UNIVERSITY OF CALIFORNIA, SAN DIEGO

Diversity and function from the ground up: Microbial mediation of wetland plant structure and ecosystem function via nitrogen fixation

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Oceanography

by

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2008

The Dissertation of Serena Maria Moseman is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

University of California, San Diego

2008

DEDICATION

I wish to acknowledge the many generations
whose strength and perseverance have enabled me
to pursue an advanced education.

My dissertation is therefore dedicated to my family,
In special honor of the Malinao women,
for lifting me up on their shoulders
in hope that I might see a brighter horizon.

I also dedicate this work to my peers and mentees who have
courageously joined me on this uncharted path.

EPIGRAPH

Mud is not always and everywhere the same, and some essential microorganisms
require kinds of muddy water that are inimical to other kinds.
Because the microbes in question are all essential,
a "microbe-oriented" policy of water use is a truly "people-oriented" policy…
that focuses on aspects of global ecology
that are genuinely important to all people.

Edward S. Deevy, Jr.
(President of the Ecological Society of America 1969-1970)

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LIST OF ABBREVIATIONS

ANOSIM	Analysis of Similarity
KF-NWP	Kendall Frost- Northern Wildlife Preserve
MDS	Multidimensional scaling
TJE	Tijuana Estuary
T-RF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
μmol	micromole
C_2H_4	ethylene
C_2H_4	acetylene

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Ecology. The dissertation author was the primary investigator and author of this paper. Chapter V is being prepared for submission to *Ecological Applications*. The dissertation author was the primary investigator and author of this paper.

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PUBLICATIONS

Moseman, S. M., Armaiz-Nolla, K., and L. A. Levin. 2008. Effects of synergistic stressors (sediment and nutrient loading) on nitrogen fixation in wetland ecosystems. Ecological Applications, *in prep.*

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Moseman, S.M., Zhang, R., Qian, P.Y. and L.A. Levin. 2008. Diversity and functional responses of nitrogen fixing microbes to three wetland invasions. Invasions Biology, *in press.*

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Moseman, S. M., Levin, L. A., Currin, C. A., and C. Forder. 2004. Infaunal colonization, succession and nutrition of macrobenthic assemblages in a restored wetland at Tijuana Estuary, California. Estuarine Coastal and Shelf Science 60: 755-770.

ABSTRACT OF THE DISSERTATION

Diversity and function from the ground up:
Microbial mediation of wetland plant structure and ecosystem function
via nitrogen fixation

by

Serena Maria Moseman

Doctor of Philosophy in Oceanography

University of California, San Diego, 2008

Professor Lisa A. Levin, Chair

Plant-dependent functions of coastal wetlands are strongly influenced by nitrogen availability. Diazotrophs, microbes that fix nitrogen, in surface sediments and rhizospheres (roots and surrounding sediments) of plants may fundamentally affect wetland ecosystems. In testing roles of nitrogen fixing microbes in niche differentiation between two key plants, *Spartina foliosa* and *Salicornia virginica,* a mensurative experiment reveals plant-specific diel patterns of nitrogen fixation (acetylene reduction). Functional disparities in nitrogen fixation rates between late- and early-successional salt marshes in Tijuana Estuary (1 pair) and Venice lagoon, Italy (2 pairs) also show roles of diazotrophs in facilitating marsh development. Nitrogen fixation rates are consistently greater in marshes with less plant growth, which is not always a function of marsh age.

Fates of fixed nitrogen are tested in isotopic enrichment experiments within an early successional marsh (Tijuana Estuary). Newly fixed nitrogen reaches *S. foliosa* roots and several animal consumers within 3-8 days. Thus, nitrogen fixation has broad significance for wetland ecosystem function.

The role of diazotroph diversity in enhancing or conferring stability to the nitrogen fixation (acetylene reduction) rates was tested in wetlands experiencing biological invasion, restoration, and sediment and nutrient stresses, via genetic fingerprinting (T-RFLP) of the *nif*H gene (coding dinitrogenase reductase). The invasive mussel, *Musculista senhousia*, salt cedar, *Tamarix* spp., and mangrove, *Avicennia marina* each produced different effects on nitrogen fixation rates, despite maintenance of diazotroph diversity. In the early successional marsh at Tijuana Estuary, positive relationships among diazotroph diversity, nitrogen fixation rates, and *S. foliosa* height during one season of plant growth (Fall) demonstrate context-dependent complementarity. Effects of anthropogenic nutrient and sediment loading on nitrogen-fixing microbes are tested by field manipulations. Ammonium nitrate additions decrease nitrogen fixation rates but increase diversity of surface diazotrophs within 17 days, while sediment inputs enhanced and prolonged ammonium concentrations. As nitrogen fixation is highly responsive to the range of explored environmental changes, concepts of functional redundancy may not easily extend to microbial realms. Wetland management should more fully consider the functional role of plant-microbe interactions in mediating ecosystem functional responses to future global changes.

CHAPTER I

Introduction

Dynamic and complex interactions translate the structure of biological communities into ecosystem functions. Nutrients, materials, and energy are transferred and transformed between and among species through a range of ecological interactions, from competition to facilitation. Conspicuous habitat-modifying plants and animals are linked not only to one another but also to myriad, mostly invisible microbial communities (Kowalchuk et al. 2002). Microbes coat living surfaces, including plant roots and shoots (Lovell 2005) as well as animal tubes, burrows, and guts (Leveille et al. 2005). They are major constituents of the soils, sediments, and aquatic and marine environments in which plants and animals live (Kristensen and Kostka 2005). These diverse communities of microorganisms perform a wide range of biogeochemical transformations, including nitrogen fixation, organic matter degradation (and nutrient remineralization), and metal transformations, upon which the more visible primary producers and consumers rely for nutrients and health (Howarth 1993). Thus, mutual negotiation of niches by macroscopic and microscopic communities manifests in functional dynamics of ecosystems across space and time (Klironomos 2002).

