



Nitrogen fixation in restored eelgrass meadows

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ABSTRACT: Biological nitrogen (N_2) fixation is the primary input of new nitrogen (N) to marine systems, and is important in meeting the N demands of primary producers. In this study, we determined whether restoration of the eelgrass *Zostera marina* L. in a shallow coastal bay 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 eelgrass following local extinction in the 1930s until restoration by seeding began in 2001. Restored meadows of different ages were compared to nearby bare sediment sites during summer peak metabolism over 2 yr. Nutrient addition by N_2 fixation was enhanced as the meadows aged. Rates of N_2 fixation in the older (7 to 8 yr old) meadows were 2.7 times more than the younger (2 to 3 yr old) meadows (average 390 and 146 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, respectively), and 28 times more than bare sediments (average 14 $\mu\text{mol N m}^{-2} \text{d}^{-1}$). Heterotrophic epiphyte bacteria fixed approximately 90% of the total N_2 in *Z. marina* meadows of both age classes. Both sediment and epiphyte N_2 fixation were strongly related to *Z. marina* density and sediment organic content, suggesting that 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. The N provided through fixation represented a large fraction (20.5 to 30%) of the total N demand to support eelgrass aboveground growth during this period of peak summertime production.

KEY WORDS: Nitrogen fixation · Seagrass · *Zostera marina* · Restoration · State change · Epiphytes · Sediments

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INTRODUCTION

In many temperate coastal marine systems, nitrogen (N) is often the limiting nutrient for benthic and pelagic primary producers (Howarth 1988, Taylor et al. 1995, Tyler et al. 2003). 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 et al. 2000) and some 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, although 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), studies have shown high rates of benthic N_2 fixation, both in bare sediments (Gardner et al. 2006, Fulweiler et al. 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), particularly in systems where pools of inor-

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ganic N in the porewater are depleted (O'Donohue et al. 1991). N fixation is among the many ecosystem services provided by organisms associated with seagrass habitats in shallow coastal systems. Given the significant loss of 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.

Seagrass-vegetated sediments often have higher rates of N₂ fixation than bare sediments, although there can be considerable overlap in rates. Studies in temperate estuarine and shallow bay systems report rates from 7 to 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 to 300 μmol N m⁻² d⁻¹ (Howarth et al. 1988 and sources therein, Nixon et al. 1996). In shallow, vegetated (sub)tropical systems, much higher N₂ fixation rates have been measured in the rhizosphere of *Zostera capricorni* (1785 to 2800 μmol N m⁻² d⁻¹; O'Donohue et al. 1991) and in *Thalassia testudinum* (360 to 10 000 μmol N m⁻² d⁻¹; McGlathery 2008 and sources therein). In vegetated sediments of some temperate systems, N₂-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 N₂ fixers are typically the dominant functional group in seagrass-vegetated sediments (McGlathery et al. 1998), although autotrophic N₂ fixers are common on illuminated surface sediments (Stal et al. 1985, Paerl et al. 1996). Heterotrophic N₂-fixing bacteria require organic carbon to fuel N₂ 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 N₂-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 et al. 1982, Marbá et al. 2006). This increase in sediment organic matter potentially provides a carbon source for sediment N₂ fixers.

The relative importance of epiphyte N₂ fixation in seagrass meadows has been less studied than sediment N₂ 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 et al. 1991). Epiphytic N₂ 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 N₂ fixation have ranged from one-third to two-thirds (Goering & Parker 1972, Capone & Taylor 1977), although most studies have been performed in the light and thus have been limited to gross autotrophic N₂ 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 epiphyte N₂ 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 N₂ fixation, restoring seagrass in a system devoid of rooted macrophytes can potentially increase the quantity of N introduced by fixation in these ecosystems. The Virginia (USA) 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 yr absence of *Z. marina*, a large-scale restoration program by seeding, begun in 2001, has resulted in approximately 4500 acres (~1821 ha) of eelgrass habitat from 200 acres (~81 ha) initially seeded (Orth et al. 2006, 2010). A restoration program of this scale provides the opportunity to quantify the effects of a state change from an algae-dominated to eelgrass-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 N₂ fixation, including those occurring in the sediments and by epiphytes in the eelgrass canopy.

MATERIALS AND METHODS

Site description

Over 200 km of shallow coastal bays extend from Delaware to Virginia, USA, bounded to the east by barrier islands and to the west by the Delmarva

