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Wetland response to sedimentation and nitrogen loading: diversification and inhibition of nitrogen-fixing microbes

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Abstract. Anthropogenic inputs of nutrients and sediment simultaneously impact coastal ecosystems, such as wetlands, especially during storms. Independent and combined effects of sediment and ammonium nitrate loading on nitrogen fixation rates and diversity of microbes that fix nitrogen (diazotrophs) were tested via field manipulations in *Spartina foliosa* and unvegetated zones at Tijuana Estuary (California, USA). This estuary is subject to episodic nitrogen enrichment and sedimentation associated with rain-driven flooding and slope instabilities, the latter of which may worsen as the Triple Border Fence is constructed along the U.S.–Mexico border. Responses of diazotrophs were assessed over 17 days using acetylene reduction assays and genetic fingerprinting (terminal restriction fragment length polymorphism [T-RFLP]) of *nifH*, which codes for dinitrogenase reductase. Sulfate-reducing bacteria performed ~70% of nitrogen fixation in *Spartina foliosa* rhizospheres in the absence of nitrogen loading, based on sodium molybdate inhibitions in the laboratory. Following nutrient additions, richness (number of T-RFs [terminal restriction fragments]) and evenness (relative T-RF fluorescence) of diazotrophs in surface sediments increased, but nitrogen fixation rates decreased significantly within 17 days. These responses illustrate, within a microbial community, conformance to a more general ecological pattern of high function among assemblages of low diversity. Diazotroph community composition (T-RF profiles) and rhizosphere diversity were not affected. Pore water ammonium concentrations were higher and more persistent for 17 days in plots receiving sediment additions (1 cm deep), suggesting that recovery of diazotroph functions may be delayed by the combination of sediment and nutrient inputs. Nitrogen fixation constitutes a mechanism for rapid transfer of fixed N to *S. foliosa* roots and a variety of primary consumers (within 3 and 8 days, respectively), as determined via $^{15}N_2$ enrichment studies with in situ microcosms of intact marsh sediment. Thus, long-term declines in nitrogen fixation rates in response to increasingly frequent nutrient loading and sedimentation may potentially alter nitrogen sources for vascular plants as well as trophic pathways in wetland ecosystems.

Key words: diazotrophs, diversity; salt marsh; Spartina foliosa; *T-RFLP; terminal restriction fragment length polymorphism; Tijuana Estuary, California, USA; Triple Border Fence; watershed.*

Introduction

Human-induced alterations of nutrient and sediment regimes on global scales threaten biological diversity and core ecosystem functions that maintain diversity. Although stress and disturbance naturally shape biological communities, multiple anthropogenic impacts can increase the magnitude, duration, and complexity of the environmental changes that affect a variety of ecosystems (Halpern et al. 2008).

The effects of nutrient loading on several ecosystems depend on responses of microbial communities, which mediate the cycling of nitrogen (Howarth 1993, Ward 2005) and their interactions with plants. In coastal wetlands, such as salt marshes, nitrogen-fixing bacteria transform N_2 into biologically available NH_3 in cyanobacterial mats as well as on plant shoots (Currin and Paerl 1998), roots, and rhizomes (Whiting et al. 1986). Nitrogen fixers, or diazotrophs, can rapidly channel reduced nitrogen to vascular wetland plants via specific, mutualistic associations (Bagwell and Lovell 2000) and are of particular importance in developing wetlands (Tyler et al. 2003). Wetlands also receive riverine, atmospheric, and marine inputs of nitrogen (Boyer et al. 2001, Traut 2005, Scott et al. 2007), which vary in their timing and are less tightly coupled to plant productivity. As nitrogen is often the limiting nutrient in coastal wetlands, its availability exerts a major influence on plant community structures and ecosystem productivity (Valiela 1983, Scott et al. 2007) and, ultimately, sustains secondary functions that depend upon the

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presence and structure of vascular plants (Whitcraft and Levin 2006).

Although coastal wetlands are maintained at the land–sea interface by natural processes that introduce sediment and nitrogen, strong anthropogenic alteration of sediment and nutrient delivery is negatively affecting diversity and function of these ecosystems (Howarth et al. 2000, Thrush et al. 2004, Deegan et al. 2007). While damming has starved some wetlands of sediment (Simeoni and Corbau 2009), the magnitude and frequency of sediment and nutrient loading have increased far beyond historical ranges in several regions due to deforestation, urbanization, habitat destruction, and hydrological alteration of watersheds along coasts worldwide (Thrush et al. 2004). Industrial fixation of nitrogen now matches global rates of natural nitrogen fixation (Galloway et al. 1995). Further, extreme climatic events, anticipated from global warming, including hurricanes and other coastal storms may exacerbate sediment and nutrient loads to coastal ecosystems (Paerl et al. 2002, Day et al. 2007).

Nitrogen of anthropogenic origin does not seem to simply substitute for that obtained via intimate ecological interactions of plants and diazotrophs. Rather, nitrogen additions to the environment from human activities, via fertilizer and atmospheric sources, are known to stimulate coastal eutrophication, the increase in organic matter that currently afflicts more than half of the estuaries in the United States (Galloway et al. 1995, Howarth et al. 2000). Eutrophication ultimately leads to loss of vascular plants through algal blooms, smothering, and associated hypoxia (Herbert 1999, Nixon et al. 2001, Duarte 2002, Diaz and Rosenberg 2008). Nitrogen addition to marsh environments also favors more competitive plant species, such as *Salicornia virginica* (*Sarcocornia pacifica*), over the more stress-tolerant *Spartina foliosa* (Boyer and Zedler 1999). These changes may occur via negative effects of nutrient loading on the microbes that otherwise introduce reactive nitrogen to specific plant species in wetlands (Moseman 2007).

Diazotrophs have been hypothesized to be less competitive among microbial communities in the presence of high exogenous nitrogen availability (Kolb and Martin 1988, Piceno and Lovell 2000). The nitrogenase enzyme with which diazotrophs catalyze nitrogen fixation is known to be inhibited by ammonium, and fixing nitrogen is energetically demanding (Yoch and Whiting 1986). Impacts of nutrient and sediment loading on diazotrophs may include not only changes in their function (nitrogen fixation rates) but also shifts in their diversity and community composition, which are thought to hold important but undefined consequences for maintenance of the biogeochemical functions that they perform (Tiedje et al. 1999, Bagwell and Lovell 2000, Smith 2007). Although the diversity of diazotroph communities has thus far seemed resistant to effects of nitrogen loading in natural environments (Piceno and Lovell 2000), less is known about the susceptibility of these microbial communities to sediment loading or multiple environmental changes in general. Predictions regarding the ecological consequences of multiple human impacts require a fundamental understanding of their synergistic interactions and mechanisms that underlie ecosystem function (Breitburg et al. 1999).

