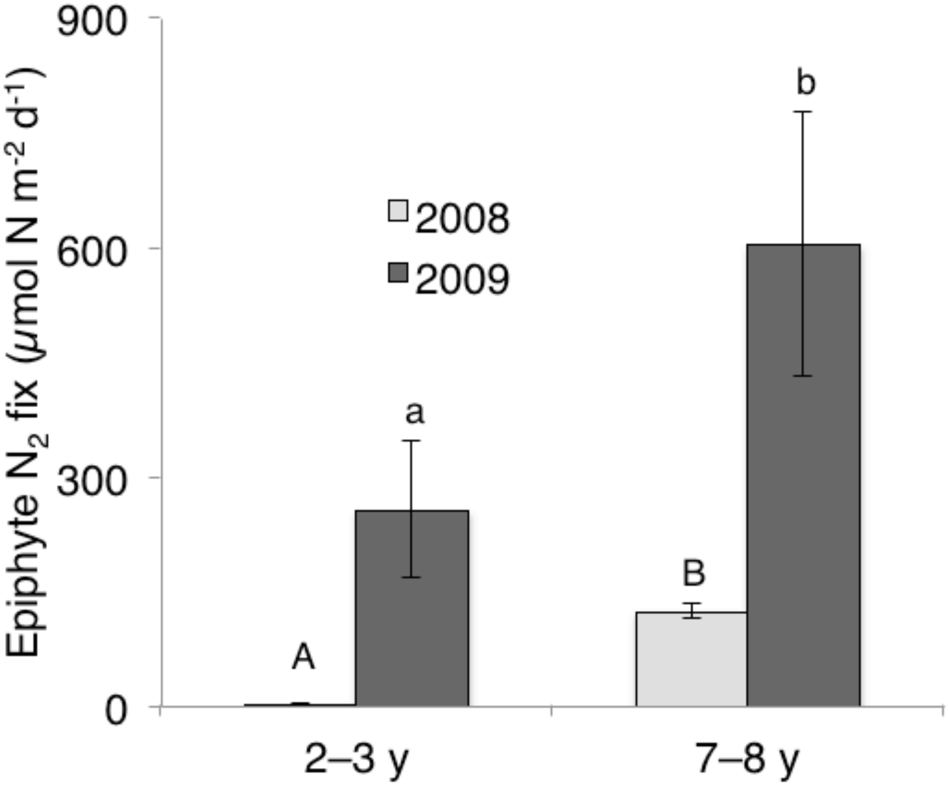

Figure 4. Epiphyte N_2 fixation in the seagrasses. Significant differences represented by difference in letter coding (ANOVA, post-hoc Tukey test); case is relative to year. Error bars represent ±1 SE.

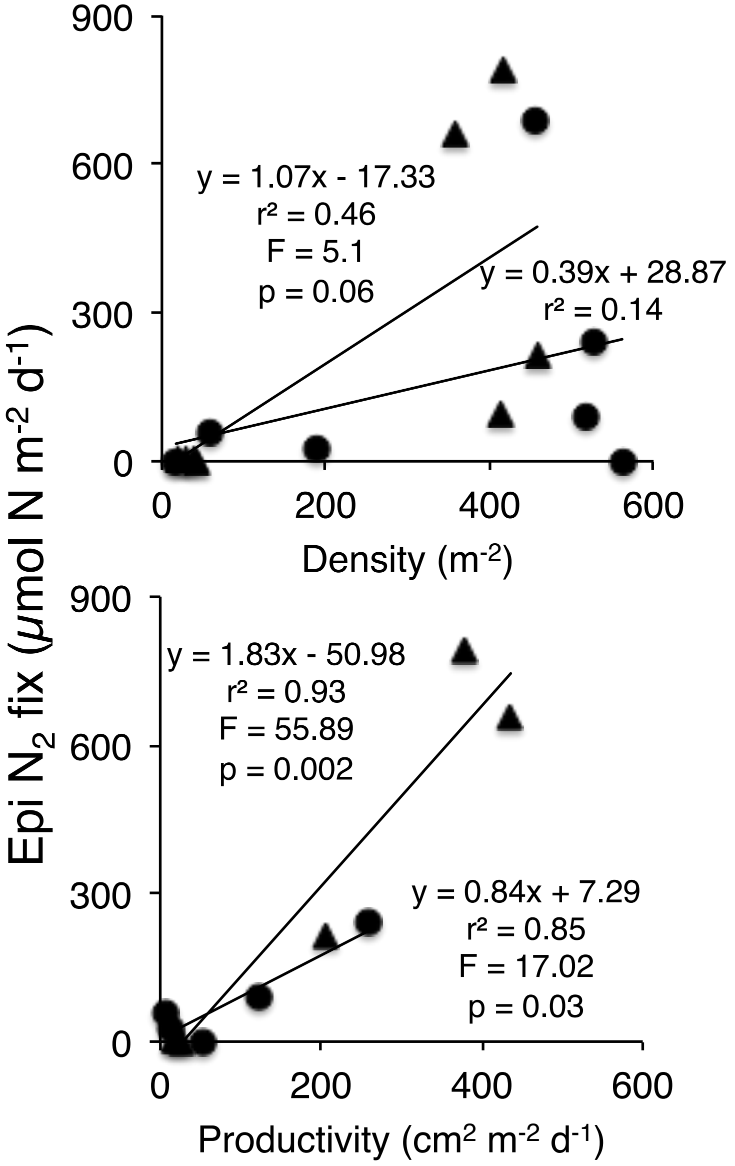

Figure 5. Regressions of epiphytic N_2 fixation with shoot density and areal productivity. Triangles represent 2008 and circles represent 2009.

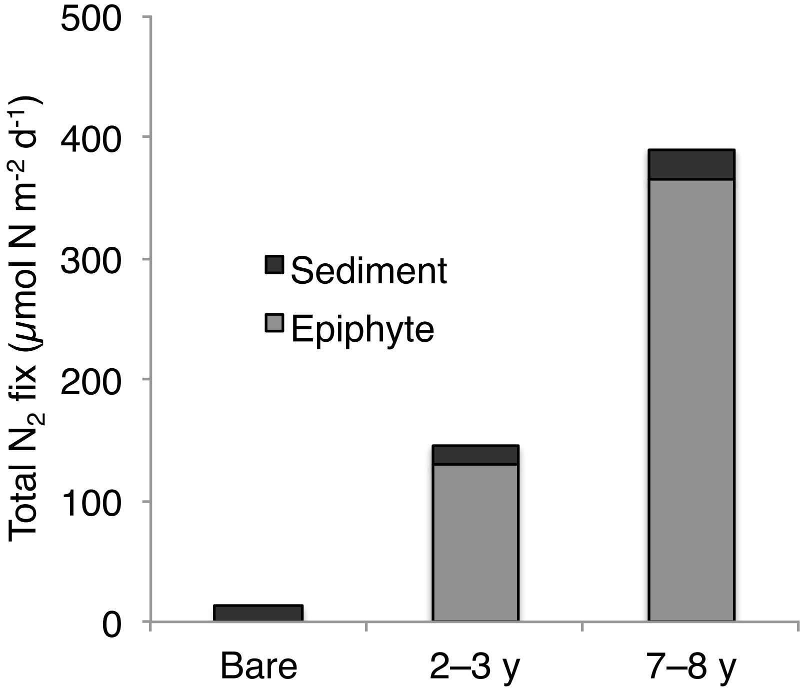

Figure 6. Total rates of N_2 fixation. "Bare" represents sediment N_2 fixation in bare sediment only, while the columns for the *Z. marina* vegetated sediments are the sum of sediment N_2 fixation and epiphyte N_2 fixation. Variance of rates and statistics are reported in the text.

Chapter Four: Depth influences N_2 fixation rates in a temperate shallow seagrass system

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ABSTRACT

Nitrogen fixation rates were measured in the sediment and epiphyte communities of restored *Zostera marina* [L.] meadows growing along a depth gradient (0.9–1.5 m, below mean seal level [MSL]) in Hog Island Bay, Virginia, USA. Total nitrogen (N_2) fixation (epiphytic + sediment N_2 fixation) decreased log-normally with depth, and heterotrophic epiphytic N_2 fixation was the dominant process in water shallower than 1 m at mean sea level (MSL). Total N_2 fixation and epiphytic N_2 fixation were most strongly correlated to shoot density, which decreased across the depth gradient. At depths greater than 1 m MSL, total N_2 fixation was markedly lower and dominated by sediment bacteria. Sediment N_2 fixation rates were low and did not correlate with any seagrass or sediment parameters. However, significant relationships between depth and sediment C, N, C:N, and organic matter were observed. Total N_2 fixation rates in shallower water were comparable to other temperate seagrass systems, but in deeper water, rates were not significantly different from bare sediment.

INTRODUCTION

Seagrasses are the dominant rooted macrophytes in many temperate coastal systems. *Zostera marina* [L.] (eelgrass) is a marine angiosperm that grows along a narrow bathymetric gradient (c.a. 0–5.5 m; Dennison & Alberte 1985, Duarte 1991) in temperate systems worldwide. Seagrass habitat availability is often limited by the light intensity reaching the seafloor, which can be affected by water column depth or by shading from epiphytes (Bulthuis & Woelkerling 1983) and / or macroalgae (McGlathery 2001). Seagrasses indirectly increase water clarity by trapping particulates in the water column, which has a positive feedback of increasing the depth of the photic zone and the spatial extent of potential seagrass habitat (Fonseca 1982, Marbá *et al*. 2006). Seagrasses can colonize deeper sediments when the light compensation point increases (Dennison 1987). This suggests that in initially turbid waters, the positive feedback whereby increased seagrass density effects an increase in water clarity, and facilitates the spread into deeper waters (Carr *et al*. 2010).

Seagrasses provide structure to the benthos that does not exist when sediments are unvegetated (*i.e.*, bare or algal-dominated). Aside from the physical effects on the water column, seagrasses provide habitat for organisms ranging from cyanobacteria to mollusks and fish species (Connolly *et al*. 2005, McGlathery 2008). Bacteria are particularly important in seagrass meadows as rhizosphere and phyllosphere bacteria are capable of converting inert N_2 gas into a biologically usable form (NH_4^+), which provides a source of nitrogen (N) to the plants and sequesters new N into the ecosystem (Cole & McGlathery *submitted*). Rates of N_2 fixation in seagrass-vegetated sediments are typically higher than in bare sediment alone (McGlathery 2008). Fixed N can satisfy 3–10% of the N requirement for seagrasses in temperate systems (McGlathery 1998, Risgaard-Petersen *et al*. 1998, Cole & McGlathery *submitted*), providing an important nutrient source in what are typically N-limited systems (Duarte 1990).

Nitrogen fixation in marine sediments and epiphyte communities is enhanced by the availability of organic matter (OM), which is often due to the presence of seagrasses (Howarth *et al*. 1988a). Vegetated sediments often have higher rates of N_2 fixation than bare sediment as a result of increased OM associated with decaying detritus (Tibbles *et al*. 1994) and the exudation of OM into the rhizosphere resulting from plant photosynthesis (McRoy *et al*. 1973, Penhale & Smith 1977, Neckles *et al*. 1994). Heterotrophic epiphytic N_2 fixers—the dominant N_2 fixers in shallow mid-Atlantic bays (Cole & McGlathery *submitted*)—use photosynthate (as OM) exuded from the leaves of seagrasses as their carbon source, implicating photosynthesis and its associated OM products as primary regulators of N_2 fixation in seagrass meadows (McRoy & Goering 1974, Penhale & Smith 1977). Heterotrophic N_2 fixers in the seagrass phyllosphere and rhizosphere are affected by the intensity of PAR and plant productivity (Moore & Wetzel 2000, Nielsen *et al*. 2002). The diurnal trends of microbial growth rates in the rhizosphere and seagrass photosynthetic rates are the same, as photosynthate pumped into the rhizosphere during plant primary production provides a carbon source for the bacteria (Moriarty & Pollard 1982).