Global declines in biodiversity, coupled with habitat degradation and climate change, warrant mechanistic understanding of links between biological diversity and ecosystem function. Several ideas are proposed to relate biological diversity to ecosystem

function; these are derived largely from studies of vascular plant communities (Tilman 1999) while microbes have been largely overlooked (but see Smith et al. 2007). Complementarity in resource use among species is proposed to increase performance of an ecosystem function within a guild (Fargione et al. 2007), manifesting in a positive diversity-function relationship. Another possible positive link between biological diversity and ecosystem function is via a sampling effect, or the increased probability of finding a highly performing species within a guild of greater diversity (reviewed in Tilman 1999, Stachowicz et al. 2007). Tests of these theories in microbial realms, and in the context of plant-microbe interactions may offer new mechanisms by which shifts in diversity affect ecosystem function.

Do microbes mediate relationships between biodiversity and ecosystem function?

The majority of biological diversity on earth is microbial (Tiedje et al. 1999), however definitions, estimations, and ecological controls of microbial species richness have not yet been fully determined (Paerl et al. 2002, Torsvik et al. 2002, Ward 2002). Though microbes have historically been assumed to be cosmopolitan in distribution, recent evidence of more restricted biogeographical patterns is emerging (Hughes Martiny et al. 2006). Nonetheless, estimates of microbial diversity are very high (Ward 2002). For simplicity, microbial diversity may be considered in terms of key functional groups, or guilds, which share capability of common biochemical transformations, such as photosynthesis or other reactions that produce energy via oxidation and reduction of inorganic molecules.

Nitrogen fixation, the conversion of dinitrogen gas (N_2) into a reduced, biologically available form (NH_3) is one major biogeochemical function that is solely performed by microbes, specifically prokaryotes (bacteria and archaea). This process is important in a range of terrestrial and marine habitats where nitrogen availability limits the growth of primary producers (including vascular plants, algae, phytoplankton, or cyanobacteria).

Though nitrogen fixing microbes (diazotrophs) are unique, being specially adapted to break the strong triple bond between the nitrogen atoms in N_2 gas, representatives are genetically and physiologically diverse (Bagwell and Lovell 2000). Nitrogen fixers range from cyanobacteria, which obtain energy via photosynthesis, to heterotrophic proteobacteria that cannot fix their own carbon and obtain energy by reducing sulfate to sulfide. Moreover, diazotrophs have been particularly noted to also display high levels of "microdiversity," the presence of many physiologically distinct but genetically similar microbial taxa within each of the broader groups (Bagwell and Lovell 2000). Microdiversity within microbial guilds has been hypothesized to confer functional redundancy (Bagwell and Lovell 2000). Diversity among multiple organisms capable of performing the same function, such as nitrogen fixation, is predicted to maintain that function in an ecosystem despite environmental changes in space or time. Functional redundancy among microorganisms is presumed to be maintained by fine-scale niche differentiation within a functional group. Optimal functional performance of a given

taxon within a diverse assemblage under distinct environmental conditions is sometimes described as the "insurance hypothesis." These hypotheses predict that microbial diversity effectively maintains stability of the functions that they mediate against environmental perturbations.

Nitrogen fixing microbes can be related to ecosystem functions not only through their roles in sustaining availability of a limiting nutrient, but also through other biogeochemical functions they perform (i.e. sulfate reduction or photosynthesis), trophic support (as bases of food chains, Moseman et al. 2004), and habitat modification roles (via cyanobacterial mat formation, facilitation of vascular plants). As plant species may differ in their reliance on nitrogen fixation as a nutrient source, dynamics of diazotroph assemblages may also influence plant diversity, affecting niche partitioning in nitrogen limited settings.

How do plant-microbe interactions affect relationships between biological diversity and function?

The nature and magnitude of non-trophic interactions can strongly influence relationships between ecosystem properties (biomass, productivity) and biodiversity (Goudard and Loreau 2008). However, theories relating biological diversity to ecosystem function have largely neglected roles and consequences of ecological interactions between microbes and plants or animals. Differential interactions of particular microbial taxa with species in macroscopic guilds (i.e. vascular plants) may maintain the

remarkable diversity that has been observed within microbial assemblages. This microbial diversity may, in turn, have functional consequences for nutrient transformations (like nitrogen fixation), resulting in positive feedbacks for ecosystem functions (plant productivity). Thus, macro-microbe interactions may influence not only the structure but also the function of biological communities.

Plants interact with microbial communities in order to meet nutrient requirements across a range of environments. In terrestrial settings (Klironomos 2002) and some wetlands (Daleo et al. 2007), plants harbor mycorrhizae fungi which aid in obtaining P and N from soils. In both terrestrial and agricultural settings, select plants can form tight, symbiotic relationships with nitrogen fixing bacteria harbored in specialized root nodules (Vessey et al. 2005). Looser interactions are also observed in terrestrial (Costa et al. 2006), aquatic (Scott et al. 2007) and coastal ecosystems (Welsh 2000, Lovell 2005) between plants and microbes in their rhizospheres (roots and surrounding sediments). Such plant-microbe interactions may facilitate ecosystem succession, influence competitive interactions between different plant species, and drive the productivity and efficiency of nutrient cycling (Kent and Triplett 2002, Reynolds et al. 2003**).** Furthermore, diversity-function relationships among microbial communities are likely to be highly affected by dynamics of plant communities in and on which they reside (Klironomos et al. 2000).