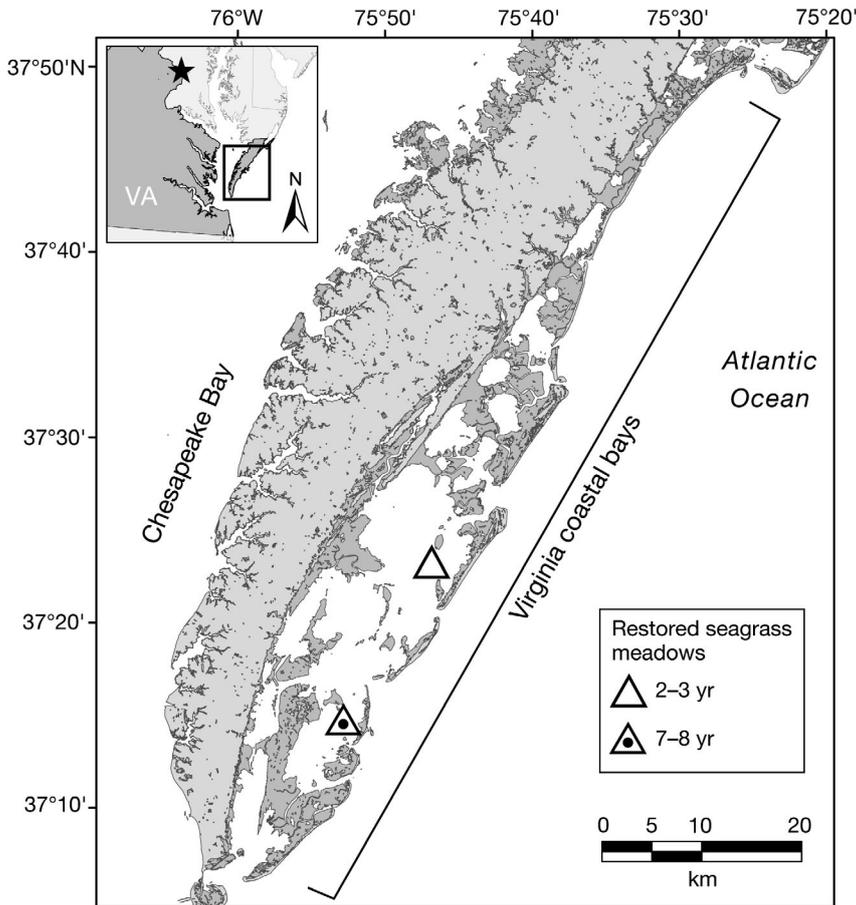


Fig. 1. Virginia (VA) coastal bays, USA. The 2 to 3 and 7 to 8 yr old eelgrass sites are within Hog Island Bay and South Bay, respectively. Land: light grey; marsh: dark grey; ★: Washington, DC

Peninsula (Fig. 1). 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 an eelgrass-dominated system was initiated in 2001 by broadcasting seeds into 1 acre (~0.4 ha) plots at 10^5 seeds acre⁻¹ (Orth et al. 2006, 2010), 4 of which were monitored in the present study. In 2006, a similar area was seeded in 1 acre plots with 10^5 seeds acre⁻¹, 4 of which were monitored in the present study. Four bare sites were identified adjacent to each eelgrass restoration site and were sampled concurrently. Here we compare the bare sites to the 2 to 3 yr old (seeded 2006; hereafter 2/3 yr old) and 7 to 8 yr old (seeded 2001, hereafter 7/8 yr old) eelgrass vegetated sites.

The study sites were located within 20 km of each other (37° 24' 47" N, 75° 43' 36" W and 37° 15' 54" N, 75° 48' 50" W) and were separated only by mudflats and a tidal channel (Fig. 1). 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 mean sea level (MSL) (Lawson et al. 2007, Carr et al. 2010). The vegetated sites were located between 0.9 and 1.4 m MSL while the bare sites ranged from 1.1 to 1.6 m MSL. The tidal amplitude was similar at both sites (1.3 versus 1.2 m; McGlathery et al. 2012, this Theme Section [TS]). Bare sediment characteristics at the sites, including organic matter, N and carbon content, exchangeable NH₄⁺, and grain size, were also not significantly different (McGlathery et al. 2012). 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 yr⁻¹ (authors' unpubl. data), which is extremely low compared to similar shallow coastal bay systems (McGlathery et al. 2007).

N₂ fixation

In June and July of 2008 and 2009, sediment and epiphyte N₂ fixation rates were quantified using the acetylene-reduction technique (methods modified from Capone & Taylor 1977, Capone 1982). Data from June and July were selected to represent peak rates of N₂ fixation based on seasonal and monthly data from 2008 and 2009, respectively. Two sediment cores were collected in each of the 4 replicate plots of each eelgrass age class and the 8 bare sites. The 2 sediment cores were taken by hand in both vegetated and bare sediment using a 1 inch (~2.5 cm) inner diameter clear acrylic corer at random locations within each replicate eelgrass plot, making sure the aboveground eelgrass 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) in the dark. For the incubation, a sub-core was taken from each large core using a 5 cm³ syringe corer down to 5 cm. The sub-core was split lengthwise to provide adequate exposure to the acetylene and to give a depth-integrated estimate of N₂ fixation