In Tijuana Estuary, a National Estuarine Research Reserve immediately north of the U.S.–Mexico Border in California, USA, heavy sediment loads are carried from destabilized hillsides by floodwaters following episodic rainfall (Zedler et al. 1992). The floodwaters also introduce high nutrient loads from ammonium-rich sewage when heavy rainfall exceeds the capacity of treatment plants. Urban and agricultural runoff also brings sediment and nutrients (ammonium and nitrate) into the estuary (King 2003). This setting is particularly timely for studies of sedimentation impacts, as construction of the congressionally mandated Triple Border Fence on the U.S.–Mexico border has required massive restructuring of the landscape along the entire southern border of this reserve and is expected to exacerbate sediment influx to the Tijuana Estuary (Altes and Snapp-Cook 2003).

The objectives of this study were to characterize the effects of two human impacts, sediment and nitrogen (ammonium nitrate) loading, on nitrogen fixation and diazotroph community structures in a coastal wetland. The following hypotheses were addressed: (1) Nitrogen loading decreases the diversity of diazotroph assemblages in both surface (0–1 cm) and rhizosphere (4–5 cm) sediments (via pore water mixing through sediment), and (2) sediment loading decreases diversity of diazotrophs in surface sediments (via physical smothering) but not in rhizospheres; (3) sediment and nitrogen additions decrease nitrogen fixation rates (via smothering of marsh surfaces and increasing availability of exogenous nitrogen, respectively). Combined effects of these impacts were hypothesized to be greater than their individual influences on nitrogen fixation. Changes in nitrogen fixation rates were hypothesized to occur independently of shifts in diazotroph community composition.

Four treatments were applied to *Spartina foliosa*-vegetated (Experiment 1) and unvegetated (Experiment 2) salt marsh sediments: (1) sediment and nitrogen loading, (2) sediment loading only, (3) nitrogen loading only, and (4) a control (no sediment or nitrogen). Diversity and functional responses of nitrogen-fixing microbes to these manipulations were contrasted among the four treatments over a period of 17 days. Responses of diazotroph assemblages were studied in both surface (0–1 cm deep) and subsurface rhizosphere (4–5 cm deep) micro-environments. The contribution of diazotrophs in *S. foliosa* rhizospheres to nitrogen fixation rates were estimated via sodium molybdate inhibitions (Experiment 3).

To assess potential consequences of changes in nitrogen fixation for primary and secondary production, an isotopic enrichment experiment using $^{15}N_2$ was performed (Experiment 4). This study tested whether newly fixed nitrogen can be a nutrient source for *S. foliosa* and macrofauna over short time scales (3–8 days). Pathways by which plants acquired newly fixed nitrogen were qualitatively characterized through comparisons of root and shoot enrichment with ^{15}N. Short-term effects of high levels of exogenous nutrients, hypothesized to decrease nitrogen fixation rates, on ^{15}N enrichments in plant tissues (from $^{15}N_2$) were also explored.

Methods

Study area

The Tijuana River National Estuarine Research Reserve, located immediately north of the U.S.–Mexico border in California, USA (32°34′ N, 117°7′ W), includes salt marshes, tidal creeks, and upland–wetland transition areas (Kennish 2004). Nutrient pollution in the form of sewage and urban and agricultural run off has caused significant impacts on water quality (Seamans 1988). The estuary has experienced an 80% reduction in tidal prism between 1852 and 1986 as a result of sedimentation (Williams and Swanson 1987), which originates from urbanized and destabilized hillsides (Zedler et al. 1992). In the southernmost portions of the estuary, deposits have been as high as 2 m in a given year. Vegetated marshes in northern regions of the estuary have been found to accrete sediment at rates of 2–8.5 cm/yr (Cahoon et al. 1996).

The 20-acre Friendship Marsh of Tijuana Estuary was restored in 2000 by excavating historic fill material to tidal elevations. The marsh supports stands of *Spartina foliosa* and *Sarcocornia pacifica* that host several endangered species including the Light-footed Clapper Rail, Belding's Savannah Sparrow, and the Snowy Plover (Zedler et al. 1992). Mean sedimentation rates of 1.3 cm/yr were measured in the restored Friendship Marsh in the southern region of the estuary (Wallace et al. 2005). Both lower vegetated and higher unvegetated zones are flushed twice daily by tides. Construction of the Triple Border Fence, along and beyond the entire southern border of the reserve, is likely to impact wetland habitat for these and other resident species through massive landscape restructuring and mobilization of sediments.

Field manipulation of nitrogen and sediment

To mimic effects of a one-time sedimentation event and associated nitrogen loading on nitrogen fixation rates and the diversity of nitrogen fixers, a manipulative experiment was conducted in the *S. foliosa*-vegetated zone of the Friendship Marsh during fall (October–November) 2006 (Experiment 1). These experiments were initiated more than 3 months after the most recent rain event in Tijuana Estuary. For this experiment, sediment was collected from a catchment basin adjacent to the Friendship Marsh that captures erosion from hillsides of the Tijuana River watershed. These sediments are typical of those that flood into the estuary during heavy rains. The sediment was filtered through a 100-μm screen, homogenized by stirring, and applied to 10 experimental plots (1 m^2; Fig. 1) that were positioned at ~20-m intervals along a transect in the *S. foliosa* zone of the Friendship Marsh.

Each experimental plot (Fig. 1) was subdivided (0.5 × 0.5 m) into four compartments that each received one of the following four treatments: (A) sediment addition (1 cm deep layer) in a slurry of ammonium nitrate (30 g N/m^2)-enriched artificial seawater (30 g NaCl, 10 g Mg $SO_4 \cdot 7\ H_2O$, 0.05 g $NaHCO_3$ in 1-L MilliQ water (E-Pure; Barnstead Thermolyne, Dubuque, Iowa, USA), (B) sediment addition in un-amended artificial seawater slurry, (C) addition of ammonium nitrate-enriched artificial seawater only, and (D) artificial seawater addition only (salinity = 40 psu, comparable to flood waters). Garden lining (~7 cm deep) divided experimental treatments and surrounded the 1 × 1 m grouped quadrat. This lining protruded ~0.5 cm above the sediment surface to help prevent experimental sediment additions from washing away. *S. foliosa* roots were also cut to a depth of 8 cm along the edge of each quadrat as this lining was installed. A set of plots with none of the four treatments was established to assess the potential for plot effects, along with control treatments (D) which were contrasted to prior studies of plant and diazotroph assemblages in the same marsh without plots (Moseman et al. 2009). Half of the replicates ($n = 5$) were initiated on the first day of the experiment, while experimental treatments were applied to the remaining replicates ($n = 5$) the following day to enable processing of the time-sensitive samples. The amount of nitrogen additions reflected levels reasonable for Tijuana Estuary as well and matched those applied in a similar experiment in *S. foliosa* marshes of southern California (Boyer and Zedler 1998).