The basics of microbial community dynamics are beginning to be revealed through advancements in technology that can be applied to discern patterns of diversity and community composition on ecologically relevant scales (Ward 2005). PCR-based approaches have revolutionized appreciation for the diversity of microorganisms in natural environments, many of which were masked by conventional culture-based studies. An array of genetic fingerprinting techniques enables rapid analyses of the diversity of natural communities across environmental gradients and/or time. Genes coding enzymes that catalyze key biochemical transformations can be targeted and amplified from environmental samples to study particular functional groups (Zehr and Capone 1996, Ward 2005). Microbial community structures (diversity, composition) can not only be examined over space and time but also compared to the dynamics of the function they perform, and the macroscopic plants and animals with which they interact, potentially revealing new insights regarding the manner in which biological diversity manifests in ecosystem function.

How does disturbance affect microbial diversity-function and plant microbe interactions?

Disturbance is known to be a major force governing the structure of biological communities (Paine and Levin 1981). Experiments in coastal ecosystems have shown that disturbance mediates ecological interactions by removing top predators or competitive dominants that otherwise regulate biological communities (Sousa 2001). Disturbance, at small temporal or spatial scales, may thus promote and maintain biological diversity,

while at larger scales (such as those often associated with anthropogenic effects), disturbance can cause rapid and widespread losses in diversity that trigger the collapse of ecosystems.

The significant role of disturbance in shaping relationships between ecosystem function and biological diversity has begun to be recognized (Thrush et al. 2008). Even in microbial realms, effects of disturbance on the structural and functional properties of biological communities may shift diversity-function relationships (Figure I-1, right).

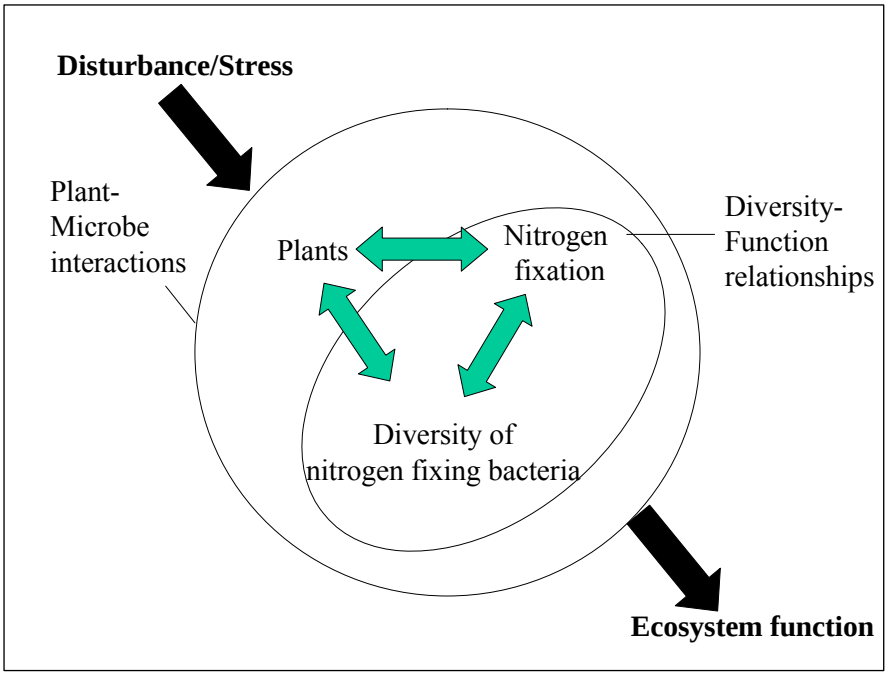

Figure I-1: Conceptual diagram of two ecological relationships (plant-microbe interactions and diversity-function relationships) that likely affect the response of ecosystem function to agents of disturbance

A major means by which disturbance may affect diversity-function relationships is via influence on the nature or strength of plant-microbe interactions (Figure I-1, left). Plants

may buffer microbes in their rhizospheres (roots and surrounding sediments) from disturbance by stabilizing sediments and soils or altering sediment chemistry (via nutrient uptake, oxygenation, exudation of labile carbon). However, not all microbe-plant interactions are beneficial, and environmental disturbances may shift the direction of ecological interactions from positive to negative, or affect their magnitude, extent, and timing, in ways that have consequences throughout the ecosystem. Efforts to understand the controls of ecosystem function, particularly changes in biodiversity, must better resolve the complexities of macrobe-microbe interactions. This can be achieved by describing basic spatial and temporal patterns of microbial distributions and activities, in order to learn fundamental environmental controls on microbial communities.

Coastal Wetland Ecosystems

Coastal wetlands are ecosystems visibly dominated by a few vascular plant species at the interface of the land and sea (Figure I-2).

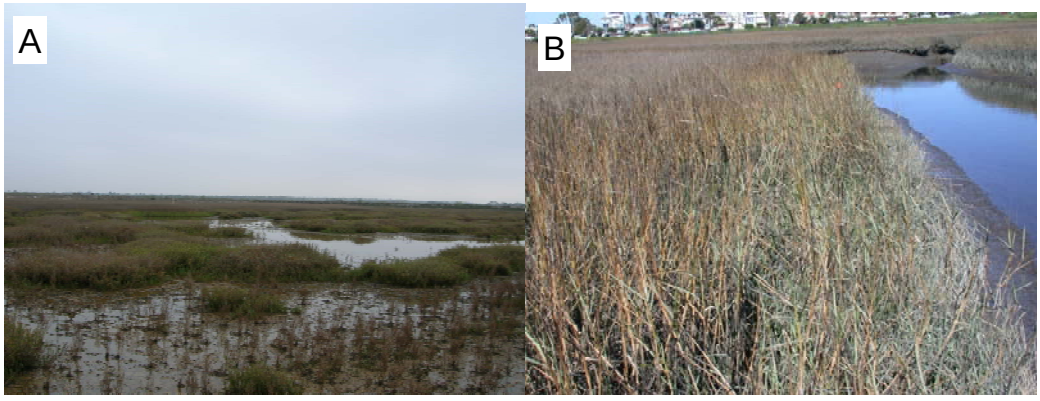

Figure I-2: Wetlands (salt marshes) in Tijuana Estuary, California dominated by (A) the pickleweed, *Salicornia virginica* (recently renamed *Sarcocornia pacifica*) and (B) the cordgrass, *Spartina foliosa*

The structure of biological communities in wetlands is thought to be largely governed by periodic inundation and exposure by tides as well as the stresses of associated salinity, temperature, and oxygen fluctuations (Bertness 1992). Plant species in wetlands are distributed along elevational gradients (Figure I-3, below), with lower limits set by tolerance of seawater inundation and upper limits set by interspecific competition for nutrients.