Our previous work has shown that, on an areal basis, *Z. marina* shoot density can be the primary driver of sediment N_2 fixation, as a higher density of shoots exuding dissolved organic matter into the rhizosphere had higher rates of sediment N_2 fixation than a less-dense meadow (Cole & McGlathery *submitted*). Our earlier results showed no difference in productivity per shoot, and suggested that areal rates of heterotrophic sediment N_2 fixation were regulated by shoot density, and that changes in light level (*e.g.*, with depth) may have concomitant changes in sediment N_2 fixation. In shallow but light-limited systems (like shallow coastal lagoons) where seagrasses grow across a very narrow depth range, seagrass productivity—and thus epiphytic N_2 fixation—may decrease across a small depth gradient.

We report epiphyte and sediment N_2 fixation associated with seagrass meadows experimentally reseeded in 2006 along a depth gradient (0.9–1.5 m MSL) in Hog Island Bay, a shallow lagoon system in the Virginia Coast Reserve Long-Term Ecological Research (VCR LTER) (Fig. 1). The Virginia coastal bays were replete with *Z. marina* until the early 1930s, when a chronic wasting disease and hurricanes extirpated the plants, resulting in a drastic increase in suspended sediments in the water column (Orth *et al*. 2006, Orth *et al*. 2010, McGlathery *et al*. *submitted*). Following the discovery of a small patch of seagrass in the late 1990s, a large-scale restoration program by seeding began in 2001 and continued through 2009, which has resulted in approximately 4500 acres of seagrass habitat from 200 acres initially seeded (Orth *et al.* 2006, Orth *et al.* 2010). The epiphyte and sediment N_2 fixation rates were correlated with physical (sediment C, N, and organic matter contents) and biological (seagrass density, productivity) characteristics known to influence N_2 fixation.

METHODS

Site description

The lagoons the of Virginia Coast Reserve are part of a 200 km long stretch of shallow coastal bays extending from Delaware to Virginia. These lagoons are bounded to the east by a chain of barrier islands and to the west by the Delmarva Peninsula. In 2006, restoration of replicate plots within a 2 km^2 (500 acre) “set aside” in Hog Island Bay was done by broadcasting *Zostera marina* seeds by hand. In this study, we measured sediment and epiphyte N_2 fixation in 7 replicate 1-acre plots seeded at a density of 100,000 seeds $acre^{-1}$ along a depth gradient of 0.6 m (0.9–1.5 m MSL) with a mean slope of 0.03% (Oertel 2001).

N_2 fixation sampling

Sediment and seagrass samples were collected in July 2009 at each replicate plot along the depth gradient to determine sediment and epiphyte N_2 fixation rates using the acetylene reduction technique. N_2-fixing bacteria are capable of substituting acetylene for N_2 gas, resulting in the evolution of ethylene (C_2H_4). While rates have been shown to be variable, we used a ratio of 3:1 when converting ethylene to NH_4^+ produced, as suggested for shallow temperate systems (Seitzinger & Garber 1987).

Sediment N_2 fixation

Paired sediment cores were collected at each replicate site using 1” ID clear acrylic tubing fitted with rubber stoppers, and were held with their tops open in site water until laboratory analysis (within 3 h of collection). In the laboratory, subcores were taken down to 5 cm using a 5 cc syringe corer. The subcore was split lengthwise, and each half was placed in a 40 cc incubation vial with a glass bead, 0.7 mL of NH_4^+-free artificial seawater, and closed with a screw-top collar and a gas-impermeable septum. Samples were purged and vented with Ar for two minutes and 10 mL of acetylene gas (C_2H_2) were injected into the vials. Following the introduction of acetylene gas, samples were swirled to slurry the sediment then vented to atmospheric pressure using a 22-gauge needle. The incubation was done in the dark at field temperature for 6–8 hours as per Cole & McGlathery (*submitted*), after which the samples were again shaken, and the headspace was injected into a pre-evacuated vacutainer. Samples were run on a Varian Star 3400 Cx gas chromatograph with a flame ionization detector and a 6' x 1/8" x 0.098 Teflon-coated column filled with Porapak N 80/100. Split-core samples were summed, and replicate cores were averaged by site. N_2 fixation rates were scaled to area using the aperture of the corer.

Epiphyte N_2 fixation

To measure epiphyte N_2 fixation, shoots were collected in triplicate at each replicate site along the depth gradient, and the top 15 cm of blades 2 and 4 from each individual plant were incubated in acetylene-saturated filtered site water. Blades were placed in a 120 mL serum vial with 90 mL of filtered site water (0.45 µm supor filter) and 10 mL of acetylene-saturated water, were crimped shut, and shaken for 30 seconds. The samples were placed in the dark at site temperature for 4–6 hours, as heterotrophic bacteria were found previously to be the dominant epiphyte functional group of N_2 fixers in the Virginia coastal bay seagrass meadows (Cole & McGlathery *submitted*). After the incubation, the samples were again shaken for 30 seconds and the headspace was injected into a pre-evacuated vacutainer. The headspace samples were analyzed on the same gas chromatograph used for sediment N_2 fixation. Leaf N_2 fixation values were averaged and multiplied by the total number of leaves on the shoot. This value was multiplied by shoot density to calculate an areal N_2 fixation rate in µmol N m^{-2} d^{-1}.

We incubated the top 15 cm of the leaf knowing that N_2 fixation is less at the base of the leaf since epiphyte colonization is a function of leaf age, and we observed little epiphyte biomass below the top 15 cm of the leaves. However, since our calculated values do not include the entire leaf surface of the plant, our method may give a conservative estimate of epiphyte N_2 fixation.

Biological and physical characteristics

In July 2009, seagrass density and productivity were measured in the 7 plots along the depth gradient. Shoot density was measured 4 times within each replicate plot by randomly throwing a 0.25 m^2 PVC frame and counting the shoots that were within the frame. Shoot productivity was measured using the leaf-marking method described in Zieman (1974). In at least 2 locations within each replicate plot, a 10 cm x 20 cm PVC frame was anchored to the sediment, shoots within the frame were marked, and remained in the field for 12–20 days. At the end of the study, the shoots in the frame were harvested, put on ice, and taken to the laboratory for analysis. Marked leaves were separated into old and new tissue (above and below the mark, respectively), and tissue area and dry weight were recorded. Shoot-specific productivity (mass or area) was multiplied by field density in corresponding replicate plots for areal productivity measurements (g DW or cm^2 m^{-2} d^{-1}).

Sediments were characterized using standard techniques for sediment organic matter and C:N. Cores were collected 5 times along a 50 m transect within each replicate plot and were placed immediately on ice. Sediment organic matter was calculated by subtracting the mass lost on ignition (in a 500 °C muffle furnace for 8 h) from the dry weight mass. Sediment C and N contents were measured using dry sediment (60 °C for > 48 h) and analyzed on a Carlo Erba Elemental Analyzer with helium as a carrier gas, a 1020°C combustion tube, and 650 °C reduction tube.

The bathymetry of the set aside in Hog Island Bay was measured in 1999 and 2000 (Oertel 2001). An Innerspace Digital Depth Sounder (Model 448) was paired with Trimble 4000SE GPS receivers and a Trimble NavBeacon XL to measure georeferenced depths. These points were interpolated using ArcGIS software using an Inverse Distance Weighted analysis to create a contour map of the seagrass meadows from which the depth at each sampling location could be determined. The depth of each replicate plot at MSL was calculated by averaging all pixels within the replicate plot using a neighborhood analysis in ArcGIS.

Statistics

All comparisons of sediment characteristics, plant productivity, and N_2 fixation with depth were done by linear regression analysis using the statistical program Minitab. All results are determined to be significant at a $p < 0.05$, unless otherwise noted.

RESULTS

Nitrogen fixation

A significant log-normal decrease in total N_2 fixation (rhizosphere + phyllosphere) was observed with increasing depth ($p = 0.045$). The greatest rate of decrease was in the plots between 0.9 and 1 m MSL (740–117 µmol N m^{-2} d^{-1}) (Fig. 2a). At replicate plots greater than 1 m deep, rates of total N_2 fixation averaged 110 (±6.5) µmol N m^{-2} d^{-1}. A functional shift between epiphyte and sediment nitrogen fixers occurs at 1 m (MSL). Epiphytes contributed the majority (76–88%) of the total N_2 fixed at depths less that 1 m; sediment bacteria fixed 90–100% of the total N_2 fixed in water deeper than 1 m (Table 1). Despite the prevalence of sediment N_2 fixation at sites greater than 1 m deep, there was no significant relationship between sediment nitrogen fixation and depth ($p = 0.5$; fig 2b) and no difference between seagrass sediments and unvegetated sediments at deeper depths. All rates of sediment N_2 fixation were within a narrow range with low variability (79–128 µmol N m^{-2} d^{-1} SE: 6.1).

The decrease in total N_2 fixation with depth was driven primarily by epiphyte N_2 fixation (Fig. 2c). The log-normal decrease in epiphytic N_2 fixation was significant, and the average rates of N_2 fixation at sites greater than 1 m deep (MSL) were < 1% of those in shallower water. The highest rate of epiphyte N_2 fixation was observed at the shallowest plot (753 µmol N m^{-2} d^{-1}, $z = 0.94$ m), and the lowest rate was observed at the deepest plot (0.4 µmol N m^{-2} d^{-1}, $z = 1.53$ m) on the depth gradient (Fig. 2c).

Seagrass density decreased linearly with depth, with maximum densities observed at the shallow end of the gradient at a depth of 0.9 up to 1.25 m MSL (Fig. 3a). Densities in water shallower than 1.25 m averaged 105 m^{-2}, which was over 5 times higher than those in deeper waters (>1.25 m MSL, avg. 20 m^{-2}). The only significant relationship observed with seagrass metrics was the strong positive correlation between shoot density and epiphyte N_2 fixation ($p = 0.009$, Fig. 4).

Several significant ($p < 0.05$) linear trends of sediment properties were evident along the depth gradient. Sediment organic matter (OM) was positively correlated with depth (Fig. 3b). Additionally, sediment C and N (both %) were positively correlated with depth, though the rate of increase of sediment N (%) with depth was over 4 times higher than that of C (Fig. 3c, d). This difference in the rate of increase in sediment C and N resulted in a negative relationship between molar C:N with depth (fig 3e). No relationships, however, were observed between any sediment parameters and N_2 fixation.

DISCUSSION

In temperate estuaries and shallow bays, light has been identified as a limiting resource to seagrass, either where shading occurs from phytoplankton blooms (Taylor *et al*. 1995), macroalgae (McGlathery 2001), epiphytes (vanMontfrans *et al*. 1984), or with increasing depth (Burkholder & Doheny 1968, Backman & Barilotti 1976, Dennison & Alberte 1982, Dennison & Alberte 1986, Neverauskas 1988). In Hog Island Bay, light has been identified as limiting to seagrasses below 1.6 m MSL (Lawson *et al*. 2007, Carr *et al*. 2010), which coincides with the current seagrass lower depth limit (McGlathery *unpublished*). Shoot density decreased significantly with depth (plots < 1.25 m deep: avg. 105 shoots m^{-2}, > 1.25 m deep: 20 shoots m^{-2}), indicating that the decrease in shoot density was the primary driver of epiphytic and total N_2 fixation across the depth gradient. The decrease in light—and as a result, shoot density—with depth, paired with the regulation of N_2 fixation by shoot density (Cole & McGlathery *submitted*), potentially explains the decrease in N_2 fixation with depth observed in this study.