Nitrogen fixation rates, diazotroph diversity, and *S. foliosa* plant properties (shoot nitrogen content, biomass, height, density) were assessed immediately prior to experiment initiation, as well as 2 and 17 d later. To determine nitrogen fixation rates and microbial diversity, two sediment cores (6 cm deep, ~2.1 cm diameter) were centered around an intact *S. foliosa* plant and collected from each quadrat. For characterization of pore water ammonium concentrations and sediment parameters (organic matter content and grain size), two additional sediment cores (6 cm deep, ~2.1 cm diameter) were collected between plants. All sediment samples were stored on ice until they could be processed or transported to the laboratory for sieving and combustion of 0–2 cm or 0–6 cm sections. Pore water salinity was measured in each quadrat by analyzing filtered seawater, extruded from the top 2 cm of sediment, on a

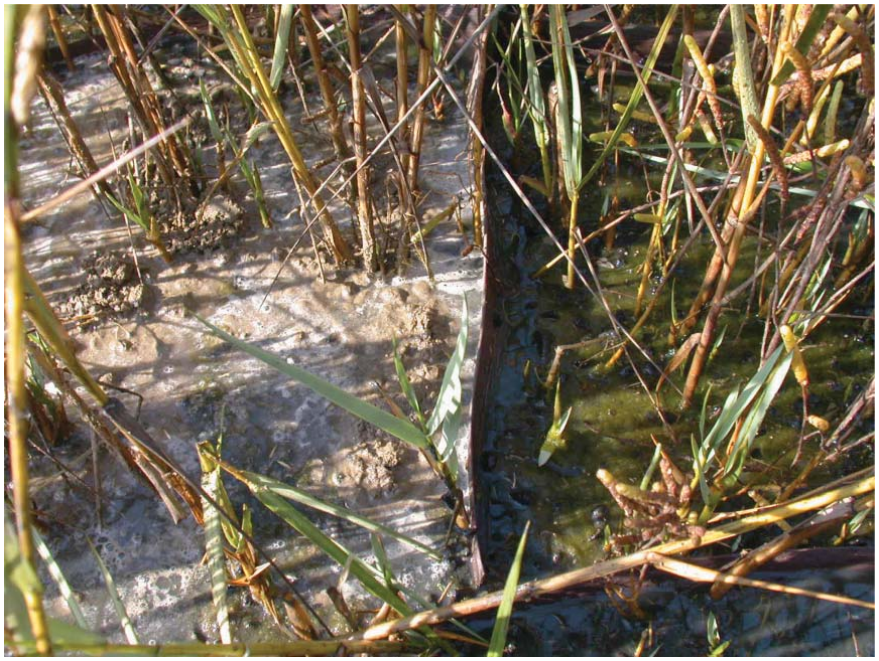

FIG. 1. Photographs of experimental treatment plots during Experiments 1 and 2 in the Friendship Marsh (Tijuana River National Estuarine Research Reserve in California, USA), representative of 10 and 16 regularly spaced replicates in the *Spartina foliosa* (Experiment 1) and unvegetated zones (Experiment 2), respectively, between late fall and early winter 2006. The photographs show differences in the benthic surface of *S. foliosa* plots to which sediment was added (left side) compared to those in which no sediment was added (right side). Only half of a 0.5×0.5 m plot (0.25 m^2) is shown. Photo credit: S. M. Moseman-Valtierra.

handheld refractometer. Light levels were also measured above and below plant canopies in each quadrat using a hand-held light meter (Apogee Instruments, Roseville, California, USA) to determine the percentage of benthic light reduction by *S. foliosa* in each quadrat. *S. foliosa* heights (mean of 10 randomly selected shoots) and densities (total number of live shoots per quadrat) were recorded on all three sampling dates. From each plant collected for acetylene reduction assays, 10-cm shoot clippings were removed after termination of the assay, washed in 5% HCl, dried, and processed to determine the percentage of nitrogen in plant tissues using a CHN elemental analyzer (Costec 4110).

To test for differences in responses of wetland diazotroph communities to sediment and nutrient additions between marsh zones, the manipulative field experiment was subsequently repeated in higher unvegetated elevations of the Friendship Marsh (Experiment 2) during March 2007 following the same design. A greater number of replicates ($n = 16$) were employed based upon power analyses from pilot studies. Samples were collected for determination of nitrogen fixation rates and sediment properties following the same procedures as in the vegetated zone. Diazotroph diversity (0–1 and 4–5 cm) was only analyzed for premanipulation conditions.

Acetylene reduction

Nitrogen fixation rates were determined in 2-h aerobic assays using the acetylene reduction method (described in Moseman 2007). Flasks containing *S. foliosa* roots and sediments were sealed with rubber stoppers, allowing plant shoots to protrude from vessels. Samples were assayed outdoors in open tubs within 3 h of their collection during evening hours (for premanipulation and 17-d samples) or the late afternoon (2-d samples). Incubation temperatures did not exceed 21°C.

Laboratory inhibition of nitrogen fixation

To evaluate effects of nutrient additions on nitrogen fixation rates while determining the relative contribution of sulfate reducing bacteria to those activities (Experiment 3), a total of 30 *S. foliosa* samples and attached sediments (2.1 cm, ~5 cm deep) were taken from an ~1-m^2 area near the mouth of the Friendship Marsh. Bottoms of the cores were sealed with plastic wrap and electrical tape and stored overnight (dark conditions for 12 h). The following day, 12 mL of one of the following three solutions was injected into the center of each vegetated sediment core ($n = 10$): (1) artificial sea water, (2) ammonium nitrate-enriched artificial sea water (30g N/L), or (3) sodium molybdate-enriched artificial sea water (20 mmol/L). Following injection of the treatment solutions, samples were immediately transferred to 125-mL flasks in which acetylene reduction assays were performed as described (with exposure to indirect sunlight). Assay temperatures did not exceed 22°C.

^{15}N: isotopic enrichment experiment (in situ)

To characterize fates of fixed nitrogen, its pathway into *S. foliosa* plants, and effects of exogenous nitrogen

FIG. 2. Photograph of mylar caps sealing sediment cores (containing *S. foliosa* plants and rhizosphere sediments) being incubated in situ during isotopic enrichment (Experiment 4). Caps are ~30 cm high. Photo credit: S. M. Moseman-Valtierra.

on plant uptake of fixed nitrogen (Experiment 4), two pairs of sediment cores (6 cm diameter, 4.5 inches depth, within 0.25 m of each other) were centered around randomly selected, intact *S. foliosa* plants in a total of three blocks (for 12 cores total; ~20 m apart) within the Friendship Marsh. In the field, each core was injected with either (1) 2 mL $^{15}N_2$-saturated artificial seawater (44.6 μmol/L) + 2 mL NH_4NO_3 (30 g/L), or (2) 2 mL $^{15}N_2$-saturated artificial seawater (44.6 μmol/L) + 2 mL unamended artificial seawater.

The $^{15}N_2$-saturated seawater was prepared by sealing 4 mL of artificial seawater (salinity of 37 psu, 20°C) into gas-tight glass vials (5.3 mL total volume; Becton Dickinson, Franklin Lakes, New Jersey, USA). From the vials, 1.3 mL of headspace air was withdrawn and then replaced by 2 mL of $^{15}N_2$ enriched gas (99.8 atom%; Cambridge Isotopes, Andover, Massachusetts, USA), which was injected directly into the seawater. This gas was shaken and allowed to equilibrate for >48 h prior to its application in the field. To distribute the ^{15}N-enriched solutions as evenly as possible throughout sediments cores, injections were performed by inserting syringes (25.5 gauge needles) ~4 cm deep into sediments, immediately adjacent to *S. foliosa* stems, then gradually squeezing contents out of the syringe as it was withdrawn from sediments.