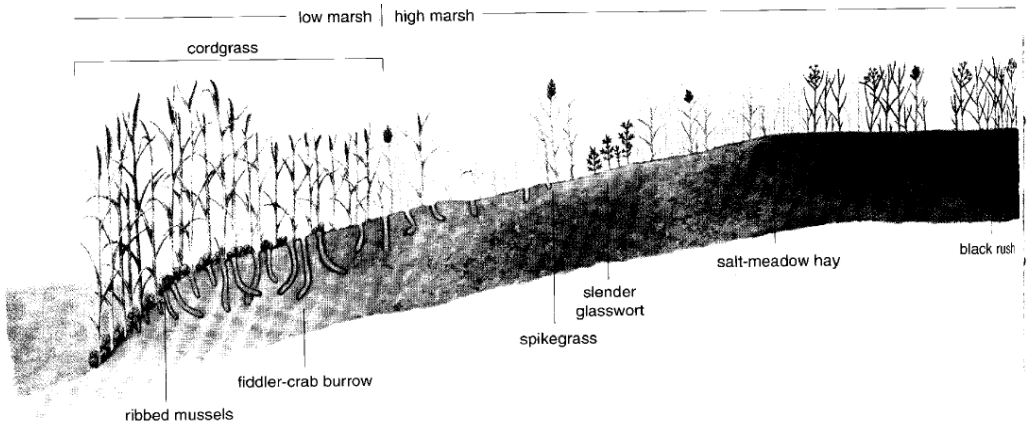

Figure I-3: Diagram, from Bertness 1992, illustrating the position of various plant species along elevational gradients of New England salt marshes.

Coastal wetlands function as critical transition zones, intercepting nutrients and pollutants traveling from land to the sea (Levin et al. 2001). Some wetlands rank among the most productive ecosystems on earth (Mitsch and Gosselink 2001), with algal and plant producers fueling food webs that support both endemic and migratory waterfowl and fish along coastlines. Vascular plants structure habitats for these mobile fauna while also stabilizing sediments and reducing water flows through wetlands, effectively buffering coasts from storm impacts.

The high primary productivity in marine wetlands is frequently nitrogen-limited (Valiela et al. 1976, Covin and Zedler 1988, Boyer et al. 2001, Scott et al. 2007). Experimental nitrogen additions have produced strong responses in plant growth and community composition in several coastal wetlands. Nitrogen limitation in coastal ecosystems is thought to be the combined result of high nitrogen demands among wetland primary producers and nitrogen losses that occur via microbially mediated denitrification, the conversion of nitrate to N_2 of N_2O gases that escape from sediments (Capone 1988).

Conventional views of wetlands have focused on either conspicuous flora and fauna or on microbially-mediated cycling of nutrients and materials, without considering interactions between macroscopic and microscopic biota. Yet over one billion bacteria occupy each gram of coastal sediment (Koster et al. 2008), and the health of major habitat-forming plants is potentially influenced by these inconspicuous microbes. Positive interactions between wetland plants and nitrogen-fixing microbes are known to occur and may hold important implications for the function of these ecosystems.

Plant-microbe interactions in wetlands

Interactions between microbes and vascular plants in wetlands likely span a spectrum that ranges from tight mutualisms to loose, indirect associations facilitations or even competitive interactions for nutrients (Figure I-4). A mutualistic interaction has

been characterized between rhizospheric diazotrophs (nitrogen fixers) and the Atlantic cordgrass, *Spartina alterniflora* (as illustrated in circle of Figure I-4, Livingstone and Patriquin 1980, Whiting et al. 1986). Nitrogen fixed via these tight associations is ecologically significant in wetlands where availability of nitrogen in exogenous sources and pools may be highly variable. Where sediments are oxygenated by burrowing animals, mycorrhizal fungi have also been found on cordgrass roots (Daleo et al. 2007).