The majority of the total N_2 fixation was the result of epiphytes growing on the *Z. marina* blades (Table 1, Fig. 2c). Epiphytic N_2 fixation, scaled to area, did have a significant and positive relationship with shoot density (Fig. 4). An increase in *Z. marina* shoots would lead to more leaf surface area per m^2, resulting in higher rates of epiphytic N_2 fixation compared to less-dense replicate plots given the consistent per-shoot rates of primary productivity.

The total amounts of N_2 fixation in the shallower plots in this study were comparable in range to *Z. marina* in other temperate systems (Capone 1982, McGlathery *et al.* 1998, Cole & McGlathery *submitted*) while seagrasses deeper than 1 m MSL were fixing nitrogen at very low rates (0.4–11.7 μmol N m^{-2} d^{-1}), much lower than peak rates observed in this system by Cole & McGlathery (*submitted*). In both studies of the N_2-fixing communities in these seagrass meadows, the epiphytes were the primary N_2-fixing community overall, and rates were correlated with shoot density. Unlike other temperate (McGlathery *et al.* 1998, Welsh *et al.* 2000) and tropical systems (Capone *et al.* 1979, Capone & Taylor 1980), sediment N_2-fixing bacteria were not dominant likely because of the low sediment OM content.

No significant relationship was observed between depth and sediment N_2 fixation, and overall rates were low (100 ± 6 μmol N m^{-2} d^{-1}) compared to epiphytic N_2 fixation (Fig. 2a, b) and to other measurements of sediment N_2 fixation (Capone 1988, McGlathery *et al*. 1998). One notable difference is that the comparable studies had significantly higher shoot densities (this study: 105 ± 19 shoots m^{-2}; McGlathery *et al*. 1998: 960–1500 m^{-2}). Because the shoot-specific primary production rates were not different among the meadows in the Virginia coastal bays (Cole & McGlathery *submitted*), we suggest that shoot density regulated the availability of OM (*i.e.*, release of photosynthate) and thus, sediment N_2 fixation rates.

We also observed differences in sediment characteristics with depth that may affect N_2 fixation rates as the restored meadows develop over time. Sediment organic matter significantly increased with depth (0.9–2.2 %), as did sediment N content (< 0.01–0.2 %) and sediment C (0.2–0.7 %; fig 3c, d). The quality of organic matter increased with depth, as represented by molar C:N (range: 45–6) and driven by the steeper rate of change of sediment N relative to C. The C:N observed in shallower sediments was higher than bare sediment alone, possibly because the amount of detrital seagrass (high C:N; Thayer *et al*. 1977) within the sediments would increase with shoot density.

Over time, however, organic matter in the rhizosphere should increase through inputs of trapped particulate carbon, seagrass burial, and exuded photosynthate, which will intensify bacterial decomposition and increase the sediment N content (Odum & de la Cruz 1967). Organic matter colonized and decomposed by bacteria will then become more labile and accessible to heterotrophs, including denitrifying and N_2 fixing bacteria (Fulweiler *et al*. 2008). Bacterially-mediated OM availability to seagrass-associated N_2 fixers in the sediment is particularly important as the terrestrial nutrient and OM inputs to the Virginia coastal bays are low compared to other shallow bays and estuaries (McGlathery *et al*. 2007, Cole & McGlathery *submitted*), and a 30-year dataset has shown no trend in water column N (0.5–4 μM NO_2+NO_3) or chl-*a* (1–6.5 μg L^{-1}) (*http://www1.vcrlter.virginia.edu/home1/?q=data_wq*).

Although light has been shown to have an indirect effect on N_2-fixing bacteria associated with seagrass plants through the release of photosynthate, (Moriarty & Pollard 1982), we found that N_2 fixation rates in seagrass-vegetated sediments were not significantly different from bare sediment (Cole & McGlathery *submitted*). This suggests in such a nascent, low-density system, N_2-fixing bacteria would likely be concentrated only at the root tips, where the photosynthate is exuded (Frederiksen & Glud 2006). Because our methods analyzed a profile of sediment within the rhizosphere, and not just at the root tips, we suspect we suspect any signal of enhanced N_2 fixation was masked (Fig. 2b). Nearby (20 km) older meadows that were restored in 2001 at the same depths (8 years since reseeding) had elevated rates of N_2 fixation when compared to both younger meadows and bare sediment, as well as higher shoot densities (400 shoots m^{-2} v. 100 shoots m^{-2} in this study; Cole & McGlathery *submitted*).

As ecosystem engineers, seagrasses create the substrate upon which N_2-fixing bacteria thrive, allowing for an increase in N sequestration as the meadows become denser and increase in area through time. Based on our results, we can infer that the reduction in irradiance is a stressor on seagrasses in deeper waters, decreasing shoot density, and minimizing N_2 fixation (fig. 2a, Carr *et al*. 2010). In shallow, low bathymetric-relief lagoon systems like the Virginia coastal bays, the potential for the spread of *Z. marina* and an associated increase in N sequestration by N_2 fixation is high. By stabilizing the surrounding sediment, seagrasses create drag on the overlying water allowing light-blocking particulates to settle out and clarify the water column (Newell &

Koch 2004, Lawson *et al*. 2007), thereby enhancing light availability. *Z. marina* has been observed within Hog Island Bay up to the 1.6 m threshold as described by Carr *et al*. (2010), though their density is patchy and survivorship is ephemeral (McGlathery *et al*. *submitted*). In nearby South Bay, 48 1-acre plots were reseeded in 2001 and 2002; by 2010, nearly the entire basin of South Bay was repopulated with *Z. marina* (Orth *et al*. 2010, *http://web.vims.edu/bio/sav*), and in many plots, shoot densities average 400 shoots m^{-2}, nearly 4 times higher than the average density in this study. The high shoot density in South Bay has resulted in clearer water, as well as significantly higher rates of both sediment and epiphyte N_2 fixation (Cole & McGlathery *submitted*). Like the replicate plots in this study, seagrasses in South Bay also exhibit little variation in primary productivity; shoot density was the driver of water clarity and N_2 fixation in both South Bay (Cole & McGlathery *submitted*) and in this study (fig 4). The 7 replicate plots in this study are part of 58 plots that were reseeded between 2006 and 2008, totaling 18 hectares (Orth *et al*. 2010, McGlathery *et al*. *submitted*). If the seagrasses in Hog Island Bay reach the same density of those in South Bay, there would likely be a significant increase in N_2 fixation. The elevated levels of fixed nitrogen would allow for an increase in seagrass above- and belowground biomass, reproductive potential, and lateral spread. A stabilized benthos and clarified water column would relieve photosynthetic light limitation and allow for colonization into deeper waters.

Seagrass-associated N_2 fixation is a new N source to this system following a 70-year absence of eelgrass, but seagrasses have been restored in only a small fraction of the habitable seafloor in Hog Island Bay. Given time, the available substrate, and continued low anthropogenic N inputs, the benthos of this system could be restored to pre-1930 conditions—replete with seagrasses, and with high rates of N sequestration via N_2 fixation.

Table 1. Proportion of total nitrogen fixation apportioned to sediment or epiphytic N-fixing bacteria along the bathymetric depth gradient.

	Sediment	Epiphyte
Depth (m MSL)	(% total N fixation)	
0.94	12%	88%
0.99	24%	76%
1.05	96%	4%
1.23	99%	1%
1.40	90%	10%
1.48	97%	3%
1.53	100%	0%

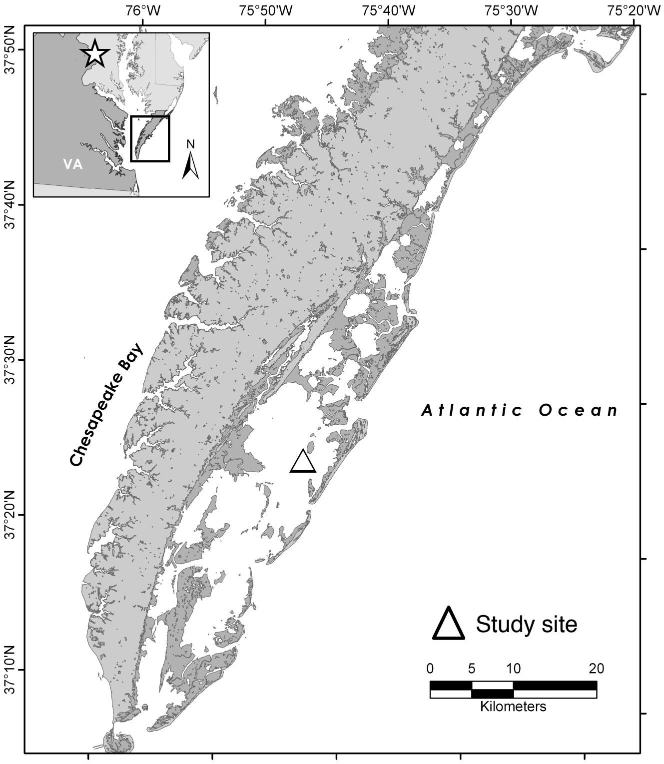

Figure 1. Locus map of the bathymetric gradient in Hog Island Bay in the Virginia coastal bays.

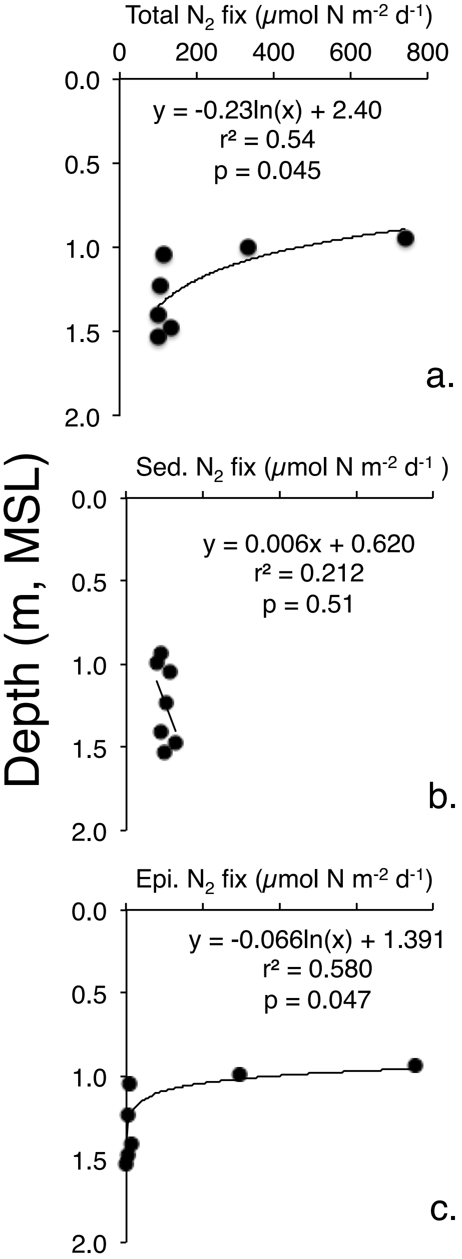

Figure 2. Nitrogen fixation rates along the depth gradient in Hog Island Bay. Total N_2 fixation values for each replicate plot represent the sum of a minimum of 2 sediment cores and a minimum of 3 *Z. marina* shoots.