Immediately following injections, sediment and plant samples were sealed with Mylar caps using Parafilm and tape and then returned in their original positions in the field (Fig. 2). These chambers were intended to prevent loss of $^{15}N_2$ label. Plants were bent to fit in the caps, but none were broken or clipped. Half of the samples (one core per treatment or two cores per plot, six cores total) were retrieved after 3 d, and the rest were collected after 8 d, to compare nitrogen uptake over these different time periods. Following field incubations, green plant shoots and roots were separated and clippings were rinsed in distilled water and 5% HCl, dried (60°C), and ground for isotopic analyses. To assess ^{15}N transfer from nitrogen fixers to consumers, sediments were sieved and macrofauna within them were sorted, rinsed in MilliQ water (Barnstead Thermolyne, Dubuque, Iowa, USA) and retained over night (to evacuate gut contents). Plant and animal samples were analyzed in the laboratory of R. Lee (School of Biological Sciences, Washington State University, Pullman, Washington, USA) using a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for determination of $\delta^{15}N$ (typical precision was ±0.5‰).

T-RFLP analysis of nitrogen fixer diversity

To assess diversity of nitrogen-fixing microbes, DNA was extracted from surface (0–1 cm deep) and rhizosphere (4–5 cm deep) sediment sections, from cores (6-cm deep), using the Power Soil DNA kit (Mo Bio Laboratories, Carlsbad, California, USA), as previously described (Moseman et al. 2008). The *nifH* gene was amplified via nested PCR with degenerate primers (Zehr and McReynolds 1998) to improve PCR yields. PCR conditions were based on Zehr et al. (1998).

PCR products were digested, in 20-μL batches, with the HaeIII restriction enzyme (4 bp) for 6 hours at 37°C. Digested products were recombined and purified via ethanol precipitation prior to resuspension in 15 μL of H_2O and submission for size analysis. Sizing of terminal restriction fragments was performed at the University of California at San Diego (UCSD) Cancer Center Sequencing Facility (San Diego, California, USA) using ABI GeneScan capillary electrophoresis (Applied Biosystems, Carlsbad, California, USA). Data were analyzed using Peak Scanner Software v1.0 (Applied Biosystems).

Statistical analyses

Effects of sediment and nitrogen additions on nitrogen fixation rates and diazotroph richness in Experiment 1 were compared across all dates using two-factor (time, treatment) repeated-measures ANOVA tests in JMP 4.0 (SAS Institute, Cary, North Carolina, USA). Time (premanipulation, 2 d and 17 d later) was found to be a significant factor in Experiment 1 ($F_{3,12} = 8.76$, $P < 0.01$). Thus, comparisons of nitrogen fixation rates or diazotroph richness (T-RFs) were drawn among treatments on a given date (premanipulation, 2 d later, and 17 d later) using multiple-factor (nitrogen, sediment, plot) ANOVA tests (Appendix B). Significant plot effects were found after 2 d but not later in Experiment 1 only (Appendix B). The effect of the sampling day (first or second, within 24 hours) was tested but not found in Experiment 1 or 2 (Appendix A). Changes in plant parameters (biomass, height, shoot N content), and environmental factors (pore water ammonium, sediment organic matter, and grain size) were also tested in this manner. Data were log-transformed prior to statistical analyses to achieve normality.

TABLE 1. Environmental properties (mean ± SE) in the Friendship Marsh in the Tijuana River National Estuarine Research Reserve in California, USA, before and after sediment and nitrogen additions in the *Spartina foliosa* zone (Experiment 1) and in the unvegetated zone (Experiment 2).

Zone and treatment	Clay (%) 2 days after manipulation		Organic matter (%) 2 days after manipulation		Pore water ammonium (μmol/L)†	
	0–6 cm	0–2 cm	0–6 cm	0–2 cm	2 d	17 d
Spartina foliosa zone‡						
A) Sediment and NH_4NO_3 added	45 ± 13	85 ± 3.7	7.7 ± 1.6	5.4 ± 1.0	420 ± 120	359 ± 90
B) Sediment added	69 ± 11	82 ± 3.2	9.9 ± 3.1	4.4 ± 0.8	25 ± 6	45 ± 16
C) NH_4NO_3 added	40 ± 15	94 ± 1.1	17 ± 5.0	8.8 ± 0.3	260 ± 99	54 ± 16
D) Control: artificial seawater added	51 ± 18	92 ± 1.6	15 ± 6.8	8.7 ± 0.9	13 ± 2	53 ± 13
Unvegetated zone§						
A) Sediment and NH_4NO_3 added	35 ± 0.14		12 ± 4		1353 ± 124	
B) Sediment added	31 ± 0.42		8.3 ± 0.8		352 ± 58	
C) NH_4NO_3 added	46 ± 0.13		8.8 ± 0.8		861 ± 109	
D) Control: artificial seawater added	48 ± 0.17		8.3 ± 0.3		396 ± 102	

Notes: No separate analyses of surface sediments (0–2 cm) were conducted in the unvegetated zone due to logical constraints, but those sediments were included in the bulk analyses of the 0–6 cm deep interval.

† Reported separately for 2 and 17 days after manipulation.

‡ Premanipulation levels (0–6 cm): clay, 70% ± 0.04%; organic matter, 8.4% ± 0.5%; pore water ammonium, 52 ± 8 μmol/L.

§ Premanipulation levels (0–6 cm): clay, 65% ± 0.09%; organic matter, 5.2% ± 0.6%; pore water ammonium, 195 ± 40 μmol/L.

In cases where significant effects of single treatments (sediment or nitrogen) were indicated by ANOVA tests (Appendix B), they were further tested for impacts on nitrogen fixation rates or diazotroph richness using paired *t* tests. Specifically, the effect of sediment on nitrogen fixation rates after 2 d and the effect of nitrogen on diazotroph properties after 17 d were tested via paired *t* tests. In contrast to the two-way ANOVA tests described above, paired *t* tests more directly compare each sample only against its counterpart in the same experimental plot. This approach thus lessens influences of spatial heterogeneity between plots (which were significant after 2 d in Experiment 1; Appendix B) on the ability to discern treatment effects. Bonferroni corrections were applied to correct for repeated comparisons (significant adjusted $P = 0.025$).

Diazotroph diversity was measured in terms of richness as the total mean number of *nifH* terminal restriction fragments (T-RFs) and evenness (as in Hewson and Fuhrman 2007, Moseman et al. 2008). Diversity was compared between dates and treatments using ANOVA tests or *t* tests as already described. Pielou's evenness (J') was also calculated from the arcsine-square-root-transformed relative fluorescence of T-RFLP profiles (peak heights) when richness changed significantly. Both richness and evenness were calculated via DIVERSE analyses with Primer 5.0 software (Clarke and Warwick 2001). Treatment effects on diazotroph community composition (profiles of which T-RF peaks were present) were visualized via nonmetric multidimensional scaling (MDS). Tests for significance of differences were performed with two-factor ANOSIM tests. MDS and ANOSIM were performed with Primer 5.0 software. These analyses were also conducted to compare diazotroph communities in different sediment depths and across dates.