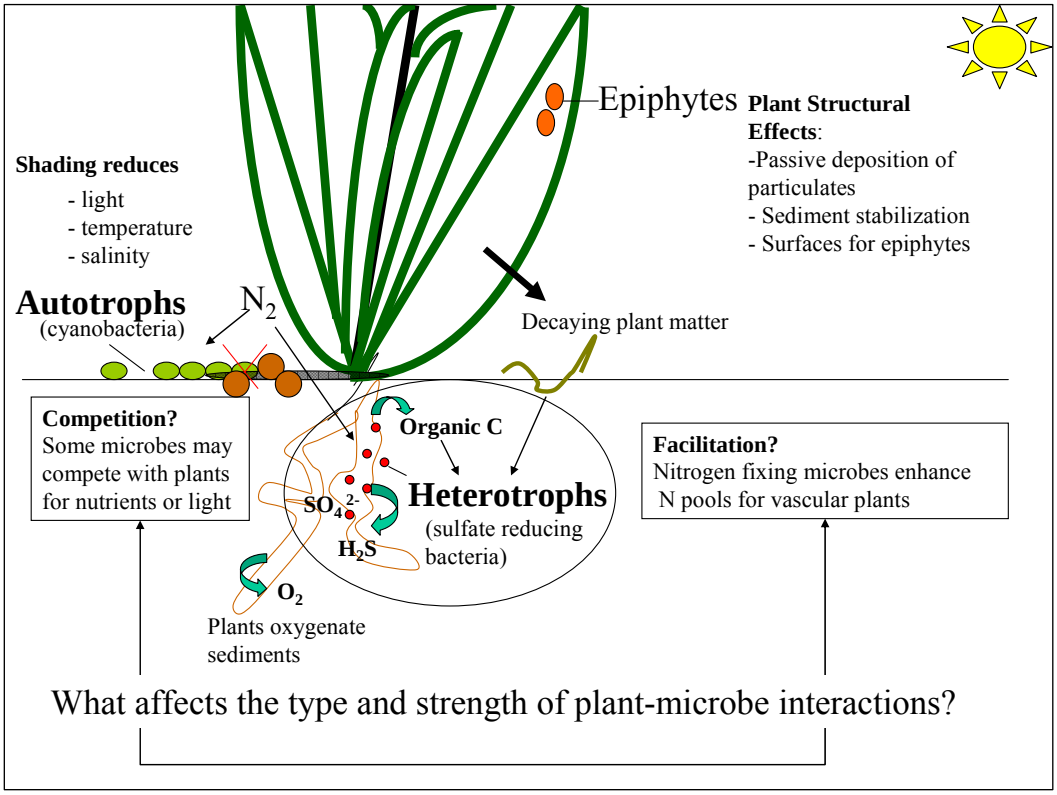

Figure I-4: Diagram of various possible interactions between wetland plants and nitrogen-fixing microbes. Direct interactions involved in mutualistic relationships between wetland plants and root-associated microbes are illustrated in the small black circle. Indirect interactions are described outside of the circle.

In addition, free-living microbes, such as cyanobacteria, on marsh surface sediments (Figure I-4) may enhance nitrogen pools of developing or restored wetlands by fixing nitrogen and indirectly facilitate vascular wetland plants. It is possible that these

autotrophs may compete with plants for nutrients or light under some circumstances. Wetland plants can vary in the nature and extent of their interaction with nitrogen fixing bacteria (Bagwell et al. 2000, La Rocque et al. 2004). Thus, the timing and magnitude of nitrogen fixation in a given habitat could strongly influence inter-specific competition between wetland primary producers.

In wetlands, nitrogen fixation can contribute significantly to the primary productivity and structure of vascular plant communities. Nitrogen-fixing bacteria in rhizospheres (roots and surrounding sediments) are particularly thought to substantially support the nitrogen demands of wetland plants (Capone 1988, Lovell et al. 2000, Bergholz et al. 2001). Nitrogen fixation by bacteria in the root zone of the seagrass, *Zostera marina* (Capone 1988) has been shown to be rapidly transferred to the plant, and that associated with the Atlantic cordgrass, *Spartina alterniflora*, was found to be tightly linked to photosynthesis (Whiting et al. 1986). Evidence suggests that a variety of other wetland plants harbor nitrogen-fixing associates, including two upper marsh pickleweed species, *Salicornia bigelovii* (Rueda Puente et al. 2003) and *Salicornia virginica* (Bagwell et al. 2000), but their interactions are poorly characterized.

Nitrogen fixation is known to play a particularly important role in early successional (disturbed, developing, or restored) marshes, which are often nutrient-poor relative to their mature, natural counterparts (Piehler et al. 1998, Tyler et al. 2003). On the Atlantic coast of the U.S., nitrogen fixation rates in young or transplanted marshes

have been found to significantly exceed those in mature, natural habitats (Currin et al. 1996, Tyler et al. 2003). Although few marsh contrasts have been made on Pacific coasts, nitrogen fixation rates in surface sediments (1 cm deep) were higher in an *S. foliosa* marsh than an early succession (4 year) restored marsh in San Diego Bay (Langis et al. 1991), suggesting the roles of diazotrophs in marsh succession may vary among geographic regions.

Better understanding of the effect of disturbance and stress on diazotroph assemblages is required to reveal relationships between the diversity and function of wetland ecosystems. Manipulations of plant cover, through clipping or increasing exogenous nitrogen levels, have suggested that nitrogen fixing microbial communities are remarkably stable (Piceno and Lovell 2000, Lovell et al. 2001). Nonetheless, differences in the diversity of nitrogen fixers that may result from stronger or larger scale disturbance and stress. Common forms of anthropogenic disturbance and stress in wetlands include biological invasions, sedimentation, and nutrient loading. Changes in nitrogen fixation rates may not only be a consequence but also a mechanism of invasion, as observed for the infamous *Caulerpa taxifolia* which invades dead seagrass beds of the Mediterranean sea by stimulating nitrogen fixation in nutrient-poor sediments (Chisholm and Moulin 2003). Thus, disturbance-induced shifts in diazotroph diversity may not only affect patterns of nitrogen fixation in space and time (Yachi and Loreau 1999, Cardinale and Palmer 2002) but also produce cascading effects for wetland ecosystems through plant-microbe (and microbe-animal) interactions.

Dissertation Objectives and Approaches

Various forms of disturbance may alter ecosystem function by shifting plant microbe interactions as well as diversity-function relationships. A general objective of this research is (1) to characterize the relationship between microbial diversity and ecosystem functioning for nitrogen fixing microbes in wetlands and (2) ask whether changes in the structure of microbial communities, resulting from different types of disturbance, translate into changes of functional significance for the vascular plants with which they interact. In particular, roles of disturbance in structuring diversity-function relationships and plant-microbe interactions are investigated by asking:

(1) How does disturbance affect the structure (diversity, composition) of nitrogen-fixing assemblages?

(2) What are consequences of structural shifts in diazotroph assemblages for the function of nitrogen fixation and vascular plant production (height, biomass) and nutrition (shoot tissue N content)?

Effects of several types of disturbance (biological invasion, restoration/succession, and sediment and nitrogen pollution) on (a) relationships between diversity and function of nitrogen fixing microbes and (b) their interactions with vascular plants are examined in coastal wetlands.