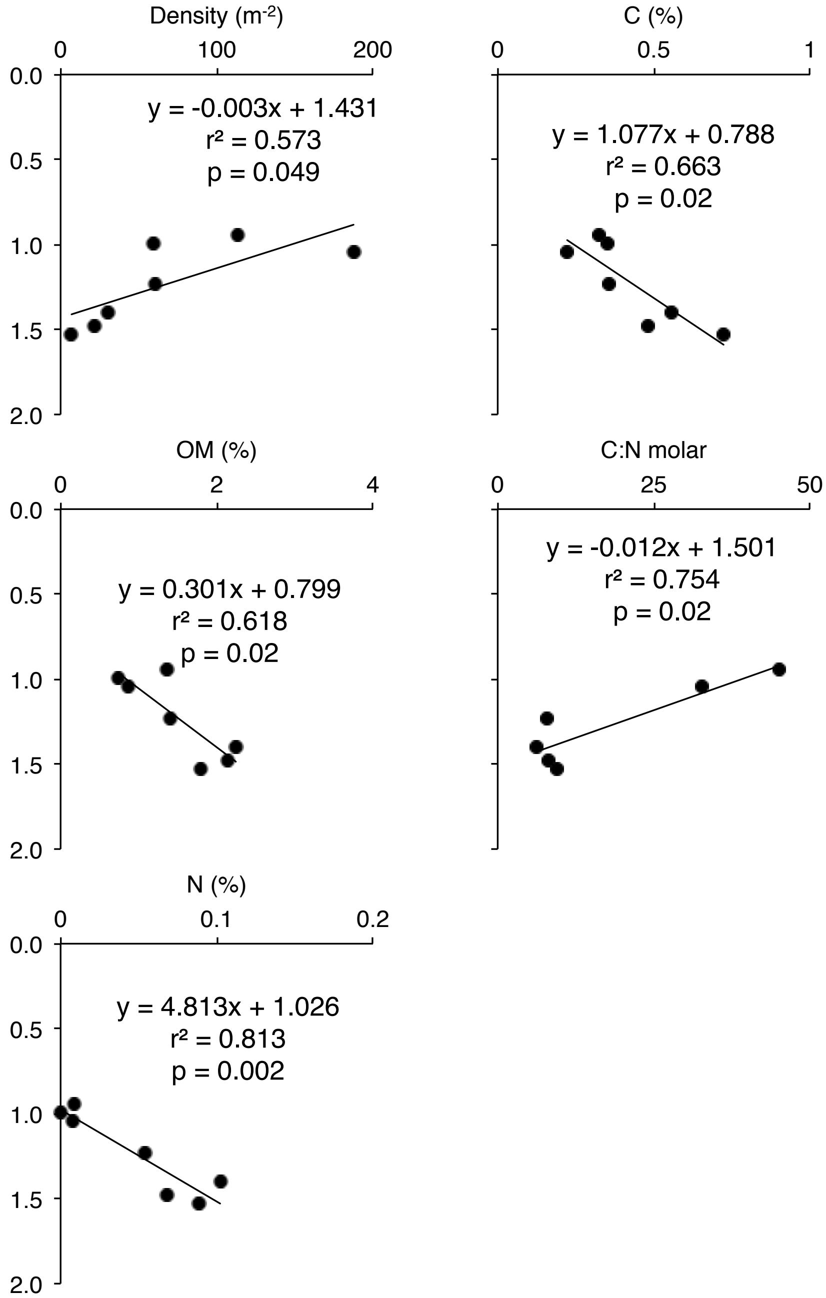

Figure 3. Seagrass and sediment parameters in replicate plots along the 0.6 m depth gradient. Density is identified as potential proxy for light effects, as no differences were observed in shoot primary productivity.

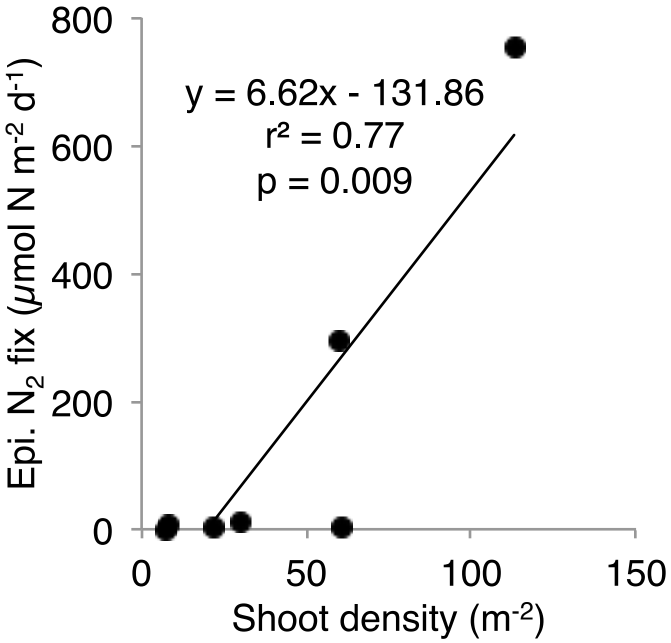

Figure 4. The observed linear increase in epiphytic N_2 fixation with shoot density reveals that density is the factor with the greatest effect on epiphytic and total N_2 fixation along the depth gradient ($p = 0.009$).

Chapter Five: Who gives a flux? Using the N_2:Ar technique to detect the balance of nitrogen fixation and denitrification in temperate, restored seagrass meadows

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ABSTRACT

Net N_2 fluxes reflect the balance of N inputs via N_2 fixation and losses via denitrification. We used the N_2:Ar technique to measure net N_2 fluxes seasonally across a chronosequence of restored seagrass (*Zostera marina* [L.]) meadows, comparing bare sediment sites (n = 8) and nearby seagrass sites restored by seed broadcasting in 2006 and 2001 in the Virginia coastal bays. Overall, the restored seagrass meadows were net denitrifying, and the rate of denitrification increased as the meadows become older (time since seeding). Denitrification rates in the older (8 year) and younger (3 year) seagrass-vegetated sites were 4.9 and 3.3 times higher, respectively, than nearby bare sediment sites, which were also net denitrifying (47.3 ± 12.7 μmol N_2-N m^{-2} h^{-1} and 31.7 ± 7.6 μmol N_2-N m^{-2} h^{-1} vs. 9.6 ± 14.6 μmol N_2-N m^{-2} h^{-1}). Water column NH_4^+ was positively correlated with N_2 fluxes. Sediment organic matter (OM) was also positively correlated

to both N_2 fluxes and *Z. marina* meadow age, suggesting that as NH_4^+ and OM build up in seagrass-vegetated sediments over time, rates of denitrification rates are enhanced relative to N_2 fixation. The N removal capacity of bare sediment was nearly equal to the terrestrial N loading rate for these coastal bays (1 g N m^{-2} y^{-1}), and vegetated sediments removed 3.9–5.8 g N m^{-2} y^{-1}, supporting the concept of seagrass beds as a "nutrient sponge". This study provides the first assessment of net N_2 fluxes for restored seagrass systems, and the rates are comparable to other shallow temperate coastal systems.

INTRODUCTION

Seagrasses throughout the world are in decline, bringing about a shift in nitrogen dynamics in shallow coastal systems (Waycott *et al*. 2009). The decline of seagrasses is driven largely by the chronic increase in anthropogenic N (Nixon 1995), which in turn, triggers a state change where shallow coastal systems become dominated by micro- and macroalgae (McGlathery 2001, McGlathery *et al*. 2007). The loss of seagrass coverage leads to a decrease in N retention, as functioning seagrass meadows bind N in the plant tissues, and ultimately senesce and are buried, often within the seagrass meadow (Risgaard-Petersen *et al*. 1998, de Boer 2007). Without seagrasses to stabilize the sediment and retain N, these nutrient pools are released into the water column disrupting and accelerating the N cycle and creating conditions suitable for a state change. Bare sediments in low-nutrient systems have been shown to shift from net denitrifying (N loss) to net N_2 fixing (N sink) following an increase in organic matter (Fulweiler *et al*. 2007). But when seagrasses become established, the associated bacteria and archaea in the rhizosphere and phyllosphere typically create a net N loss, denitrifying N at a rate higher than, or in equilibrium with, N_2 fixation (Eyre *et al*. 2010), though the opposite trend has been observed (Hemminga *et al*. 1991, Risgaard-Petersen *et al*. 1998).

The high rate of primary productivity in seagrasses corresponds to a large N requirement to maintain growth (Den Hartog 1970, Eyre & Ferguson 2002). To satisfy this N requirement, seagrasses use a variety of mechanisms. Seagrasses take up as much as 80% of their N requirement from sediment porewater and the water column (Short & McRoy 1984, Pedersen & Borum 1993, Risgaard-Petersen *et al*. 1998). Seagrasses also create drag on the overlying water column, indirectly trapping particulate matter and organic material (Chen *et al*. 2007, de Boer 2007, Wicks *et al*. 2009) that can be broken down and remineralized within the sediment to be taken up through the roots (Risgaard-Petersen *et al*. 1998). Nitrogen fixation, primarily by bacteria in the rhizosphere, can provide < 5–12% of the annual N requirement for temperate seagrasses (Welsh *et al*. 1996a, McGlathery *et al*. 1998) and up to 50% of the seagrass N demand in tropical systems (O'Donohue *et al*. 1991), but heterotrophic epiphytes also have been observed as the dominant N_2-fixing functional group in restored *Zostera marina* populations (Cole & McGlathery *submitted*).

N_2 fixation rates characteristically higher in seagrass meadows, compared to bare sediment. Highly productive seagrasses passively diffuse photosynthate into the rhizosphere triggering bacterial activity (e.g., N_2 fixation: McRoy & Goering 1974, Penhale & Smith, 1977, Oremland & Taylor 1977, Moriarty & Pollard 1982; and denitrification: Risgaard-Petersen *et al*. 1998, Welsh *et al*. 2000). Peak (spring / summer) rates of N_2 fixation in temperate seagrass-vegetated sediment range from 70–520 µmol N

m^{-2} d^{-1} (Capone 1988, Welsh *et al.* 1996b, McGlathery *et al.* 1998, Welsh *et al.* 2000, Cole & McGlathery *submitted*), 3–30 times higher than associated unvegetated sediment (McGlathery *et al.* 1998, Cole & McGlathery *submitted*). The elevated rates of photosynthetically-catalyzed N_2 fixation provide a N source to the seagrasses, creating a positive feedback. However, increases in water column NH_4^+ concentrations (anthropogenic or natural), potentially inhibit N_2 fixation by suppressing the formation of nitrogenase (Howarth *et al.* 1988).

Seagrasses-associated microbes balance N_2 fluxes primarily through N_2 fixation (N_2 sink) and denitrification (N_2 loss), though anammox has also been identified as a mechanism for N_2 loss (Mulder *et al.* 1995). Denitrifying bacteria are heterotrophic, requiring anoxic sediment and a source of NO_3^- (Rysgaard *et al.* 1996, Eyre & Ferguson 2002). Photosynthetic O_2 from seagrass lacunae is transported to roots, which is passively diffused into the sediment (Christensen *et al.* 1994). In these oxic microsites, nitrifying bacteria convert NH_4^+ into NO_3^-, which can then be converted into N_2 gas by denitrifying bacteria in the anoxic sediment (Rysgaard *et al.* 1994) at rates comparable to N_2 fixation (30–670 μmol N m^{-2} d^{-1}, though rates have been observed as high as 5000 μmol N m^{-2} d^{-1}; compiled from McGlathery 2008). The N_2 balance in seagrasses is variable depending upon location and season, suggesting that processes associated with seagrasses regulate and balance N_2 fluxes (Welsh *et al.* 2000). Because seagrasses elevate sediment organic carbon concentrations (Penhale & Smith 1977, de Boer 2007), providing a carbon source for N_2-fixing, nitrifying, and denitrifying bacteria, resource competition may limit any one process from dominating (Pedersen & Borum 1993, Risgaard-Petersen *et al.* 1998, Welsh *et al.* 2000).