rates. Each half was placed in a 40 cm³ 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 argon gas for 2 min, after which time 10 ml of acetylene (C₂H₂) gas was injected into the vials. The vials were then shaken for 10 s and vented to atmospheric pressure. Vials were kept in the dark at field temperature and incubated for 6 to 8 h. Transferring the gas samples into a pre-evacuated vial ended the incubation. Gas samples were run on a Varian Star 3400 Cx gas chromatograph equipped with a flame ionization detector and a 1.86 × 0.32 × 0.25 cm 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 N₂ (as NH₄⁺) using a 3:1 molar conversion (Seitzinger & Garber 1987). An initial analysis to determine the potential for autotrophic sediment N₂ fixation in vegetated sediments showed no differences within a 2 × 2 design for oxic versus anoxic and light versus dark incubation ($F = 1.76$, $p = 0.2$), with all rates comparable to N₂ fixation rates in dark anoxic sediment. Autotrophic N₂ fixation was calculated as the difference between incubations in the light (reflecting activity of both autotrophs and heterotrophs) and dark (heterotrophic activity only). An additional analysis to measure the percentage of N₂ fixation by sulfate-reducing bacteria was performed on replicate cores using the presence/absence of molybdate (40 mM Na₂MoO₄) to suppress sulfate reduction (Howarth et al. 1988), and the treatment resulted in no significant differences between replicates. Based on these findings, and to increase the number of replicates, we used anoxic and dark conditions without molybdate in subsequent incubations.

A profile of sediment N₂ 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 C₂H₂ and incubated in the same manner as described above.

Shoots of *Zostera marina* were collected haphazardly in each of the 4 replicate plots in the 2/3 and 7/8 yr 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 N₂ fixation, chosen randomly from the 5 shoots collected in each replicate plot (2 per replicate plot were analyzed). For each shoot, the length and width of each leaf was recorded to calcu-

late 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 s, 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 N₂ fixation was heterotrophic (dark incubations), and so subsequent incubations were done in the dark. Incubations lasted for 4 to 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 vial 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 fixed N₂ (as NH₄⁺) using a 3:1 molar conversion (Seitzinger & Garber 1987). The fixed N₂ was representative of the leaf area of the top 15 cm of the leaves incubated. To scale to an areal rate of N₂ fixation, the average of the 2 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 N₂ 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 N₂ fixation. This value was multiplied by shoot density to calculate an areal N₂ fixation rate in $\mu\text{mol N m}^{-2} \text{d}^{-1}$.

Site characterization

Eelgrass density and plant productivity were measured concurrently with the N₂ fixation measurements in both 2008 and 2009. At each of the 4 replicate plots for each age category, 10 PVC quadrats (0.25 m²) were thrown and the shoots were counted. Plant productivity was measured on all shoots ($n = 5$ to 20) within 2 anchored grids (20 × 10 cm) 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 to 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 yr old and 7/8 yr old eelgrass meadows and the 8 adjacent bare sites. Five cores were collected equidistant along a 50 m transect in the middle of each plot using a 60 ml syringe, bagged, and placed immediately on ice. Sediment organic content was calculated as loss on ignition from sediment dry weight after combustion in a 500°C muffle furnace for 8 h. Carbon and N contents were measured on dried sediment (60°C for at least 48 h) using a Carlo Erba Elemental Analyzer with a 1020°C combustion tube and 650°C 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 eelgrass age class). An initial test showed that the sediment characteristics of the bare sites at the 2 locations were not significantly different, so all bare sites were pooled for further comparisons with the eelgrass sites in each age class. For the eelgrass characteristics, replicate measurements of densities and productivities within each plot were also averaged, and all replicate plots for each age class were then averaged. Differences in

pooled sediment and seagrass parameters were tested using analysis of variance (ANOVA); all parameters conformed to ANOVA assumptions (heteroscedasticity and normality). Epiphyte N₂ 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 2 eelgrass age classes. Analyses of epiphyte and sediment N₂ fixation, eelgrass characteristics, and sediment profiles as a function of *Zostera marina* meadow age (time since seeding) were done using a 2-way ANOVA, and pairwise differences were tested using post hoc Tukey's tests (SAS version 9.2). Linear regressions were used to analyze relationships between N₂ fixation rates and plant characteristics or chemical variables using statistical software (Minitab). For all comparisons, p -values ≤ 0.05 were considered significant.

RESULTS

Sediment N₂ fixation

Heterotrophs were the dominant functional group fixing N₂ in the sediments, as indicated by the similar rates between light and dark incubations. Sulfate-reducing N₂-fixing bacteria were responsible for a relatively small percentage of the sediment N fixation, contributing 16% of the sediment N₂ fixation in both bare and vegetated sites. Sediment N₂ fixation rates were significantly higher in 2008 than in 2009, possibly due to low temperatures in 2009 (Fig. 2). In 2008, peak sediment N₂ fixation rates (June to July) in the 7/8 yr old eelgrass meadows (mean \pm SE = $32.5 \pm 1.2 \mu\text{mol N m}^{-2} \text{d}^{-1}$) were significantly higher than in the 2/3 yr old meadows ($21 \pm 2.0 \mu\text{mol N m}^{-2} \text{d}^{-1}$) and bare sediments ($18 \pm 1.5 \mu\text{mol N m}^{-2} \text{d}^{-1}$; Fig. 2). In 2009, however, the only significant differences were between the 7/8 yr old meadows ($18 \pm 1.0 \mu\text{mol N m}^{-2} \text{d}^{-1}$) and bare sediments ($9.7 \pm 3.0 \mu\text{mol N m}^{-2} \text{d}^{-1}$; Fig. 2). There were no significant differences between the bare sediments and 2/3 yr old meadows in either year. Depth profiles of sediment N₂ fixation showed that rates increased with depth up to 5 cm in the eelgrass-vegetated sediments and decreased with depth in the bare sediments (Fig. 3).