Basic patterns of nitrogen fixation rates in space (intra-marsh) and time (day-night contrasts) are described between two major salt marsh environments, *Spartina foliosa* and

Sarcocornia pacifica zones in Mission Bay, CA (Chapter II). This research improves basic understanding of the biotic and abiotic controls on nitrogen fixation and suggests a role of microbes in niche differentiation between these dominant, habitat-forming plant species. Mensurative experiments then address changes in nitrogen fixing microbial communities in response to 3 distinct biological invasions by a mussel, (*Musculista senhousia*), salt cedar (*Tamarisk* spp.), and a mangrove (*Avicennia marina*) in southern California wetlands (Chapter III). Marked differences in the functional responses of nitrogen fixers to these invaders, even between the structurally similar invasive trees, highlight the importance of environmental contexts for nitrogen fixation while also challenging notions of functional redundancy among diazotroph assemblages. Functional disparities in nitrogen fixation functions between an early and late succession marsh are tested by employing marsh restoration at Tijuana Estuary (CA) as a large-scale mensurative experiment (Chapter IV). Strong functional roles of diazotroph composition emerge from this 2 year study, and a novel positive relationship between plant structure and diazotroph diversity are found in the early successional ecosystem. To further elucidate key controls on nitrogen fixation and plant-diazotroph interactions, manipulative experiments (including isotopic enrichments) then address consequences of sediment and nutrient stresses on plants, diazotroph assemblages, and exchanges of nitrogen between them (Chapter V). Although sedimentation is suggested to exacerbate nutrient stress, nitrogen loading emerges as a key controller of nitrogen fixation, diazotroph diversity, and plant-microbe interactions.

To determine diversity of diazotrophs, a genetic fingerprinting technique (T-RFLP) is applied to the *nif*H gene, coding dinitrogenase reductase, the enzyme that catalyzes nitrogen fixation (Zehr and Capone 1996). Functional responses of these microbes are determined in parallel measurements of nitrogen fixation rates via acetylene reduction assays (Capone and Montoya 2001). Structural (height, biomass, density) and nutritional (shoot N content) properties of plants are tested for relationships to nitrogen fixer activity and diversity via regression analyses across the range of spatial scales (intra-marsh zone contrasts to inter-marsh comparisons) explored in this research.

These studies constitute new approaches to understanding basic functioning of wetland ecosystems, and diversity-function relationships in general, by explicitly considering dynamics of plant-microbe interactions. The investigated impacts of stress and disturbance in wetlands may not only reveal patterns and mechanisms of degradation but also pathways and processes that are central to ecosystem function and resilience. Both ecological theory and conservation efforts can benefit from improved understanding of mechanisms by which macroscopic and microscopic communities engage one another to maintain diversity and ecosystem function amidst environmental change. In the concluding chapter (VI), consequences of nitrogen fixation and diazotroph diversity for wetland plant structure and trophic function are reviewed across spatial scales, and potential roles of microbes in mediating wetland ecosystem response to changing environments are discussed in the context of management and conservation.

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CHAPTER II[1]

OPPOSITE DIEL PATTERNS OF NITROGEN FIXATION ASSOCIATED WITH SALT MARSH PLANT SPECIES (*SPARTINA FOLIOSA* AND *SALICORNIA* SPP.) IN SOUTHERN CALIFORNIA

Abstract

In marine wetlands, nitrogen fixation is a potentially important nutrient source for nitrogen-limited primary producers, but interactions between nitrogen fixers and different vascular plant species are not fully understood. Nitrogen fixation activity was compared in sediments vegetated by 3 plant species, *Spartina foliosa, Salicornia virginica,* and *Salicornia bigelovii* in the Kendall Frost Reserve salt marsh in Mission Bay (CA). This study addressed the effects of plant type, day and night conditions, and sediment depths on nitrogen fixation. Higher rates of nitrogen fixation were associated with *S. foliosa* than with either of the two *Salicornia* spp., which are known to compete more effectively than *Spartina* for exogenous nitrogen in the salt marsh environment. Rates of nitrogen fixation, determined by acetylene reduction, in sediments vegetated by *Salicornia virginica* were low during the day (7.7 $\pm$ 1.2 μmol C_2H_4 m^{-2} h^{-1}) but averaged 13 $\pm$ 6.6 μmol C_2H_4 m^{-2} h^{-1} at night, with particularly high rates in samples from locations with

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visible cyanobacterial mats. The opposite diel pattern was found for sediments containing *S. foliosa* plants, in which average daytime and nighttime rates of nitrogen fixation were 62 $\pm$ 23 μmol C_2H_4 m^{-2} h^{-1} and 21 $\pm$ 15 μmol C_2H_4 m^{-2} h^{-1}, respectively. For *S. foliosa,* nitrogenase activity of rinsed roots and different sediment sections (0-1 cm, or 4-5 cm depths) were measured. Although nitrogen fixation rates in vegetated sediment samples were substantial, all but one of rinsed *S. foliosa* root samples (n=12) and subsurface sediments at 4-5 cm depths failed to show nitrogen fixation activity after 2 hours, suggesting that the most active nitrogen fixers in these systems likely reside in surface sediments. Further, nitrogenase activity in shaded and unshaded *S. foliosa* samples did not differ, suggesting that nitrogen fixers may not rapidly respond to changes in plant photosynthetic activity. Average nitrogen fixation rates in *S. foliosa*-vegetated samples from the Mission Bay salt marsh were on the same order as those of highly productive Atlantic coast marshes, and this microbially-mediated nitrogen source may be similarly substantial in other Mediterranean wetlands. Sediment abiotic variables seem to exert greater control upon nitrogen fixation activity than the effects of particular plant species. Nonetheless, dominant plant species may differ substantially in their reliance on nitrogen fixation as a nutrient source, with potentially important consequences for wetland conservation and restoration.