The loss of seagrasses and the resulting state change to bare or algal-dominated sediment, and conversely, the restoration of seagrass habitat, have the potential to strongly affect the relative rates of N_2 fixation and denitrification in marine systems. Bare sediment in shallow, temperate coastal waters exhibits variable N_2 fluxes, depending largely on temperature and the supply of organic C to the benthos, but are typically net denitrifying (Fulweiler *et al*. 2007, Nixon *et al*. 2009). In shallow coastal systems where macroalgae are the dominant primary producers, thick algal mats can effectively form a barrier between the sediment and water column. The high absorption of water column nutrients by the algal mats reduces the exchange of sediment / water NH_4^+ (McGlathery *et al*. 1997), and in a low-nutrient water column this process can inhibit denitrification (Krause-Jensen *et al*. 1999).

This study provides the first dataset quantifying the N_2 flux in an ecosystem in transition from bare to a seagrass-dominated state. Restoration efforts began in the Virginia coastal bays in 2001 following a 70-year absence, and continued through 2008, resulting in seagrass meadows of different ages (time since seeding). Overall, the seagrass restoration efforts have resulted in the largest areal recovery of seagrasses (*Z. marina*) worldwide (Orth *et al*. 2010). Within this restored system, we have shown that older seagrass meadows fix more N than both younger meadows and bare sediment (Cole & McGlathery *submitted*). The dominant N fixing functional group has been identified as heterotrophic epiphytes living on the surface of the seagrasses, contributing over 90% of the fixed N. Sediment N fixation rates were low compared to literature values for established seagrass meadows; and whilst no differences were seen between bare sediment and the younger meadows, sediments in the older meadows fixed more N than bare sediments. This present study used the same chronosequence of replicate plots from Cole & McGlathery (*submitted*)—older (8 year) vegetated, younger vegetated (3 year), bare sediment—to determine if there was an effect of seagrass bed age on the balance of sediment N_2 fluxes via N_2 fixation and denitrification, and to identify potential drivers of the net N_2 flux.

METHODS

Study area

The Virginia coastal bays are at the southern end of the Delmarva Peninsula, part of a 200 km long string of shallow coastal bays on the east coast of Virginia, 200 km southeast of Washington, DC (Fig. 1). The Virginia coastal bays are barrier-built, bounded to the east by a string of barrier islands separated by inlets to the Atlantic Ocean and to the west by the mainland. The bays are shallow (average 1 m MSL), and have a 1 m tidal amplitude (Oertel 2001). There is no riverine input to this system, and circulation is tidal and wind driven (Oertel 2001, Lawson *et al*. 2007). The two clusters of newly-restored *Zostera marina* meadows were within the same body of water and were 22 km apart, separated only by tidal channels, interspersed mudflats, and marsh. The two seagrass sites were restored by seed broadcasting in 2001 and 2006 (Orth *et al*. 2010) and in this study, we monitored 4 replicate 1-acre plots at 10^5 seeds acre^{-1} at each site. At the time of this study, the restored beds were 8 years old and 3 years old. The chemical and physical characteristics of the bare sediments associated with these two seagrass sites were not statistically different, with the exception of sediment organic matter, which was higher in the older meadow (2.3% ± 0.1 SE) than the younger (1.7% ± 0.3 SE) (full comparison in McGlathery *et al. submitted*). An on-going 17-year water quality dataset collected in this study area by the Virginia Coast Reserve Long-Term Ecological Research program shows that the water column N (0.5–4 µM NO_2+NO_3, 3.2–3.6 µM NH_4^+) and chl-*a* (1–6.5 µg L^{-1}) are quite low relative to other shallow coastal systems and show no trends of changing water quality during the period of record (VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq). The low water column nutrients and chl-*a* concentrations are consistent with the very low levels of N loads to this system (1 g N m^{-2} y^{-1}) compared to most other shallow coastal bays and estuaries (McGlathery *et al*. 2007, Cole & McGlathery *submitted*).

Water column, sediment, seagrass sampling

During each sampling event, salinity, water temperature, and dissolved O_2 (DO) measurements were made at 10 cm above the sediment surface within each replicate plot. Salinity was measured using a hand-held refractometer, while temperature and DO were recorded using a Hach HQ40d Portable Multi-parameter meter with an LDO probe. Water samples were collected using a 60 cc syringe and filtered through 0.45 µm supor filter into a sterile Whirlpac bag that was sealed and frozen until analysis. Water samples were analyzed for NO_3^-, NH_4^+, and PO_4^{3+} using a Lachat QuikChem 8500 equipped with an autosampler. Sediment organic matter was collected from each core at the completion of the N_2 flux incubation. Using a cut off 60 cc syringe, the sediment was cored down to 5 cm to coincide with the depth of maximum biomass in the seagrass rhizosphere. The sample was weighed, then dried at 60 °C for 48 h before being reweighed and ashed in a muffle furnace at 500 °C for 6–8 h. Organic content was calculated as the difference between sediment dry weight and ash-free dry weight. *Zostera marina* shoot density and productivity were measured in each replicate plot coincident with the N_2:Ar core collection. Shoot density was measured by enumerating the number of shoots in a randomly thrown 0.25 m^2 PVC quadrat in at least 2 locations in each replicate plot, and shoot productivity was measured by the leaf-marking technique (Zieman 1974).

Core collection and pre-incubation

Sediment cores were collected seasonally on four occasions—in June, August, and October 2009 and April 2010. The 30 cm long, 10.2 cm i.d. clear PVC cores were inserted into the sediment using a push corer, extracted, capped at both ends, and held at field water temperature until returned to the flow-through laboratory facility. Cores contained approximately15 cm of sediment and a 15 cm water column. Prior to coring in vegetated sediment, the seagrass was trimmed to the sediment surface using scissors, taking care to not disturb the area surrounding the shoot. The purpose of removing aboveground biomass was to prevent oxygen bubble evolution (photosynthesis) in the cores, which would disrupt the N_2:Ar measurements. Also, the seagrass blades routinely became tangled in the water circulation mechanism during trial runs. We recognize that this allows us only to address the long-term effect of seagrass presence on the balance of N_2 fixation and denitrification, and not the immediate effect of DOC release by photosynthesizing vegetation on bacterial activity (Paerl *et al*. 1987, Tibbles *et al*. 1994, McGlathery *et al*. 1998). At each site, we collected two sets of triplicate cores, one set in vegetated sediment and another in bare sediment. In addition, 40 L of site water was filtered through a three-stage system, with 0.45 µm as the finest mesh size. Cores were capped and brought to the flow-through facility in Oyster, VA where they were placed in a water bath with the 40 L carboy of site water at field temperature. In the water bath, the core caps were vented (though water was not exchanged with the water bath), oxygenated overnight using an aquarium pump and diffusers, and kept in the dark to ensure steady-state conditions.

N_2:Ar method

Prior to the incubation, the height of the water column was measured at 4 locations in each core, and approximately 90% of the water was siphoned off and replaced with filtered site water from the 40 L header tank without disturbing the sediment surface. Cores contained no air bubbles and were sealed airtight at the start of the incubation, and the lids were outfit with a free-spinning magnetic stir bar, an inlet port (connected to the 40 l header tank) and an outlet port (for sampling). Strong magnets on the outside of a rotating carousel placed in the center of the core array caused the magnetic stir bars to spin, circulating the water inside of the cores. In addition to the 3 replicate vegetated sediment cores and 3 replicate bare sediment cores, 3 cores were filled with the filtered site water as a control. Incubations lasted for 5–10 h, depending on the rate at which dissolved oxygen (DO) was drawn down; ideal study conditions were to draw the DO concentration down at least 20% from initial in the 6 cores with sediment, but not allowing the DO to drop below 2 mg O_2 L^{-1}. The water bath and cores were kept completely dark during the incubation to measure N_2 fluxes under heterotrophic conditions, as well as to inhibit bubble formation in the cores either by photosynthesis or by temperature increases.

Water samples for dissolved gas analysis (DGA) were collected in duplicate from each of the 9 cores at 5 points throughout the incubation period in 12 mL clear soda glass tubes. Opening the header tank reservoir and sampling port on each core allowed for gravity-fed flow and replacement while samples were being taken. Tubes were collected until overflowing, at which point the samples were poisoned with 0.2 mL of a saturated $HgCl_2$ solution and the tubes were screw capped without trapping any bubbles. Samples were immediately placed atop ice in a cooler and kept cold and in the dark until the gas analysis. Water temperature and dissolved oxygen concentrations were measured during the 1st, 3rd, and 5th (*i.e.*, final) sampling events using a Hach HQ40d Portable Multi-parameter meter with an LDO probe.

N_2:Ar concentrations were measured following the method of Kana *et al*. (1994) using a Balzers quadrupole membrane inlet mass spectrometer system (MIMS) outfit with a ± 0.1 °C water bath and an in-line 600 °C furnace with a copper reduction column. The furnace stripped the sample of O_2 in order to dissociate any N_2 that may have bound as NO or N_2O during the incubation period as per Eyre *et al*. (2002). The water bath was programmed to the same temperature as the incubation (*i.e.*, field temperature at time of collection) to maintain dissolved gas concentrations.

Flux calculations

Dissolved gas values from the MIMS were corrected using linear regression of machine drift during the analysis. These corrected values were used to calculate the flux of N_2 across the sediment-water interface using linear regression as a function of N_2 and Ar concentrations (as N_2:Ar), incubation time, solubility table-predicted N_2 and Ar, core area, and core water volume. Only linear portions of the regression were used to calculate the net N_2 flux; non-linear curves or curves with any change in Ar concentration were excluded. Net N_2 flux values from the control (water only) cores were subtracted from the bare and vegetated core fluxes, though the average control core flux was significantly smaller than either vegetated or bare cores, and had a mean ~ 0 µmol N_2-N m^{-2} h^{-1}, indicating that there were no fluxes attributable to water column N_2 fixation or denitrification.

Statistical analyses

N_2 fluxes, water column NH_4^+, and sediment organic matter were grouped by cover type (vegetated vs. bare), and seagrass meadow age (0 y, 3 y, 8, y) for comparative analyses. For all statistical analyses, the aforementioned parameters were assembled by replicate plot and all parametric assumptions were confirmed (including heteroscedasticity and normality). A linear regression was fit to data pooled by meadow age to detect an overall effect of time since seeding on net N_2 fluxes. Spearman correlations were performed to test whether these sediment and water column parameters were related to changes in net N_2 flux with meadow age.

RESULTS

During this study period (June 2009–April 2010), water temperatures varied by 15 °C, while salinity varied by no more than 2 ‰ (Table 1). A Spearman correlation revealed that sediment organic matter (%) was significantly correlated to *Zostera marina* meadow age (Table 2). The 8 y meadow did not have significantly more OM than the 3 y meadow during any of the 4 individual sampling periods, but when data were pooled, the older meadow had significantly more OM than the younger meadow ($p = 0.03$), indicating that meadow age (time since seeding) influence sediment OM content. There were no differences in the sediment OM in the bare and vegetated sediment in the younger meadow, though significant differences were observed between the 8 y meadow and bare sediment ($p < 0.001$).