For additional understanding of diazotroph distributions, comparisons of diazotroph communities were made between *S. foliosa* vegetated and unvegetated zones prior to experimental manipulations.

Relationships of measured environmental (plant or sediment) factors with nitrogen fixation rates and diazotroph richness were examined via linear or quadratic regression analyses, with Bonferroni corrections applied for multiple comparisons of nitrogen fixation rates, diazotroph richness, and each environmental factor to each other ($P = 0.05/3 = 0.017$). Data families were considered to be distinct between experimental treatment and date.

RESULTS

Field manipulations

Nitrogen additions (treatments A and C) increased pore water ammonium concentrations ($F_{3,24} = 5.73$, $P = 0.01$) in the *S. foliosa* zone (Experiment 1) after 2 d to levels roughly eight times greater than premanipulation conditions (Table 1). After 17 days, pore water ammonium concentrations were significantly greater in plots of treatment A (sediment and nitrogen added, 350 μmol/L) than all other treatments ($F_{3,31} = 8.58$, $P < 0.01$, ~50 μmol/L; Table 1). Experimental sediment additions (treatments A and B) decreased the clay content of surface (0–2 cm) sediments ($F_{4,30} = 3.57$, $P = 0.02$) and organic matter content ($F_{4,29} = 3.91$, $P = 0.01$; Table 1) relative to treatments C and D, but did not affect this factor in sediments in greater depths (0–6 cm deep) (clay, $F_{4,21} = 1.00$, $P = 0.43$; organic content, $F_{4,23} = 1.33$, $P = 0.29$; Table 1). Plant, microbial, and environmental properties in control plots were within ranges observed in prior studies in the same marsh (Moseman et al. 2009) suggesting no significant short-term plot effects.

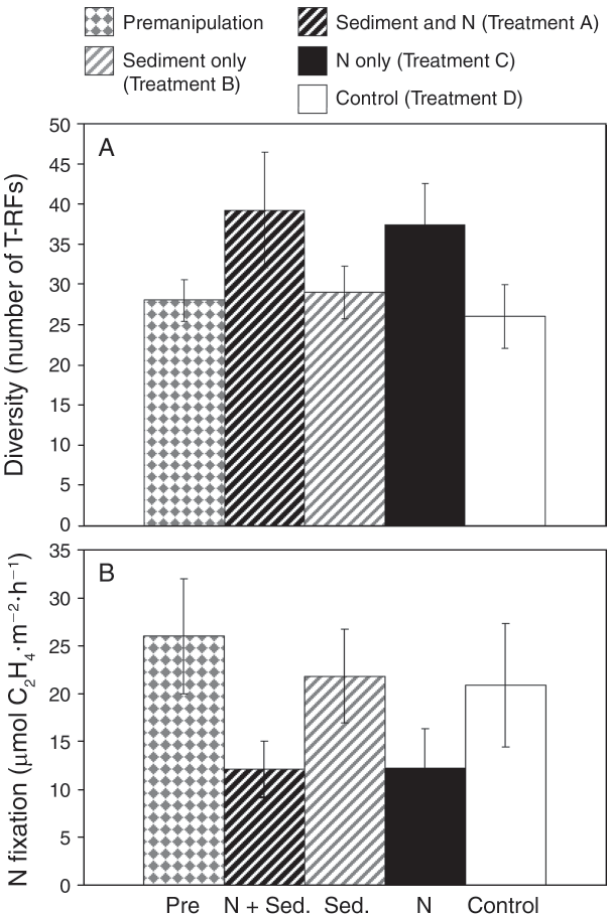

FIG. 3. (A) Diversity (mean ± SE) of diazotrophs in surface (0–1 cm) sediments (determined as the number of terminal restriction fragments [T-RFs] from analysis of the *nifH* gene) among treatments in the *S. foliosa* zone (Experiment 1) after 17 days; (B) nitrogen fixation (acetylene reduction) rates (mean ± SE) among treatments in the *S. foliosa* zone after 17 days (Experiment 1).

Environmental manipulations had similar effects in the unvegetated zone (Experiment 2) where pore water ammonium concentrations in treatments A and C were greater than B and D ($F_{3,60} = 21.32$, $P < 0.01$; Table 1) and exceeded premanipulation levels (195 μmol/L) by almost an order of magnitude (1353 μmol/L). Ammonium concentrations were also higher in treatment A (sediment and nitrogen added) than treatment C (nitrogen only) after 17 d ($t_{13} = 2.96$, $P < 0.01$). As in the *S. foliosa* zone, experimental sediment additions did not affect organic content ($F_{4,24} = 1.47$, $P = 0.24$) or grain size ($F_{3,8} = 0.79$, $P = 0.53$) of the whole top 6 cm of sediments.

Responses of diazotrophs to sediment and nutrient loads

Two days following experimental manipulations, nitrogen fixation rates varied significantly among plots (Appendix B), although there was a trend of lower nitrogen fixation rates among plots receiving sediment than those that did not ($t_{14} = -2.01$, $P = 0.06$; Appendix B). Diazotroph richness (total mean number of T-RFs) did not vary between treatments after only 2 d (0–1 cm, Appendix A; 4–5 cm, $F_{3,28} = 0.13$, $P = 0.94$).

After 17 d, diazotroph richness (number of T-RFs) in surface sediments was higher among plots with nitrogen additions than those without nitrogen additions (paired $t_{14} = 2.11$, $P = 0.05$, Fig. 3A; Appendix B). Conversely, nitrogen fixation rates were significantly lower in plots with nitrogen additions than those without them after 17 d (paired $t_{16} = -3.16$; Fig. 3B; Appendix B). Among all treatments, diazotroph diversity in surface sediments was positively related to pore water ammonium concentrations after 17 d ($r^2 = 0.32$, $P < 0.01$; Fig. 4). No such relationships existed prior to ($r^2 = 0.02$, $P = 0.41$) or 2 d following experimental manipulations ($r^2 = 0.06$, $P = 0.40$).

The evenness of diazotroph communities in treatment A alone, as reflected by relative fluorescence of each terminal restriction fragment (T-RF), increased significantly within the first 2 d of the experiment from $J' = 0.71 \pm 0.06$ to $J' = 0.82 \pm 0.09$ (all values are mean ± SE; $t_8 = -3.13$, $P = 0.01$), but declined to premanipulation levels ($J' = 0.75 \pm 0.04$) by 17 d ($t_5 = -1.28$, $P = 0.26$). Evenness of diazotroph T-RFs in treatment C increased significantly from $J' = 0.72 \pm 0.09$ to $J' = 0.85 \pm 0.09$ only after 17 d ($t_5 = -6.63$, $P < 0.01$). Diversity of rhizosphere diazotrophs (4–5 cm) did not change within 17 d among any treatments ($F_{4,53} = 0.82$, $P = 0.51$).