Introduction

Nitrogen is known to be a limiting nutrient for primary producers in many marine wetlands (Valiela and Teal 1974, Covin and Zedler 1988, Boyer and Zedler 1998, Tyler *et al.* 2003) although bacteria are possibly limited by phosphorus (Sundareshwar *et al.*

2003). In vegetated wetlands such as salt marshes, the conversion of dinitrogen gas into biologically available nitrogen by bacteria, via nitrogen fixation, may be an important control not just on primary productivity but also on the health of habitat-forming vascular plants. For instance, *Spartina alterniflora* (Atlantic cordgrass), a dominant plant of Atlantic coast marshes, has a tight, mutualistic relationship with nitrogen-fixing bacteria in its rhizospheres. The microbes respond rapidly to changes in plant photosynthetic activity and consume labile organic substrates produced by the cordgrass in exchange for rapid direct provision of fixed nitrogen (Boyle and Patriquin 1981, Whiting et al. 1986). Although much is known about interactions between nitrogen fixers and *S. alterniflora* (Boyle and Patriquin 1981, Whiting *et al.* 1986, Piceno and Lovell 2000), less is known about associations of nitrogen fixers with other vascular plant species that dominate coastal regions worldwide, such as those in salt marshes of Mediterranean climates.

Several wetlands worldwide experience Mediterranean climates, including those on coasts of the Mediterranean sea, central Chile, south Africa, southwestern Australia, and the western U.S., which are characterized by dry summers that can be stressful for vascular plants. Mediterranean salt marshes constitute a major category of wetlands in which nitrogen fixation has been understudied but is likely to be significant. Most Mediterranean marshes experience relatively rare rainfall events (coastal mean in southern California = 25 cm y^{-1}, Langis *et al.* 1991). As a consequence, rain rarely washes nutrients into wetlands from surrounding watersheds (Langis *et al.* 1991). Further, the hypersalinity, characteristic of Mediterranean marsh sediments, may increase

nitrogen demands by vascular plants, as nitrogen-containing compounds such as proline and glycinebetaine are thought to be used in osmotic regulation by halophytes (Stewart and Lee 1974, Cavalieri and Huang 1979).

In salt marshes of southern California, nitrogen fixation is likely to be a significant ecosystem function. Southern California marshes are much smaller than their Atlantic coast counterparts, partly due to extensive urban development (Schoenherr 1992), so any watershed-based nutrients are thought to pass quickly through the wetlands (Langis *et al.* 1991). Experimental nitrogen additions suggest vascular plant productivity is nitrogen limited including that of the dominant plant at low marsh zones on the Pacific coast*, Spartina foliosa,* and of *Salicornia virginica* and *Salicornia bigelovii* which comprise most of the upper marsh zone in the Kendall Frost Reserve (McCray 2001). More specifically, nitrogen has been found to limit the height of *S. foliosa* plants, which directly impacts the ability of these plants to provide adequate nesting habitat for the endangered Clapper rail (Boyer & Zedler 1998). Also, nitrogen fixers have been isolated from roots of *Salicornia virginica* (Bagwell *et al.* 2001) and *Salicornia bigelovii* (Rueda-Puente *et al.* 2003) and shown to be physiologically distinct from those of other salt marsh plants (Bagwell *et al.* 2001), although *in situ* rates of nitrogen fixation associated with this species have not been reported. The abundance of cyanobacteria in southern Californian salt marshes has been noted (Zedler 1980), further suggesting these sites have high nitrogen fixation potential. Research is needed to characterize interactions between nitrogen fixers and vascular plant species of southern because nitrogen dynamics are known to regulate competition between *S. foliosa* and *Salicornia* species (Covin & Zedler

1988; Boyer & Zedler 1999; McCray 2001). Therefore, nitrogen fixation may play be an important factor affecting interspecific plant interactions. Further, studies of nitrogen fixation associated with plant species other than *S. alterniflora* can test the prevalence of mutualistic interactions between vascular plants and nitrogen-transforming microbes.

Many studies have examined nitrogen fixation activity associated with only one species of salt marsh plant and have not compared the influence of different plant species on this microbial function. One pioneering study revealed moderate nitrogenase activities associated with excised roots of 33 plant species from 13 elevational zones of a Nova Scotian salt marsh (Patriquin & Keddy 1977). Another study found higher nitrogen fixation in low marsh dominated by *Spartina alterniflora* than in high marsh zones where *S. patens* and *Distichlis spicata* grew, although rates supported by each species were not specifically compared (Valiela & Teal 1979). A more recent study found that distinctions between physiological profiles of nitrogen fixers associated with three different plant species (*Spartina patens, S. alterniflora, Juncus roemerianus*) were greater than those of microbes isolated from different habitats occupied by one species, *S. patens* (Bergholz *et al.* 2001). This research suggested that, under some circumstances, plant species can have stronger influences on nitrogen fixation than environmental factors (Bergholz *et al.* 2001). More work is needed to determine how salt marsh plant species differ in their interactions with nitrogen fixers and should not only include studies in Mediterranean environments but also more detailed assessment of temporal dynamics of nitrogen fixation.

In addition to the influence of plant species, nitrogen fixers are affected by plant by dramatic environmental changes associated with shifting day and night conditions. Previous salt marsh research has indicated that nitrogen fixation activity in sediments of *S. alterniflora* marshes can display dramatic diel patterns, with peak times of activity varying seasonally and with dominant cyanobacterial species (Currin *et al.* 1996). Diel or other temporal patterns of nitrogen fixation have not been described in such detail in Mediterranean or southern Californian marshes, although one study documents seasonal variation in two *S. foliosa* marshes in San Diego Bay (Langis *et al.* 1991). A basic characterization of diel patterns of nitrogen fixation is important for accurate estimates of the magnitudes of nitrogen fixation. Also, diel patterns of nitrogen fixation associated with various plant species may help to elucidate the nature of plant-microbe interactions, as nitrogen fixers intimately associated with vascular plants likely display highest nitrogen fixation rates during the daytime, when plant photosynthesis occurs, rather than at night (Whiting *et al.* 1986).