Water column nutrients (NH_4^+, NO_3^-, PO_4^{3+}) did not exhibit seasonal patterns during the study (Table 1) and no significant differences were seen between the water column parameters and the two seagrass meadows. The low concentrations of water column nutrients were consistent with values reported by the Virginia Coast Reserve Long Term Ecological Research water quality database (VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq). *Zostera marina* shoot density was significantly higher in the older meadow during each sampling period and also when all data were pooled ($p < 0.001$). We observed that shoot primary productivity showed some seasonal pattern, particularly that the lowest rates for both the younger and older meadows were observed during the August sampling period (0.18 ± 0.1 cm^2 d^{-1} and 0.18

± 0.08 cm^2 d^{-1}, respectively) when temperatures were highest (Table 3). Pooling productivity rates from both sites revealed no statistical differences.

Net N_2 fluxes increased significantly with *Z. marina* bed age (Fig. 2). There were no significant differences between sites, but the pooled N_2 flux data showed that the restored seagrass meadows in Virginia coastal bays were net denitrifying (positive N_2 flux), and that the magnitude of net denitrification increased as the seagrass beds matured (Fig. 3). Average fluxes in the 3 y meadow ranged from 13–57 μmol N_2-N m^{-2} h^{-1}, while fluxes in the 8 y meadow were generally larger, ranging from 6–98 μmol N_2-N m^{-2} h^{-1} (Fig. 3). Minimum N_2 fluxes were observed in October in the 3 y meadow, and in August in the 8 y meadow (Fig. 4), coincident with the lowest water column NH_4^+ in each meadow, respectively (Table 1).

Sediment organic matter was correlated with seagrass meadow age and with N_2 fluxes (Table 2), suggesting that the buildup of OM as seagrass meadows age may influence N_2 fluxes. As a required nutrient for nitrification–denitrification, water column NH_4^+ was positively correlated with N_2 fluxes (Table 2).

Oxygen uptake rates from the incubation cores did not show a strong seasonal pattern in the 3 y old *Z. marina* meadow (Fig. 5). Vegetated sediment O_2 uptake rates in the younger meadow did not differ across sampling dates, nor were the vegetated sediments different from the associated bare sediment at any time. However, O_2 uptake in vegetated sediment was higher than in bare sediment when all data were pooled (-55.0 ± 0.2 mmol O_2 m^{-2} d^{-1} and -34.1 ± 0.3 mmol O_2 m^{-2} d^{-1}, respectively; $p = 0.02$). At the older (8 y) meadow, the highest O_2 uptake rates were observed in August 2009 (-88.9 ± 9.0 mmol O_2 m^{-2} d^{-1}), and were significantly different from the bare sediment. The sediment

O_2 uptake rates in vegetated sediments in the 8 y *Z. marina* meadow in October and April were (-42.3 ± 1.2 mmol O_2 m^{-2} d^{-1} and -57.9 ± 0.7 mmol O_2 m^{-2} d^{-1}, respectively) and were significantly greater than the bare sediment O_2 uptake for both dates (Fig. 5).

DISCUSSION

The N_2 flux rates were increasingly net denitrifying across a restored seagrass chronosequence, and this correlated with increasing concentrations of sediment organic matter (OM) and water column NH_4^+ (Table 2, Fig. 2). Average net N_2 flux rates in this study (8 y meadow: 47.3 ± 12.7 µmol N_2-N m^{-2} h^{-1}; 3 y meadow: 31.7 ± 7.6 µmol N_2-N m^{-2} h^{-1}; bare: 9.6 ± 14.6 µmol N_2-N m^{-2} h^{-1}) were comparable to N_2 fluxes observed in other temperate seagrass meadows (Welsh *et al*. 2000, Eyre & Ferguson 2002, Eyre *et al*. 2010) and associated with submerged vegetation in temperate lakes (Risgaard-Petersen & Jensen 1997). In addition, the difference between N_2 fixation and denitrification rates in another *Zostera marina*-vegetated system (Risgaard-Petersen *et al*. 1998) was within the same range as rates observed in this study.

The differences in average rates of N_2 flux between vegetated and bare sediment for all seasons (Fig. 3) suggest that the mechanisms regulating net N_2 fluxes were plant-mediated. This is supported by related work in the Virginia coastal bays by Cole & McGlathery (*submitted*) where N_2 fixation in *Z. marina*-vegetated was significantly higher than bare sediment during peak growth (June–July).

The water column NH_4^+ concentrations—collected 10 cm above the sediment surface—were lower than the 17-year mean reported by the Virginia Coast Reserve

LTER (this study: 2.58 ± 0.58 µM NH_4^+, 30-y mean: 3.40 µM NH_4^+ ± 0.23) (VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq). Additionally, water column NO_3^- was 0.37 µM NO_3^- ± 0.11, over 13 times less than the 30-year average (5.06 µM NO_3^- ± 0.15). Despite these low concentrations, we observed that water column NH_4^+ was correlated to N_2 fluxes (Table 2), and peak fluxes corresponded to peak NH_4^+ values in each of the two seagrass meadows (Table 1, Fig. 4). In vegetated sediment with elevated OM concentrations, NH_4^+ production through mineralization can be as high as 16–34 mmol N m^{-2} d^{-1} (Iizumi *et al*. 1982, Dennison *et al*. 1987), though rates in the Virginia coastal bays range from 1–10 mmol N m^{-2} d^{-1} (Anderson *et al*. 2010). We believe that sediment mineralization is the most likely source of NH_4^+ in the water column. In a low-nitrogen system like the Virginia coastal bays, we expect high competition for NH_4^+ and NO_3^- between microbes and seagrasses (Risgaard-Petersen 2003, McGlathery 2008). The correlation between NH_4^+ concentrations and N_2 fluxes indicates that the tight coupling between nitrifying and denitrifying bacteria may be out-competing *Z. marina*, providing a mechanism for the N limitation in seagrass tissue at this site (C:N: 21–37; Cole & McGlathery *submitted*).

In contrast to most estuarine systems where sediment organic matter is abundant (Nixon 1995, Nixon *et al*. 2001), the Virginia coastal bays have low sediment OM, driven primarily by low N loading rates of 1 g N m^{-2} y^{-1} (Cole & McGlathery *submitted*) and subsequently, water column primary production (organic carbon) (McGlathery *et al*. 2007). The low water column chl *a* concentrations (1–6.5 µg chl *a* l^{-1}) in the Virginia coastal bays indicate that the sediments likely received little organic carbon from the water column, potentially constraining N_2 fixation and denitrification activity. We found the lowest N_2 flux rates in bare sediment, which also had the lowest amount of sediment organic matter (Table 1, Fig. 6). Additionally, organic matter was positively correlated with both *Z. marina* meadow age and N_2 flux (Table 2), indicating as organic matter builds up in the sediments of restored meadows with time, while the rates of N loss (via denitrification) increase (Fig. 3, Fig. 6). Organic carbon addition experiments in shallow temperate systems have shown similar relationships between net denitrifying sediments and OM using both NO_3^- flux experiments (Caffrey *et al*. 1993) and the N_2:Ar technique (Fulweiler *et al*. 2007, 2008). Tibbles *et al*. (1994) demonstrated that an addition of labile organic matter (plant storage polysaccharides, laminarin, and glycogen) stimulated nitrogenase activity and thus, N_2 fixation while Dahllöf & Karle (2008) found that *Z. marina* beds became net denitrifying with an increase in OM. Cole & McGlathery (*submitted*) showed an increase in N_2 fixation as *Z. marina* beds age, a process driven primarily by shoot density which results in both the release of photosynthate into the phyllosphere and rhizosphere and an increase in sediment OM. We suggest that the increase in sediment OM would enhance mineralization at rates exceeding N_2 fixation, increasing NH_4^+ availability, which is nitrified in the oxygenated rhizosphere, and denitrified into N_2 gas. This shift in the N_2 balance is supported by the linear increase in net N_2 fluxes that we observed with an increase in seagrass bed age and the positive correlation of the N_2 fluxes with sediment OM (Table 2, Fig. 2).

Since our experimental design excluded aboveground biomass and measured fluxes under heterotrophic (dark) conditions, we cannot include short-term effects of plant photosynthesis on bacterial activity. We suspect that had we left *Z. marina* aboveground tissues in the sediment cores and performed the incubation under light conditions, the seagrasses may have added O_2 into the root zone, increasing the potential for coupled nitrification–denitrification. However, in a low-nutrient environment like the Virginia coastal bays, primary producers may outcompete the bacteria for NH_4^+, thus reducing overall nitrification–denitrification (Ottosen *et al.* 1999, Risgaard-Petersen & Ottosen 2000). Furthermore, the exudation of photosynthate into the rhizosphere and phyllosphere could have increased rates of N_2 fixation and denitrification, potentially altering the overall net N_2 flux. Therefore, the N_2 flux rates presented in this study may be over- or underestimates, depending on whether N_2 fixation or denitrification has a stronger response to seagrass photosynthetic products. Net N_2 fluxes from the N_2:Ar method for light incubations with vegetated cores have proven problematic, as dissolved N_2 gasses preferentially absorb into photosynthetically-derived O_2 bubbles (A. Giblin *personal communication*). Denitrification measurements using the isotope pairing method (IPM) allow for the presence of seagrasses (*e.g.*, Pinardi *et al.* 2009), but IPM has been shown to underestimate denitrification by 35–85% (Ferguson & Eyre 2007). In this study, we chose to identify the overall N_2 flux balance along an age chronosequence, and we were interested in relative differences between meadows.

Anammox (anaerobic ammonium oxidization) is a less understood pathway whereby ammonium is oxidized using NO_3^- as an electron acceptor under anaerobic conditions (Mulder *et al.* 1995, Koop-Jakobsen & Giblin 2009), and like denitrification, anammox results in a net loss of N_2 gas. Annamox typically represents < 10% of total N_2 produced in both shallow marine systems (Risgaard-Petersen *et al.* 2003) and temperate salt marshes, and its contribution decreases with salinity (Koop-Jakobsen & Giblin 2009). Few studies of anammox exist in vegetated marine systems, though seagrass sediments represent a potential anammox hotspot. Net N_2 fluxes from the N_2:Ar method used in this study include inputs from anammox but cannot distinguish specific sources and sinks, unlike the isotope pairing technique (Risgaard-Petersen *et al*. 2003).

System-wide scaling

The net N_2 fluxes for bare sediment in this study were 1.57 g N m^{-2} y^{-1}, indicating that bare sediment alone is capable of removing over 100% of the anthropogenic N inputs to this coastal bay system (1 g N m^{-2} y^{-1}) (Cole & McGlathery *submitted*). This net N loss may partially explain the exceptionally low water column DIN concentrations in this system (VCR LTER data base, www1.vcrlter.virginia.edu/home1/?q=data_wq). This is also consistent with the nutrient budget created for Hog Island Bay by Anderson *et al*. (2010) indicating that high rates of primary production were supported mostly by remineralization in the sediments and efficient nutrient cycling. The recovery of *Zostera marina* in the Virginia coastal bays after a nearly 70-year absence (Orth *et al*. 2006) may further intensify the N removal capacity, as the vegetated sediments were more net denitrifying than bare sediment alone (Fig. 3). If seagrass coverage was scaled to the habitable portion of the Virginia coastal bays ($-0.6 \text{ m} \geq z \leq -1.6$ m MSL, Carr *et al*. 2010), N removal capacity would be 3.9–5.8 g N m^{-2} y^{-1}, far exceeding the anthropogenic N inputs.

Conclusions

Based on these results, we can conclude that restored *Zostera marina* meadows were net denitrifying, and became more so as the meadows aged. The seagrass-associated increases in sediment organic matter and water column NH_4^+, paired with the oxygenating of the rhizosphere by the seagrasses likely enhanced coupled nitrification–denitrification, resulting in net denitrifying benthos. Previous studies of these same *Z. marina* meadows have shown that N_2 fixation increased with seagrass bed age (Cole & McGlathery *submitted*), and while this present study does not dispute this finding, we can say that denitrification increased at an even greater rate. Our results confirm the concept of seagrasses as a "nutrient sponge" and that the continued recovery of seagrasses in the Virginia coastal bays will restore and enhance the ecosystem service of nutrient removal as watershed N moves across the land-sea margin.