Shoot density, areal rates of primary productivity, and sediment organic matter were identified as environmental variables that could influence sediment N₂ fixation rates in eelgrass-vegetated sediments of different ages (time since seeding; Table 1). The in-

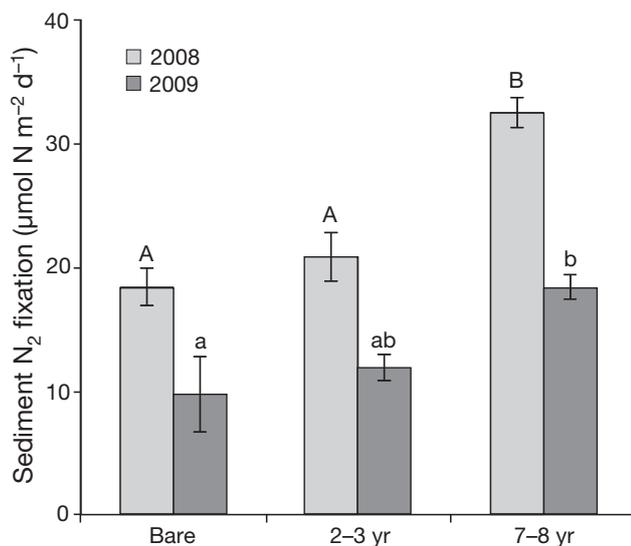


Fig. 2. Sediment N₂ fixation in bare sediment (bare) and in the rhizosphere of restored *Zostera marina* meadows (2 to 3 and 7 to 8 yr old). Different letters indicate statistically significant differences (ANOVA, post hoc Tukey's test; $p < 0.05$); case of letter is relative to year. Means \pm 1 SE

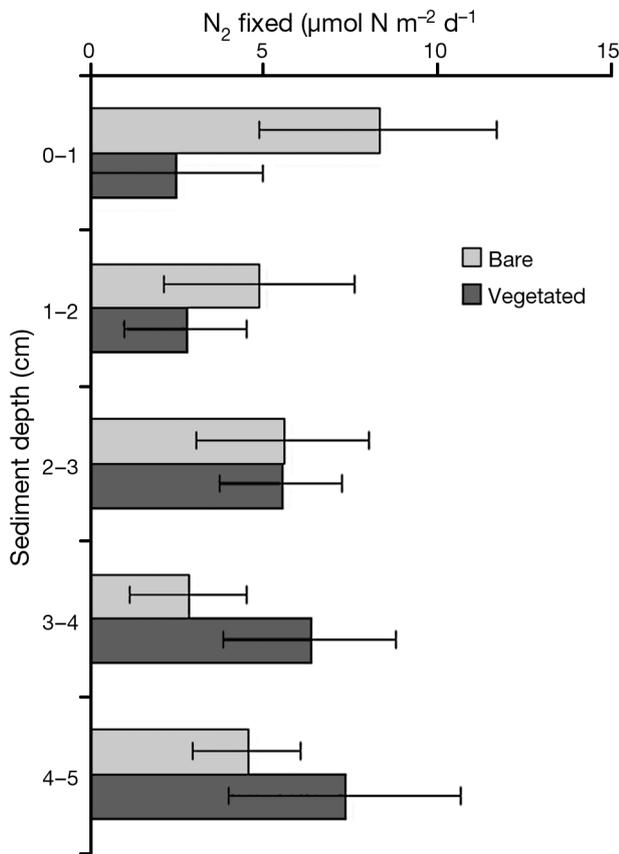


Fig. 3. Depth profile of sediment N_2 fixation in bare and *Zostera marina* vegetated sediment. No significant differences across depth or sediment type are apparent, although peak rates and depth relationships are different in the presence versus absence of eelgrass. Means \pm 1 SE

crease in *Zostera marina* shoot density was positively correlated with sediment N_2 fixation in both 2008 and 2009 (Fig. 4). Additionally, the higher rates of areal productivity were positively correlated with higher rates of sediment N_2 fixation, though only significant in 2008 (Fig. 4). Lastly, higher sediment organic mat-

ter was positively correlated with sediment N_2 fixation, though again only significant in 2008 (Fig. 4). Despite having an $r^2 = 0.93$, the low number of samples in 2009 yielded a linear regression that was not significant.