In the unvegetated zone of the marsh (Experiment 2), nitrogen fixation rates did not differ significantly among treatments after 2 d ($F_{4,58} = 0.63$, $P = 0.64$) or 17 d ($F_{4,56} = 0.72$, $P = 0.58$), and thus responses of diazotroph diversity to sediment and nitrogen additions were not investigated. Nitrogen fixation rates in the unvegetated zone were 12 ± 2.5 μmol $C_2H_4 \cdot m^{-2} \cdot h^{-1}$, which were marginally lower than those in the *S. foliosa* zone ($t_{70} = -1.60$, one-tailed $P = 0.06$), while pore water ammonium levels were significantly higher ($F_{1,77} = 9.00$, $P < 0.01$). Sediment organic matter was lower than in the *S. foliosa* zone ($F_{1,27} = 11.53$, $P < 0.01$).

Composition of diazotrophs communities

Composition of diazotroph assemblages (reflected in T-RF identity and relative fluorescence) consistently differed between surface and rhizosphere sediments (premanipulation, ANOSIM Global $R = 0.413$, $P = 0.01$; 2 d later, Global $R = 0.79$, $P < 0.01$; 17 d later, Global $R = 0.65$, $P < 0.01$) in Experiment 1. Diazotroph composition changed over time among all treatments (Table 2A, Fig. 5) but did not vary among treatments after 2 or 17 d or across all three dates in the study (Table 2B, Fig. 5).

Although the richness (total number of T-RFs) of diazotroph assemblages did not differ between *S. foliosa*-vegetated and unvegetated zones ($t_{102} = -0.62$, $P = 0.54$), the diazotroph composition was distinct (ANOSIM Global $R = 0.279$, $P < 0.01$).

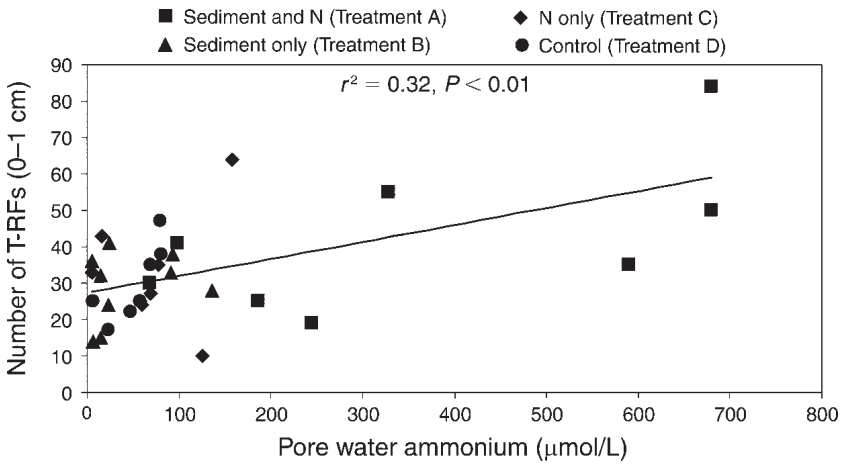

FIG. 4. Relationship between surface (0–1 cm deep) diazotroph diversity (number of T-RFs) and pore water ammonium concentrations (μmol/L) among all four experimental treatments.

Resistance of plants to sediment and nitrogen additions

No significant effects of treatment or time were observed on plant height (treatment, $F_{4,23} = 0.017$, $P = 0.98$; time, $F_{2,22} = 1.29$, $P = 0.29$), aboveground biomass (treatment, $F_{4,14} = 0.49$, $P = 0.74$; time, $F_{2,13} = 1.32$, $P = 0.30$) or belowground biomass (treatment, $F_{4,20} = 0.94$, $P = 0.46$; time, $F_{2,19} = 1.42$, $P = 0.27$) in Experiment 1 (among all dates). Nitrogen content of *S. foliosa* shoots increased over the 17-d time period only for treatment C (nitrogen addition; $t_2 = -21.84$, $P < 0.01$). *S. foliosa* density declined significantly between premanipulation conditions and 17 d later (but was not measured 2 d following the experiment) in treatment A only ($t_7 = 4.21$, $P < 0.01$).

Laboratory inhibition of nitrogen fixation by ammonium

Nitrogen fixation (acetylene reduction) rates of *S. foliosa* samples in Experiment 3 were reduced by 70% in the presence of sodium molybdate (7.6 ± 2.2 μmol $C_2H_4 \cdot m^{-2} \cdot h^{-1}$; mean ± SE for all values shown), a known inhibitor of sulfate reducing bacteria (Welsh 2000), compared to controls with artificial seawater additions (25 ± 5.2 μmol $C_2H_4 \cdot m^{-2} \cdot h^{-1}$). Rates were also inhibited by additions of ammonium nitrate (4.1 ± 2.8 μmol $C_2H_4 \cdot m^{-2} \cdot h^{-1}$, $F_{2,28} = 9.82$, $P < 0.01$).

Fates of fixed nitrogen among S. foliosa *plants and animal consumers*

S. foliosa shoots ($\delta^{15}N$, 3–52‰ above controls) and roots ($\delta^{15}N$, 22‰ to >3300‰ above controls) were isotopically enriched within 3 d. Roots showed significantly greater ^{15}N enrichment than shoots (3 days, paired $t_2 = 4.15$, $P = 0.05$; 8 d, paired $t_3 = 5.85$, $P = 0.01$). *S. foliosa* roots exposed to $^{15}N_2$ in the absence of ammonium nitrate additions were ~13 times more enriched in ^{15}N than roots in the presence of exogenous nitrogen after 8 d (Fig. 6; paired $t_1 = -3.83$, $P = 0.08$), but no difference was observed among shoot tissues (paired $t_2 = 1.29$, $P = 0.32$) or roots after only 3 d ($t_2 = -0.40$, $P = 0.72$).

The uptake of fixed nitrogen by animal consumers was assessed only after 8 d. At that time, significant enrichment was noted among three of the five taxa that were collected (Table 3). Insect larvae and some, but not all, capitellid and spionid polychaetes showed substantial ^{15}N enrichment relative to controls, while most individuals of the gastropod C*erithidea californica* and the amphipod *Corophium* sp. were not enriched (Table 3).

DISCUSSION

Diazotroph responses to sediment and nitrogen loading

Our observations that ammonium nitrate additions decreased nitrogen fixation rates (Experiments 1, 2, and 3) are consistent with known roles of ammonium as an inhibitor of the nitrogenase enzyme (Yoch and Whiting 1986) and with the high energetic costs of fixing

TABLE 2. Comparisons of diazotroph composition (across all treatments) in Experiment 1 over time based on ANOSIM tests and comparisons of diazotroph composition between treatments in Experiment 1 at different periods after experiment initiation.

Comparison of composition	Global R	P
A) Across all treatments		
Dates after experiment initiation		
0 days vs. 17 days	0.024	0.19
0 days vs. 2 days	0.136	<0.01
2 days vs. 17 days	0.089	0.01
0, 2, and 17 days	0.176	0.01
B) Between treatments		
Date after experiment		
2 days	−0.019	0.77
17 days	−0.021	0.78
0, 2, and 17 days	0.003	0.40

Note: No significant differences were observed for the between-treatment comparisons.