Table 1. Water column and sediment parameters measured during core collection. All water column parameters were sampled 10 cm above the sediment surface, and sediment organic matter (OM) was sampled at the completion of N_2 flux incubations. Values in parentheses ( ) represent standard error.

		Water	Salinity	Vegetated	Bare	Vegetated	Bare	Vegetated	Bare	Vegetated	Bare
		(°C)	(‰)	Sediment OM (%)		NH_4^+ (μM)		NO_3^- (μM)		PO_4^{3+} (μM)	
June 2009	3 y	27	31	1.13 (0.30)	1.39 (0.20)	10.87 (3.20)	— —	1.26 (7.03)	— —	16.42 (7.03)	— —
	8 y	25	32	1.37 (0.17)		3.61 (1.01)	— —	0.91 (0.48)	— —	2.22 (0.80)	— —
August 2009	3 y	29	32	- -	0.82 (0.13)	3.65 (1.12)	— —	0.17 (4.76)	— —	5.28 (4.76)	— —
	8 y	29	30	1.71 (0.13)		0.79 (0.27)	1.13 (0.06)	0.15 (0.08)	0.18 (0.04)	0.73 (0.08)	0.67 (0.08)
October 2009	3 y	20	30	1.01 (0.18)	1.08 (0.20)	0.00 (0.00)	0 (0.00)	0.51 (0.19)	0.09 (0.02)	0.71 (0.19)	0.61 (0.02)
	8 y	22	30	1.39 (0.11)		1.39 (0.03)	1.34 (0.49)	0.08 (0.05)	0.77 (0.15)	0.55 (0.09)	0.71 (0.02)
April 2010	3 y	14	32	1.23 (0.28)	0.84 (0.15)	3.59 (1.88)	1.21 (0.17)	0.55 (0.09)	0.05 (0.05)	0.42 (0.09)	0.35 (0.05)
	8 y	17	32	1.21 (0.61)		1.29 (0.64)	0.90 (0.45)	0.04 (0.04)	0.03 (0.03)	0.17 (0.01)	0.15 (0.00)

Table 2. Spearman correlations between *Zostera marina* meadow age, net N_2 fluxes, and water column parameters revealed that sediment organic matter (OM) increased with meadow age, while both OM and water column NH_4^+ were positively correlated with N_2 fluxes, indicating that they are the likely drivers of the N_2 fluxes across the chronosequence.

	NH_4^+		OM	
	coeff.	p	coeff.	p
N_2 flux	0.478	0.018	0.393	0.047
Meadow age	0.085	0.691	0.497	0.010

Table 3. Seagrass-specific measurements from the restored meadows were measured at the time of N_2:Ar core collection. Unlike shoot-specific productivity, shoot densities were significantly different between the 3 y and 8 y sites, and neither parameter correlated to net N_2 fluxes.

		Density (m^{-2})	Productivity (cm^2 d^{-1})
June 2009	3 y	84 (30)	1.93 (0.31)
	8 y	406 (25)	0.30 (0.08)
August 2009	3 y	77 (22)	0.18 (0.10)
	8 y	397 (11)	0.18 (0.08)
October 2009	3 y	45 (16)	1.13 (0.11)
	8 y	339 (20)	— —
April 2010	3 y	34 (10)	0.63 (0.20)
	8 y	277 (48)	1.14 (0.08)

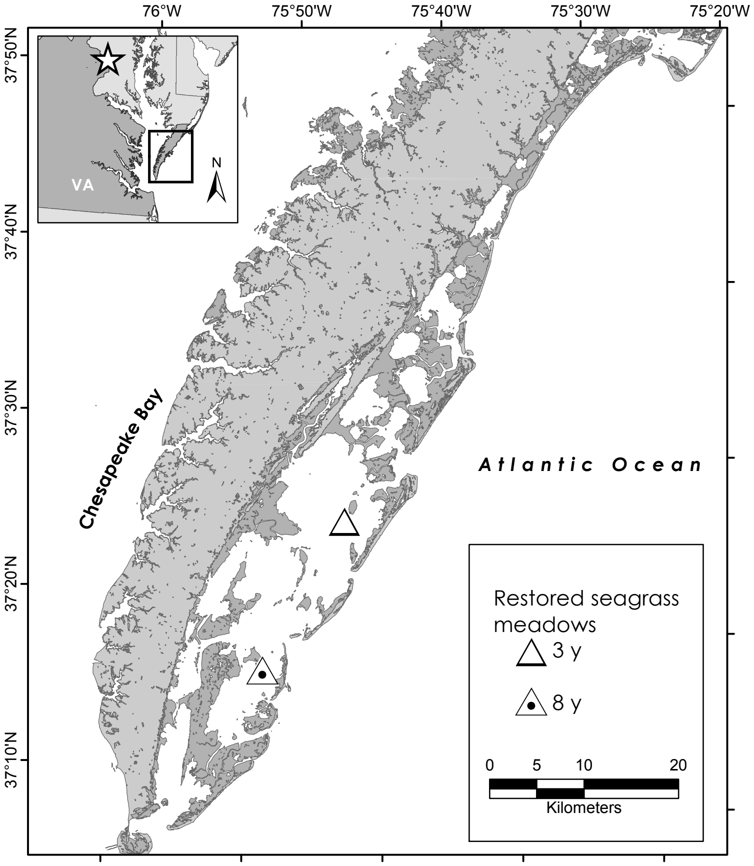

Figure 1. The Virginia coastal bays including locations of *Zostera marina* restoration plots. Replicate vegetated (n = 4) and bare (n =4) plots exist at each of the 2 meadows. No significant differences were found between the 2 sets of bare plots, resulting in a pooled data set for bare sites (n = 8).

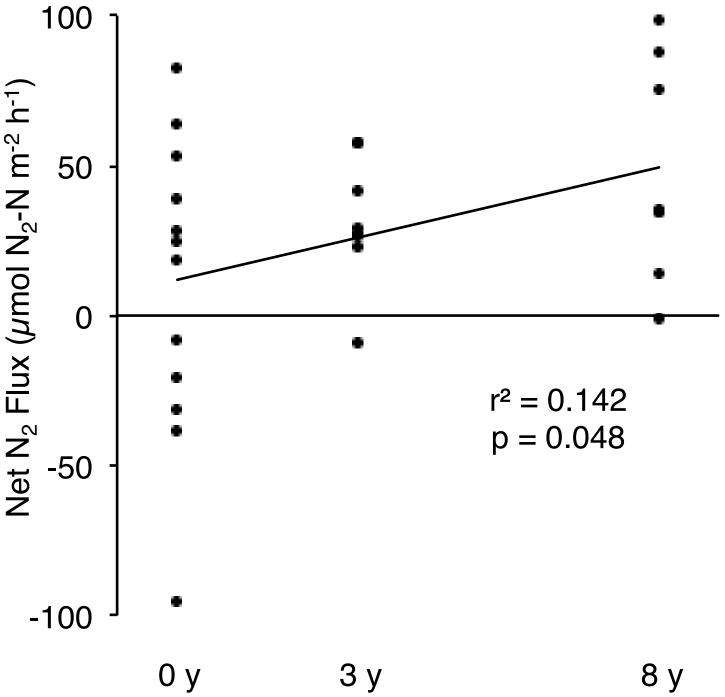

Figure 2. A linear regression of all N_2 fluxes across the chronosequence shows that the Virginia coastal bays are net denitrifying (N_2 loss), and that N_2 loss intensifies as seagrass meadows increase in age. "0 y" data represent bare sediment. Replicate plots are presented as a single point.

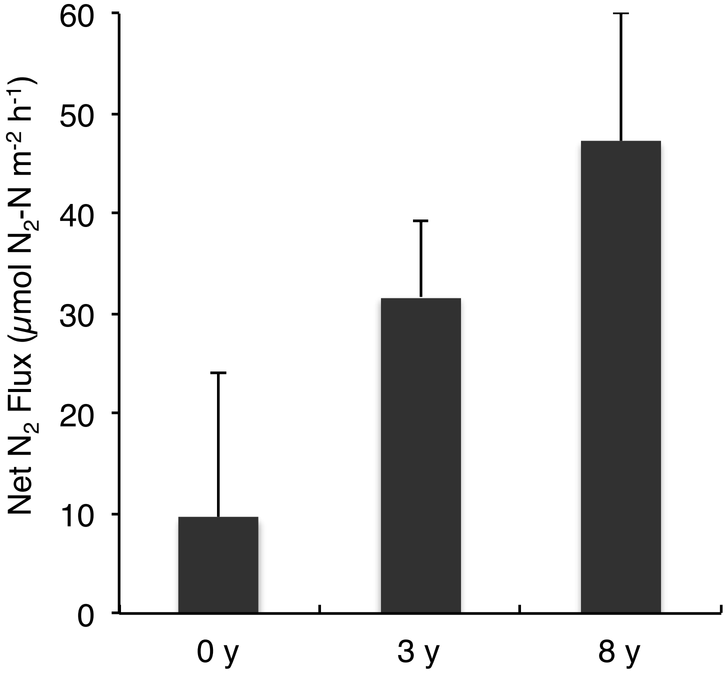

Figure 3. Averaged yearly rates of net N_2 fluxes indicate that seagrass bed age increases the potential for net denitrification. Error bars display standard error (±1 SE).

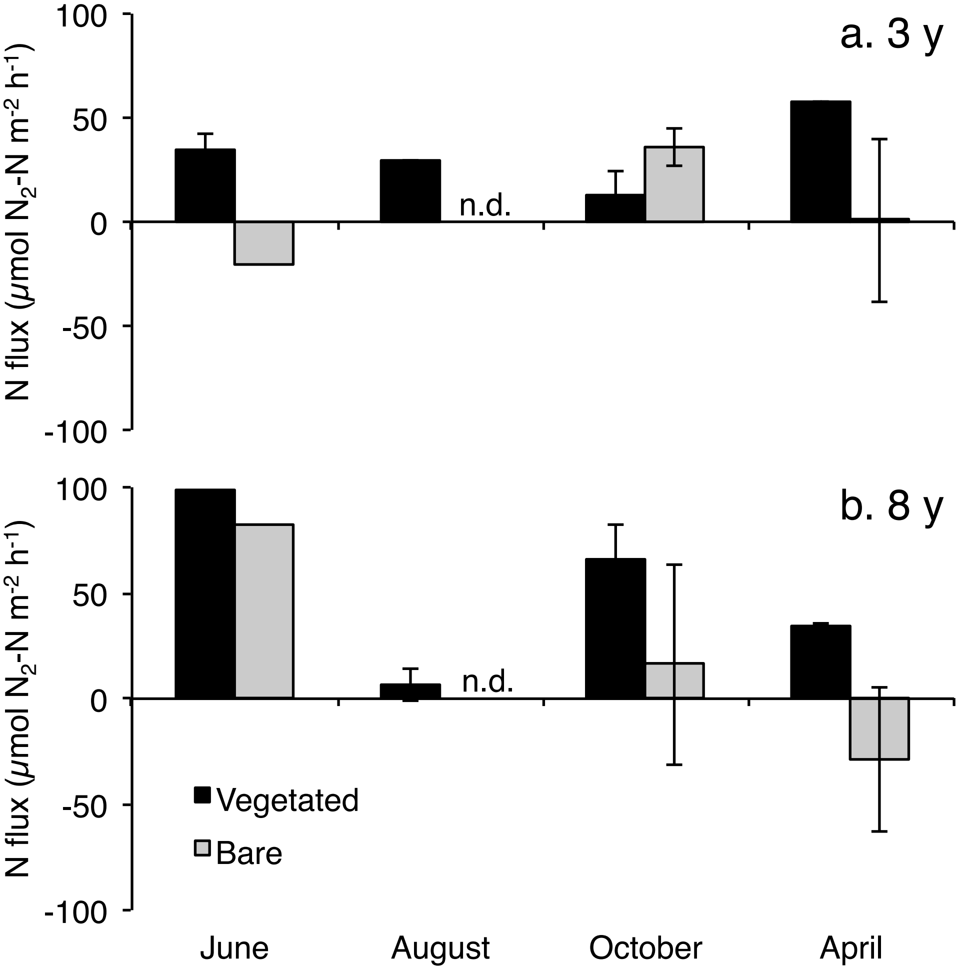

Figure 4. Seasonal net N_2 flux measurements using the N_2:Ar technique. Error bars display standard error (±1 SE). Values without error bars represent single core incubations.