Epiphyte N_2 fixation

Rates of epiphyte N_2 fixation were higher in the 7/8 yr old *Zostera marina* meadows than in the 2/3 yr old meadows during both years (Fig. 5). Heterotrophs were the dominant functional group of the epiphytic N_2 fixers in both the 7/8 and 2/3 yr old meadows; our initial test indicated that autotrophs fixed $<1\%$ of the total N_2 fixed. Rates of epiphyte N_2 fixation in 2009 were significantly higher than in 2008 (Fig. 5), an opposite trend than what was measured for sediment N_2 fixation (Fig. 2).

Areal rates of epiphyte N_2 fixation were positively correlated with *Zostera marina* shoot density, though not significantly in either year (Fig. 6). Furthermore, shoot-specific productivity did not vary between sites of different ages, but did vary by year (Table 1). Because epiphyte N_2 fixation rates were not significantly correlated with shoot density, but were positively correlated with areal productivity rates in both 2008 and 2009, we show that the areal productivity effect occurred independently of shoot density (Fig. 6). The slopes of the regressions for epiphyte N_2 fixation versus density and productivity were considerably steeper in 2008 than in 2009 (Fig. 6).

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 and July in 2008 and 2009, with a higher mean temperature in June and July 2008 of 0.81°C (VCR LTER, www.vcr.lter.virginia.edu/data/metdata/index.html).

Table 1. *Zostera marina*. Eelgrass and sediment parameters of restored *Z. marina* meadows and associated bare sediment. Means \pm SE

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

Eelgrass and sediment parameters

Eelgrass and sediment parameters for the bare sediments and different-aged eelgrass meadows showed significant differences by both meadow age and sampling year (Table 1). The 7/8 yr old *Zostera marina* meadows had significantly higher shoot densities than the 2/3 yr old meadows in

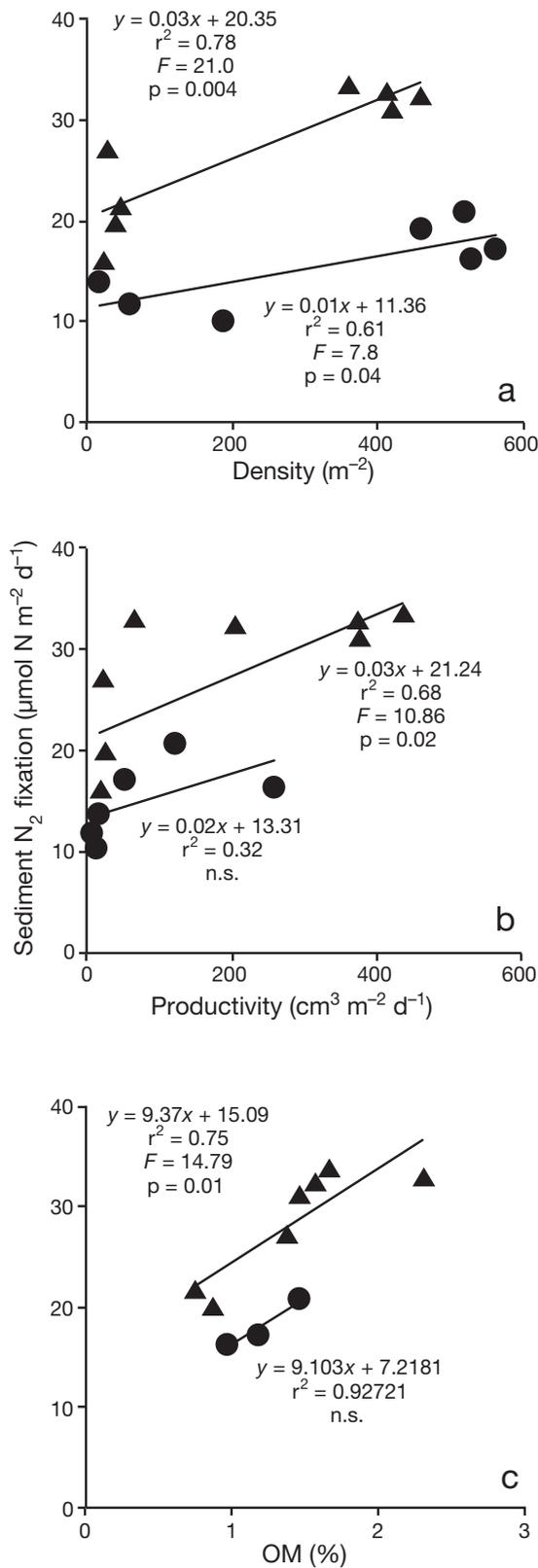


Fig. 4. Sediment N₂ fixation regressed with (a) eelgrass *Zostera marina* shoot density and (b) areal productivity, and (c) sediment organic matter (OM) for the years 2008 (▲) and 2009 (●)