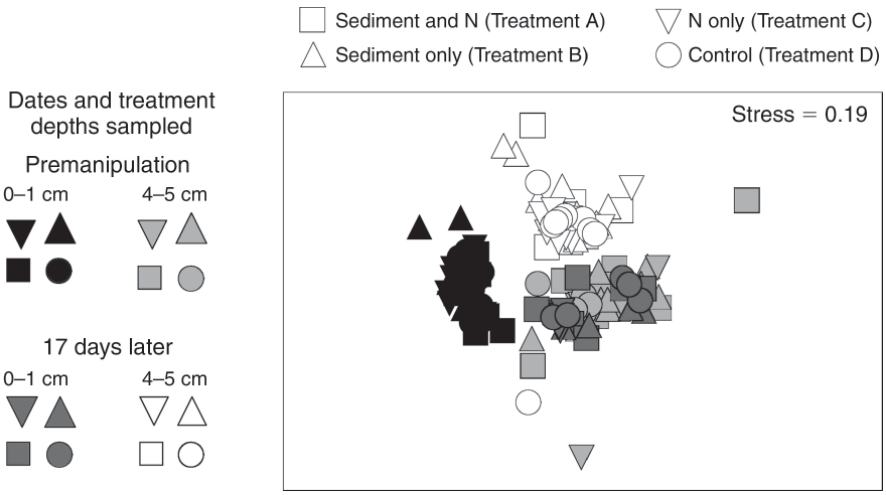

FIG. 5. Multidimensional-scaling plot of diazotroph community composition (based on which T-RFs were present) in surface and subsurface sediments of all four treatments prior to experimental manipulations (black and light gray) and 17 days following the experiment (dark gray and white). Data from two days after the experiment (not shown) would be positioned between those from these two dates. In multidimensional scaling, similarity in community composition is represented by greater proximity in unitless, two-dimensional space. Thus, points clustering more closely together in this figure represent samples with more similar T-RFLP profiles.

nitrogen. Declined nitrogen fixation rates may have reflected shifts not only in the physiological state of the diazotrophs but also in their relative abundance (but see Zehr et al. 2007).

Sediment additions, comparable to the annual vertical accretion estimated (1.3 cm) in the Friendship Marsh during a 5-yr period with floods (Wallace et al. 2005), did not significantly affect nitrogen fixation rates (Experiments 1 and 2) or diazotroph richness. In the *Spartina foliosa* zone (Experiment 1), this may have been small due to a dominant contribution of subsurface microbes to nitrogen fixation, as suggested by results of sodium molybdate inhibitions (Experiment 3). Similar observations of significant nitrogen fixation by diazotrophs in wetland plant rhizospheres relative to epibenthic counterparts have been made (reviewed in Welsh 2000, Lovell 2005). In unvegetated sediments (only ~30 m from the *S. foliosa* zone), higher exogenous ammonium concentrations likely maintained low nitrogen fixation rates and minimized response to sediment and nutrient additions (Experiment 2). These ammonium concentrations could result from less frequent tidal flushing and/or less uptake of nutrients from the environment by plants.

Although no significant short-term interactions between sediment and nitrogen treatments on nitrogen fixation rates or diazotroph diversity were observed (Appendix A), the combination of sedimentation and nitrogen loading was found to enhance pore water ammonium concentrations and their persistence. Yet sediment additions alone did not affect pore water ammonium levels and thus did not constitute a direct input of nitrogen (Table 1). These results suggest that by trapping nutrient additions, sedimentation can delay and reduce the recovery of diazotroph activities, which may eventually facilitate shifts in microbial community composition.

Responses of diazotroph community structure to higher nutrient concentrations

Nitrogen fixing assemblages in surface sediments that received ammonium nitrate additions increased in richness relative to controls while those in *S. foliosa* rhizospheres were not affected (Fig. 3A). Nonetheless, nitrogen fixation rates declined, demonstrating the loss of a microbially mediated function independently of compositional changes or declines in assemblage richness, as also observed for diazotrophs in the context of biological invasions (Moseman et al. 2008). Thus, the relative abundance or physiological states of a few dominant diazotrophs seem more significant to the performance of nitrogen fixation than diversity, as observed in oceanic environments (Goebel et al. 2007,

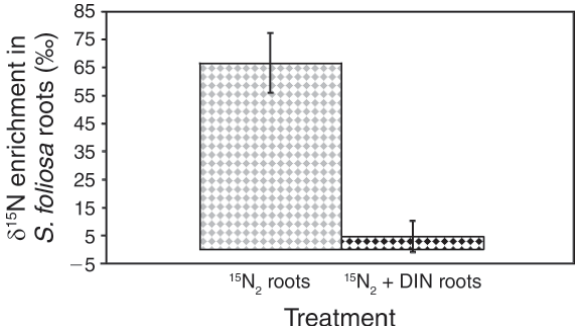

FIG. 6. Isotopic enrichment ($\delta^{15}N$) of *S. foliosa* roots (mean ± SE) exposed to $^{15}N_2$ in the presence or absence of (unenriched) ammonium nitrate or dissolved inorganic nitrogen (DIN; $n = 2$) (Experiment 4). Mean background values, 11‰ ± 4.0‰ (roots), have been subtracted.

Zehr et al. 2007), although shifts in composition of microbial communities may become more important on longer time scales (Piceno and Lovell 2000). These results contribute to findings of high functional activity among microbial communities of low diversity (Janousek et al. 2007) and support general ecological models by showing some parallels of diversity function relationships among microbes with those of plant and animal communities (Tilman 1999, Sousa 2001, Smith 2007).

The increased richness of diazotroph assemblages in Experiment 1 following nitrogen loading reflects responses of macroorganisms to small-scale disturbances, in which the removal of dominant species can increase community diversity by decreasing competition (Sousa 2001). Surface diazotroph assemblages exposed to nitrogen loading increased not only in richness (total mean number of T-RFs [terminal restriction fragments]) but also in evenness (a metric based on relative heights of T-RF peaks among samples), which may suggest that nutrient enrichments differentially affected competitive dominants in the diazotroph assemblages. Such functional dominance among diazotrophs has been previously reported in coastal ecosystems (Short and Zehr 2007). Another nonexclusive possibility is that nutrient additions in this study may have benefited some diazotrophs, which can grow in cultures with ammonium (Fritzche and Niemann 1990). Thus declines in nitrogen fixation may not have represented disturbance or stress to these diverse microbial assemblages.

Ammonium is considered to be a key regulator of competitive abilities of diazotrophs (and thus their diversity) (Kolb and Martin 1988). In sorghum rhizospheres, nitrogen treatments (12 kg N/ha and 120 kg N/ha) were more important than plant cultivar in affecting diazotroph community structure (Coelho et al. 2008). The diversity (Shannon-Weiner index) and evenness of clone libraries (representing >90% of total diversity) was greatest among soils treated with higher amounts of nitrogen (Coelho et al. 2008), as with surface-dwelling diazotrophs in this wetland study. In contrast, the robustness of diazotroph communities in *S. foliosa* rhizospheres is consistent with minor diversity declines observed among diazotrophs in rhizospheres of a related cordgrass, *S. alterniflora*, after 10 years of repeated nitrogen loading at 16.3 g N/m^3 (Piceno and Lovell 2000). The communities of rhizosphere microbes may have been less strongly impacted than surface-dwelling diazotrophs, as the former were not directly smothered by sediments added to marsh surfaces, and plant uptake potentially ameliorated impacts of added nitrogen in the rhizosphere. The diversity of rhizosphere diazotrophs also may not have been affected since no major changes were observed among plants in this short-term study. However, on longer time scales, diazotroph diversity may decrease in response to chronic nutrient loading, as observed in coastal wetland rhizospheres after 10 years (Piceno and Lovell 2000) and in sandy soils after 27 years (Ruppel et al. 2007).