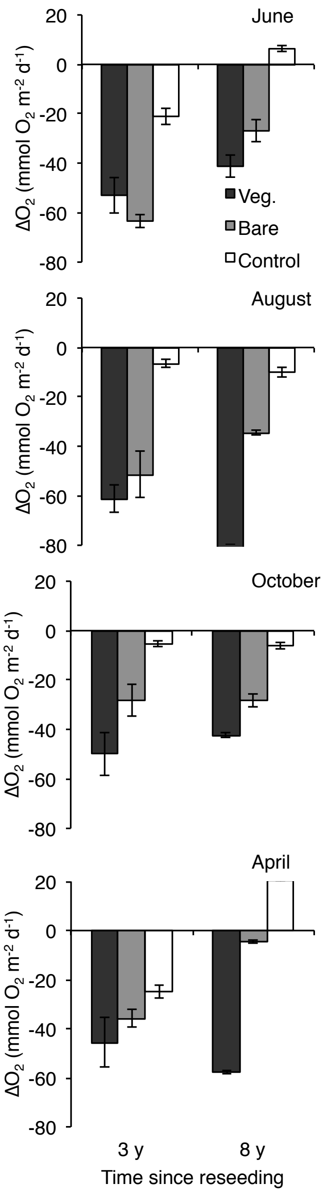

Figure 5. Oxygen uptake measurements from N_2:Ar incubation cores. While no differences were observed between the restored seagrass meadows during any sampling period, differences between vegetated and bare sediment were observed in the 8 y vegetated plots in August ($p = 0.003$), October ($p = 0.01$), and April ($p < 0.001$), confirming that under heterotrophic conditions, vegetated sediment respire at a higher rate than bare sediment alone. Error bars display standard error (±1 SE).

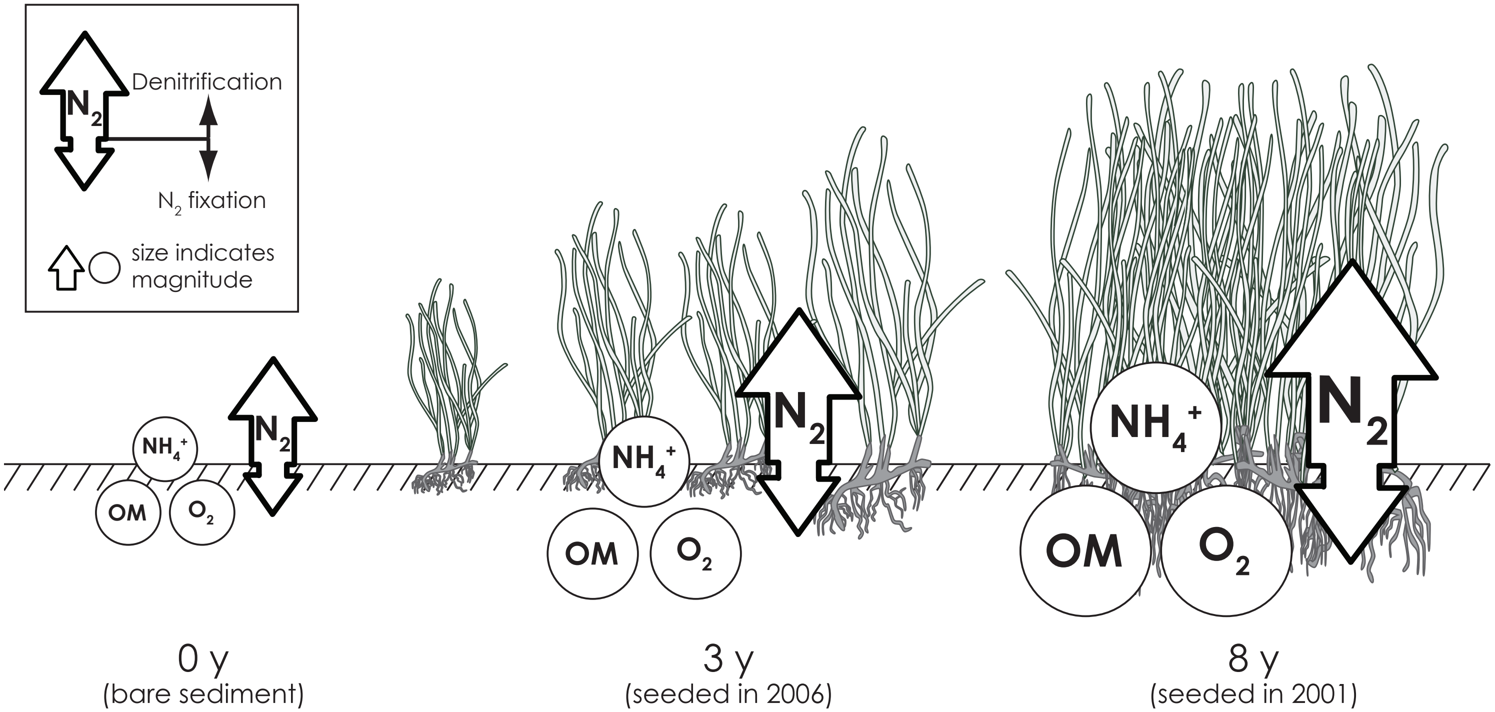

Figure 6. Conceptual diagram of N_2 fluxes across a chronosequence of restored seagrasses in the Virginia coastal bays. In both bare and vegetated sediment, denitrification is the dominant process regulating net N_2 fluxes. Cole & McGlathery (*submitted*) have demonstrated that sediment N_2 fixation increases with seagrass meadow age, regulated by organic matter (OM) availability in the sediment. In this study, we have confirmed that net N_2 fluxes are driven by sediment OM and water column NH_4^+. The increased oxygenation potential of the rhizosphere by seagrasses may increase denitrification, resulting in net denitrification.

Chapter Six: Conclusions

Summary

This dissertation is apposite to the growing field of research showing that seagrasses are essential in mediating the flow of nitrogen (N) as it moves from terrestrial into marine systems. The low N loading rates to the Virginia coastal bays coupled with the ability of seagrass-associated microbial communities to remove N from the system as N_2 gas supports the long-term datasets that show that the Virginia coastal bays have some of the lowest water column N and chl-*a* concentrations of any coastal water body along the east coast of the United States. The major inputs of N to the Virginia coastal bays were agricultural fertilizers and direct deposition onto the surface of the bays; the major determinant of which source was larger depended on the land to bay area ratio (Chapter 2). The N input rates into the Virginia coastal bays are the lowest in the mid-Atlantic, and among the lowest along the east coast of the US. The microbes associated with newly restored seagrass (*Zostera marina*) meadows in this system fixed N_2 gas at increasing rates as the meadows age, and the oldest meadows fixed N_2 at rates comparable to established seagrass meadows, but is unique in that the dominant functional group of N_2 fixers was heterotrophic epiphytes (Chapter 3). These heterotrophic epiphytes dominated the N_2-fixing community in water shallower than 1.25 m MSL, but as the seagrasses reached further toward the 1.6 m MSL seagrass depth limit, total rates of N_2 fixation dropped off logarithmically, and sediment N_2-fixing bacteria became the dominant functional group (Chapter 4). Denitrifying bacteria removed N from the system at higher rates as the *Z. marina* meadows aged. If the seagrass meadows in the Virginia coastal bays were restored to all available habitats within the system, the bacterial communities associated with the seagrasses would be capable of removing N at rates twice as high as the current loading rates measured in Chapter 1 (Chapter 5).

Future research

The role of N in the marine environment is complex, particularly in the presence of seagrass beds (Fig. 1). To date, nearly all measurements of N_2 fluxes in seagrass meadows have come from either the Isotope Pairing Technique (IPT) (Welsh *et al.* 2000, Pinardi *et al.* 2009) or mass balance of individually-measured fluxes (Hemminga *et al.* 1991, Pedersen & Borum 1993), though other techniques exist (Devol 1991, Groffman *et al.* 2006). The results presented here dovetail nicely with a recent study published by Eyre *et al.* (2010) in which the authors calculate a N budget for a shallow oligotrophic subtropical system and measure N_2 fixation and net N_2 fluxes in seagrass meadows using the same methods of this dissertation. Yet while this dissertation provides net N_2 fluxes in *Zostera marina* meadows, the grasses were removed, and the incubation was done *ex situ* in ambient conditions. A non-destructive, closed-system method for

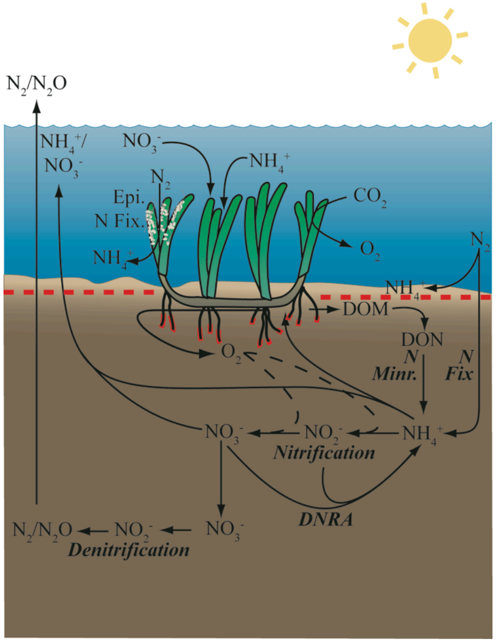

Figure 1. The nitrogen cycle in a seagrass meadow.

measuring N_2 fluxes in vegetated sediment still does not exist for both light and dark conditions. Additional research currently underway using a combination of stoichiometry and the N_2:Ar method may soon solve this methodological shortcoming (A. Giblin, *personal communication*).

Little is known about the ecological impact that newly restored seagrasses have on their environment. The restoration efforts in the Virginia coastal bays are possible because of the rare conditions that caused and followed the removal of *Z. marina* in the late 1920s. Most seagrass loss worldwide is the result of anthropogenic influences (Waycott *et al*. 2009), but the lack of such influences in the Virginia coastal bays presents a unique opportunity to observe and quantify the ecological effects that new seagrass meadows induce. Many of the assumptions in this dissertation focus on the effects that *Z. marina* has on its microbial community. In order to provide a more complete budget of N processes in these meadows, information quantifying: DOC exudation rates into the phyllosphere and rhizosphere, N burial of detritus, oxygenation rates and associated nitrification rates, sediment porewater profiles, and benthic infauna would be useful, and warrant further research. As seagrasses continue to decline worldwide (Orth *et al*. 2006, Waycott *et al*. 2009), continued advancement of the field of seagrass ecology becomes all the more important in the conservation and preservation of these ecosystem engineers.

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