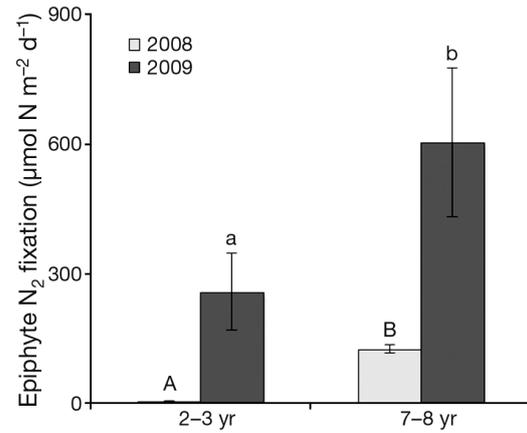


Fig. 5. *Zostera marina*. Epiphyte N₂ fixation in the eelgrass meadows of different ages (2 to 3 and 7 to 8 yr old). Significant differences represented by difference in within-case letter coding (ANOVA, post hoc Tukey's test; $p < 0.05$); case is relative to year. Means \pm 1 SE

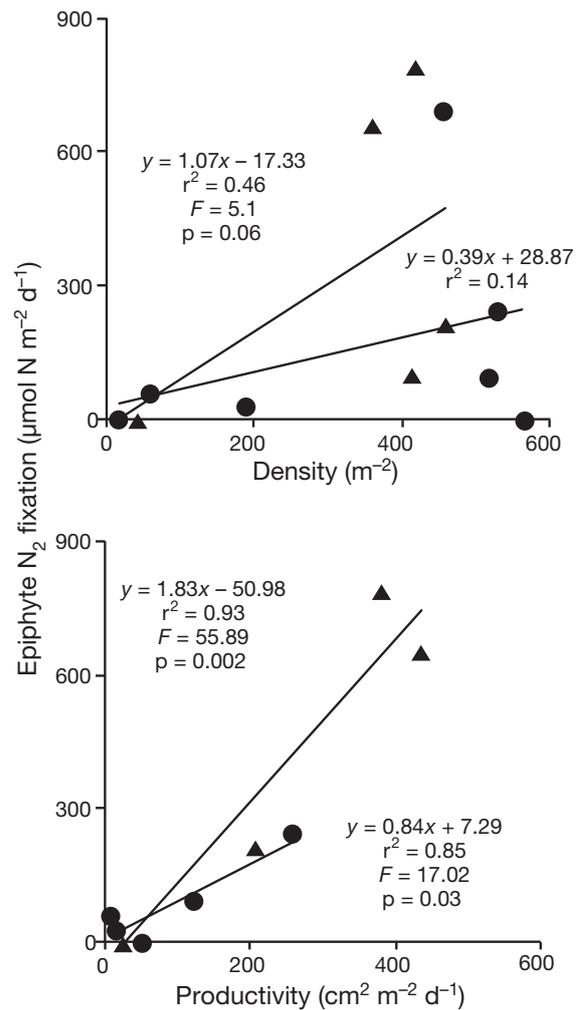


Fig. 6. *Zostera marina*. Regressions of epiphyte N₂ fixation with eelgrass shoot density and areal productivity for the years 2008 (▲) and 2009 (●)

Table 2. *Zostera marina*. ANOVA table of p-values ($\alpha = 0.05$) for biological and physical eelgrass characteristics between meadow ages (2 to 3 and 7 to 8 yr old) and over the course of this study

Block	Comparison	Density	Productivity	Foliar N	Foliar C	C:N ratio
2–3 yr	2008 vs. 2009	0.26	0.005	–	–	–
7–8 yr	2008 vs. 2009	0.01	0.04	–	–	–
2008	2–3 vs. 7–8 yr	<0.001	0.54	–	–	–
2009	2–3 vs. 7–8 yr	<0.001	0.56	–	–	–
All	2–3 vs. 7–8 yr	<0.001	<0.001	<0.001	0.33	<0.01

both 2008 and 2009 (Table 2). The rates of productivity per shoot were not different between the two meadow types in 2008 or 2009. However, productivity rates per shoot were higher at both eelgrass 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 yr old meadows (2.3% versus 1.2% in the older meadows). Average C:N indicated that the younger meadows were significantly less N-limited than the older meadows (C:N 20.6 versus 37.3). The 7/8 yr 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 yr old meadows and bare sediments ($p = 0.32$).

DISCUSSION

Total rates of N₂ fixation

Our data show that N₂ fixation in restored eelgrass meadows was enhanced as the meadows aged. The markedly higher rates of N₂ fixation in the 7/8 yr 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. N₂-fixing bacteria in the older meadows fixed on average 390 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, 2.7 times more than the younger meadows (146 $\mu\text{mol N m}^{-2} \text{d}^{-1}$), and 28 times more than bare sediment sites (14 $\mu\text{mol N m}^{-2} \text{d}^{-1}$), and the majority of the N₂ fixed was by heterotrophs (Fig. 7). The higher variance at the vegetated sites was a result of the high and variable rates of epiphyte N₂ fixation. Our findings are in agreement with previous studies showing a positive feedback between seagrass presence and rates of N₂ fixation (Capone 1988, Welsh et al. 2000, Carpenter & Capone 2008), but show for the first time that this

feedback is based on seagrass density. N₂ 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 N₂ fixation rates than bare sediments, our data show that by 7 to 8 yr since seeding when densities were >400 shoots m^{-2} , rates were significantly greater.