TABLE 3. Observed values of $\delta^{15}N$ for macrofaunal taxa in enclosures exposed to subsurface $^{15}N_2$ for eight days (Experiment 4).

Taxon and plot number	$\delta^{15}N$ (‰)	
	Control†	Observed
Polychaete: capitellid	12	
1		127
2		1864
3		13
3		14
Polychaete: spionid	10	
2		1642
3		18
Insect: chironomids and dolichopodids‡	19	
2		599
Gastropod: *Cerithidea californica*	7	
2		14
3		8
3		9
3		7
Crustacean: *Corophium* sp.	77	
3		12

Notes: Each value reflects the signature of a single individual (or portion thereof) except where noted. Not all taxa were found in each enclosure, but those plots from which each individual was collected are indicated in the column labeled "plot number." High levels of enrichment in plot 2 may have been due to abundance of cyanobacteria which can have patchy distributions, though no mats were specifically noted. Enrichment chambers may also have been more effectively sealed.

† Control values were not available in this experiment and are thus presented from prior work in the Friendship Marsh during 2002 (L. Levin, *unpublished data*).

‡ Both insect taxa were combined in one sample (for sufficient mass for analyses), and control values are available from dolichopodids only.

Microbial communities in the unvegetated zone (Experiment 2) were distinct from those in the *S. foliosa* zone (Experiment 1), possibly due to adaptation to higher ammonium concentrations. Thus, the role of microbial community structure cannot be separated in this study from effects of distinct abiotic environments on functional (nitrogen fixation) responses of diazotrophs to sediment and nutrient impacts across vegetated and unvegetated zones (Reed and Martiny 2007).

Influence of sediment and nitrogen additions on plants

Little plant response to nitrogen input was evident within 17 d of this study (Experiment 1) except in treatment C (nitrogen addition only) plots in which *S. foliosa* shoot nitrogen content increased after 17 d. Declines in pore water ammonium concentrations in plots of treatment C between 2 and 17 d were possibly due to plant uptake, suggesting potential for *S. foliosa* to facilitate recovery of nitrogen fixation, or tidal flushing alone. Mechanisms for the decline in *S. foliosa* density in treatment A only (nitrogen and sediment addition) are

unclear but could have involved plant death and loss to tidal export or grazing. More substantial plant responses to nitrogen additions would likely occur over longer terms (>17 d). In previous nutrient enrichments (at 30 g N/m^2) of *S. foliosa* marsh in San Diego Bay, earliest plant structural responses were noted only after 2 months (Boyer and Zedler 1998). Further, timing of nutrient additions has been found to affect plant responses, as *S. foliosa* nutrient demands peak in spring months (Boyer and Zedler 1998). Yet, experiments (1 and 2) were timed for mimicking sediment and nitrogen inputs typically associated with heavy winter rains.

Broader consequences of declines in nitrogen fixation

Fixed nitrogen is known to be transferred on short time scales (7 d) to marsh plants (Capone 1988, O'Donohue et al. 1991), although extents vary among plant species (Jones 1974). Fixed nitrogen quickly reached *S. foliosa* tissues in our study, despite potential negative effects of mylar caps, including elevated temperatures and reduced light availability, which may have decreased plant photosynthesis. The >10-fold decline in ^{15}N enrichment of *S. foliosa* roots (from $^{15}N_2$) in the presence of ammonium nitrate additions (Fig. 6) was likely due not only to a decline in nitrogen fixation rates, but also to dilution of ^{15}N by un-enriched $^{14}NH_4^+$ which *S. foliosa* plants were also able to acquire. The extent to which vascular plants, particularly those in mutualisms with diazotrophs, are able to "switch" from nitrogen fixation to the use of exogenous nitrogen in response to nutrient loading warrants further study as it holds consequences for ecosystem sustainability, agricultural practices (Rueda-Puente et al. 2003, Oberson et al. 2007), and restoration (Bashan et al. 1998).

The resilience of nitrogen fixers, and their potential to recover functions once environmental nitrogen pools decrease, will also affect ecosystem response to episodic nitrogen inputs. This study demonstrates that simultaneous sedimentation increases the magnitude and extends the duration of high nutrient concentrations (Experiments 1 and 2), which delay the recovery of nitrogen fixation activities. Further, longer-term nitrogen enrichment may disfavor plant species, such as *Spartina foliosa*, that have developed intimate associations with diazotrophs in nitrogen-limited environments (Moseman 2007), and favor strong competitors for exogenous nitrogen, including *Salicornia* (or *Sarcocornia*) species and algae (Boyer and Zedler 1999), which differ in productivity, canopy structure, and ability to support higher-level (including endangered) species.

Broader consequences of declines in nitrogen fixation in response to sediment and nutrient loading also include effects on ecosystem food webs, which are known to be affected by nitrogen inputs (Breitburg et al. 1999, Deegan et al. 2007). Fixed nitrogen, traced via ^{15}N, was quickly received by most of the macrofaunal species collected (Table 3), perhaps by consumption of cyanobacteria, which are major food sources particularly in invaded (Levin et al. 2006) and developing wetlands (Currin et al. 1995, Moseman et al. 2004). Rhizosphere diazotrophs could also be consumed by subsurface-feeding macrofauna, such as *Capitella* spp. (Table 3). Faunal grazing on ^{15}N-labeled microbial biomass was similarly observed (via isotopic enrichment of dissolved inorganic nitrogen, urea, and amino acids) in an intertidal mud bank (Veuger et al. 2007). These results suggest changes in diazotroph communities can affect wetland ecosystems via shifts in trophic interactions.

A growing volume of studies highlights the role of microbes in mediating functions of aquatic (Gutknecht et al. 2006), coastal (Bergholz et al. 2001, Daleo et al. 2007), and terrestrial ecosystems (Klironomos et al. 2000, Reynolds et al. 2003). As sedimentation (Thrush et al. 2004) and anthropogenic inputs of nitrogen increase on global scales (Galloway et al. 1995, Howarth et al. 2006), shifts in the function and richness of inconspicuous microbial groups such as diazotrophs may have cascading consequences for dynamics of coastal ecosystems.

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APPENDIX A

Lack of significant effect of experimental treatment or day of experiment initiation on nitrogen fixation rates in Experiment 1 and 2 determined via two-factor repeated measures ANOVA tests (*Ecological Archives* A020-059-A1).

APPENDIX B

Independent and overall effects of nitrogen, sediment, and their interaction as well as plot (location) on nitrogen fixation rates and diazotroph richness in surface sediments (0–1 cm) in Experiment 1, based on multiple-factor ANOVA tests (*Ecological Archives* A020-059-A2).