The rates of N₂ 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 to 8 yr 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 N₂ fixation (279 to 464 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) comparable to our 7/8 yr old site. The results of 2 European studies of *Z. marina* and *Z. noltii* are also consistent with our findings, with rates of N₂ fixation ranging from 300 to 429 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (McGlathery et al. 1998) and 143 to 521 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (Welsh et al. 1996a), respectively. It is important to note that the total N₂ fixation rates were equivalent primarily because our epiphyte N₂ fixation rates were very high. Our rates of total N₂ fixation only include heterotrophic N₂ fixation based on our find-

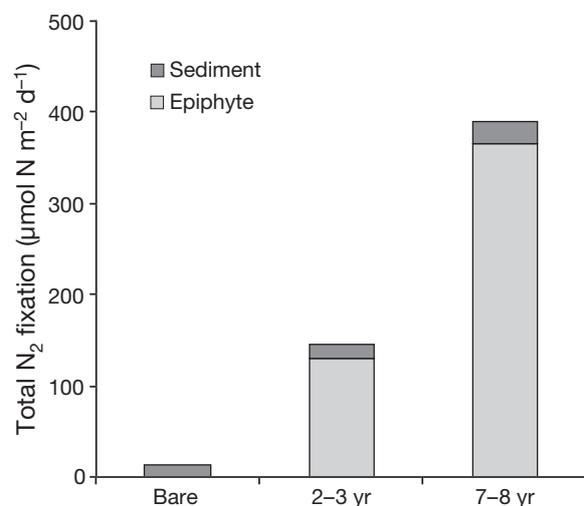


Fig. 7. *Zostera marina*. Total rates of N₂ fixation. Bare: sediment N₂ fixation in bare sediment only. Epiphyte: *Zostera marina* vegetated sediments; sum of sediment N₂ fixation and epiphyte N₂ fixation. Variance of rates and statistics are reported in 'Results' and 'Discussion'

ings that autotrophic N₂ fixation contributed a minor amount of N₂ fixed, and thus may be a slightly conservative estimate of total N₂ fixation rates.

Contributions of epiphyte and sediment N₂ fixation

The majority of total N₂ fixation (epiphyte + sediment N₂ fixation) in the eelgrass meadows of both age classes was attributed to epiphytes on the eelgrass blades, which accounted for 89 and 93% of the total N₂ fixed in the younger and older meadows, respectively (Fig. 7). On an areal basis, epiphyte N₂ fixation was higher in the older *Zostera marina* meadows in both years. Because shoot-specific rates of N₂ fixation and primary productivity were not different between meadows in the different age classes, we attribute the higher epiphyte N₂ fixation in the older meadows to differences in shoot densities that were a function of eelgrass meadow age (Table 1, Fig. 6).

The dominance of N₂ fixation by epiphytes compared to sediments has not been shown previously in other temperate seagrass meadows. We know of only 1 study in a tropical system, the Gulf of Carpentaria, Australia, where N₂ fixation was dominated by epiphytes for the seagrass *Enhalus acoroides* (300 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ compared to rinsed roots at 21 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ and rhizomes at 7 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) during summer months (Moriarty & O'Donohue 1993). In our study, summertime epiphyte N₂ fixation rates ranged from 2 to 256 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (mean 129 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) in the younger meadows, and rates were significantly higher (123 to 604 $\mu\text{mol N m}^{-2} \text{d}^{-1}$; mean 364 $\mu\text{mol N m}^{-2} \text{d}^{-1}$) in the older seagrass meadows (Fig. 5). For temperate seagrass meadows, previous studies have shown that *Zostera marina* leaves collected in North Carolina, USA, had low to undetectable rates of epiphyte N₂ fixation (McRoy et al. 1973), and *Ruppia maritima* collected from brackish water in Chesapeake Bay had rates of 5 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (Lipschultz et al. 1979). A comparison with tropical systems shows some similar rates to those we measured, where N₂ fixation associated with *Thalassia testudinum* leaves ranged from 82 to 228 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (Capone et al. 1979) to 285 to 357 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ (Capone & Taylor 1977). The high rates of epiphyte N₂ fixation in the Virginia coastal bays may be in part related to the low nutrient status and high water clarity of these coastal bays. External nutrient loading rates to the Virginia coastal

bays are very low compared to other shallow coastal bay systems (McGlathery et al. 2007, Giordano et al. 2011, authors' unpubl. data), and the 16 yr database from the VCR LTER shows that water column chlorophyll was very low (chl *a*: 1 to 6.5 $\mu\text{g l}^{-1}$) and has shown no trends of decreasing water quality during this period (www1.vcr.lter.virginia.edu/home1/?q=data_wq). Additionally, Orth et al. (2012, this TS) report median turbidity values to be very low, typically <10 NTU from 2003 to 2009.

Sediment N₂ fixation measured in the rhizosphere of *Zostera marina* only comprised on average 11% of the total N₂ fixed in the younger meadows and 7% in the older meadows. Rates of N₂ fixation in the 7/8 yr old *Z. marina* meadows were significantly higher than in the younger meadows in 2008, but not in 2009, although the trend of increasing sediment N₂ fixation with eelgrass meadow age was evident that year. This difference may be due to the lower temperatures in 2009 compared to 2008, which could have translated into lower eelgrass productivity rates. We found that rates of sediment N₂ 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 to 25 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, present study; 279 to 464 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, Capone 1982). Peak sediment N₂ fixation rates reported from temperate European seagrass meadows were also consistently higher than those found in the present study (143 to 521 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, Welsh et al. 1996a; 300 to 429 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, McGlathery et al. 1998). We suspect that the rates of sediment N₂ fixation in the present 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 (our Table 1; Neckles et al. 1993, Moore & Wetzel 2000), and this influences the positive feedback between seagrass presence and N₂ fixation.

Drivers of N₂ fixation

The mechanisms behind the positive relationship between *Zostera marina* shoot density and areal sediment and epiphyte N₂ fixation rates that were apparent in the meadows of different ages during both years are likely the stimulation of bacterial activity in the rhizosphere by the excretion of organic carbon from eelgrass roots and also by accumulation of organic matter in the sediments

(Figs. 2 & 6). The majority of the N_2 fixation (both sediment and epiphyte) 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 eelgrass on N_2 fixation, showing peak rates in the rhizosphere of vegetated sediments where maximum root biomass occurred (4 to 5 cm below surface), while the peak in bare sediment cores was at the surface (0 to 1 cm; Fig. 3). 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 eelgrass meadows, where the sediment organic content was significantly higher, also had higher sediment N_2 fixation than bare sediments (Fig. 2). 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 to 20% of the total N_2 compared to >80% (Capone 1982, Welsh et al. 1996b), although 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 to 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 Virginia coastal bays (Giordano et al. 2011, authors' unpubl. data, VCR LTER database, www1.vcr.lter.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 (e.g. Herbert 1975, Welsh et al. 2000). 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 et al. 1982, Moore 2004, Marbá et al. 2006, Chen et al.

2007, Gruber & Kemp 2010, Hansen & Reidenbach 2012, this TS). Our findings suggest the mechanism of drag-induced particle deposition can be affected by meadow age as seagrass density and sediment organic matter increase because both of these variables correlated positively to an increase in sediment N_2 fixation (Fig. 4).

Contribution to *Zostera marina* N demand

N demand was calculated from the N content of the eelgrass leaves, the shoot-specific primary production rate, and shoot density. During the study period, the N demand for the 2/3 yr old eelgrass meadow was $488 \mu\text{mol N m}^{-2} \text{d}^{-1}$ and $1903 \mu\text{mol N m}^{-2} \text{d}^{-1}$ for the 7/8 yr old meadow. The 16.4 and $25.8 \mu\text{mol N m}^{-2} \text{d}^{-1}$ supplied by sediment N_2 fixation in the younger and older meadows, respectively, had the potential to supply 3.4 and 1.4% of the total N demand of each eelgrass meadow. The larger amount of N_2 fixed by epiphytes had the potential to provide 19 to 26.5% to the total N demand in the older and younger meadows, respectively. Combining the sediment and epiphyte N_2 fixation rates, we see that N_2 fixation could supply 29.8% of the total *Z. 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 C:N ratios for the eelgrass tissues in the restored meadows (younger: 21, older: 37) and the low N content of the leaves (younger: 2.3%, older: 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 eelgrass 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 to 150 yr, 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 yr period when the coastal bays of the VCR were devoid of seagrasses, restored *Zostera marina* meadows (7 to 8 yr old in this study) fixed N₂ at rates comparable to established mature meadows primarily because of high rates of heterotrophic epiphyte N₂ fixation, and these rates were nearly 30-fold higher than bare sediments. If the rates of N₂ fixation for the older eelgrass meadows are scaled to *Z. marina* potential habitat in the VCR coastal bays (depths at MSL between 0.6 and 1.6 m; Carr et al. 2010, 2012, this TS), we see the potential for nearly 3.3×10^5 kg N to be fixed each year, which equates to 77% of the allochthonous N from the watersheds of the VCR (authors' unpubl. data). In comparison, if the same potential habitat was to remain unvegetated, the N₂ 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 N₂ fixation that supplements the plant and bacterial communities, potentially supporting continued expansion of the eelgrass meadow into uncolonized, bare sediment.

Acknowledgements. Financial support for this study was provided by the National Science Foundation grant DEB-0621014 to the Virginia Coast Reserve LTER project. We thank L. Reynolds, C. Buck, S. Barry, K. Hondula, M. Miller, and C. Wolner for field, laboratory, and statistics support.

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