The nature of plant-microbe interactions can also be characterized by experimental determination of microhabitats in which nitrogen fixers are most active. Specific sites of nitrogen fixation among microhabitats of salt marshes have best been identified in wetlands dominated by *S. alterniflora.* They include plant rhizospheres (roots and surrounding sediments) (Teal *et al.* 1979; McClung *et al.* 1983), dead plant culms and shoots (Currin and Paerl 1998, Newell *et al*. 1992, Moisander *et al.* 2005) as

well as surface sediments (Whitney et al. 1975, Patriquin and Keddy 1977, Currin et al. 1996, Piehler *et al.* 1998). Few investigations have attempted to detail the nature of interactions of *S. foliosa* and nitrogen-fixing microbes (Langis *et al.* 1991) although this species is likely to harbor nitrogen fixers in the same microhabitats as its close relative, *S. alterniflora.*

The purpose of this research was to compare nitrogenase activity in sediments vegetated by three plant species in the Kendall Frost Reserve salt marsh, Mission Bay (CA) and to characterize diel (day versus night) patterns of nitrogen fixation for *S. foliosa*- and *S. virginica*-vegetated sediments. I tested the null hypotheses that (a) daytime nitrogen fixation rates do not differ in rhizospheres of *Spartina foliosa*, *Salicornia virginica,* and *Salicornia bigelovii* and (b) daytime nitrogen fixation rates in *S. foliosa*- and *S. virginica*- vegetated sediments do not differ from night-time rates (April- May 2005). As oxygen concentrations can affect nitrogen fixation rates, an experiment in May 2005 with *Salicornia virginica* addressed the null hypothesis that aerobic, anaerobic, and microaerobic treatments in the headspace of flasks did not affect nitrogen fixation rates of *S. virginica*-vegetated sediments during the day or night.

In experiments with *S. foliosa*, which was predicted to support higher rates of root-associated nitrogen fixation compared to *Salicornia* species, major sites of microbial activity in *S. foliosa* samples were characterized by: (1) comparing rates in different

sediment depth intervals, (2) assaying rinsed roots, and (3) testing effects of plant shading on nitrogen fixation. The null hypotheses addressed by these experiments (in April 2005) were (a) nitrogen fixation rates do not vary between different sediment depth intervals (0-1, 4-5 cm) of *S. foliosa*-vegetated cores, (b) nitrogen fixation activity does not differ between rinsed *S. foliosa* roots and *S. foliosa*- vegetated sediment cores and (c) shading of *S. foliosa* shoots does not affect nitrogen fixation activity in rhizosphere sediments. For *Salicornia virginica,* an experiment in August 2005 tested simply whether leaf and stem surfaces were as important sites of nitrogen fixation as rhizospheres by comparing epiphytic rates of nitrogen fixation on *S. virginica* to those of *S. virginica*-vegetated sediments during the day and night.

Methods

Study Area

This research took place within the University of California Kendall Frost Reserve in Mission Bay, San Diego, California (32^{o} 47'35' N, 117^{o} 13'00" W). This reserve consists of 16 acres of salt marsh dominated by *Salicornia virginica* and *Salicornia bigelovii* in upper elevations and *S. foliosa* at low elevations. This reserve represents the last vestiges of a wetland that once spanned more than half of the bay, prior to its transformation in the late 1940s to a recreational water park (City of San Diego, Parks and Recreation). Low *S. foliosa* elevations of the salt marsh fall within the boundaries of the adjacent Northern Wildlife Preserve of the City of San Diego. The vegetation of the Kendall Frost Reserve and Northern Wildlife Preserve collectively provide nesting habitat for several bird species including the federally endangered

clapper rail (*Rallus longirostris levipes*) and the state endangered Belding Savannah Sparrow (*Passerculus sandwinchensis beldingi*).

Nitrogen fixation in *Spartina foliosa* marsh

To characterize nitrogen fixation rates, twelve experimental blocks (10 m X 5 m) were established parallel to the shoreline in the *Spartina foliosa* zone on the border of the Kendall Frost Marsh and Northern Wildlife Preserve. In April (2005), triplicate vegetated cores (approximately 2 cm diameter, 6 cm deep), centered around and containing the culms and attached roots of live *S. foliosa* plants, were taken from random positions within each of the twelve blocks for determination of nitrogen fixation (acetylene reduction) rates. One additional unvegetated core of the same dimensions, not centered around a plant culm, was also extracted within 0.25 m of the triplicate vegetated cores from blocks 1-6 for nitrogen fixation assays of surface and sub-surface sediment sections. These cores were sealed, and processed *in situ* for determination of acetylene reduction rates as described below. On the same date, twelve *S. foliosa* samples were collected in the evening (just prior to sunset) and assayed *in situ* just after sunset. These samples were processed in the same manner as samples used in day-time assays except flasks were not wrapped in foil.

From each block, one sediment core (4.8 cm diameter, 6 cm deep) was extracted in April (2005) for determination of sediment grain size and organic matter content. These cores were kept on ice while being transported to the laboratory and were then

frozen at -17 ^{o}C. A sediment core (2 cm diameter, 6 cm deep) was also extracted from each block, sealed in a 50 ml centrifuge tube, and stored on ice until processing for porewater nutrient measurements (described below). Plant density was determined by counting the number of *S. foliosa* stems in a small quadrat (0.25 m x 0.25 m) randomly positioned within each block. The average plant height in each quadrat was also determined by measuring heights of up to 10 *S. foliosa* plants. The heights and biomass of individual *S. foliosa* plants collected in the sediment cores that were used for measurement of nitrogen fixation rates were also recorded. Following completion of acetylene reduction assays, plant biomass was separated into above- and below-ground components by clipping the plant stem at the top of the sediment core. Both fractions were then rinsed with water on a 0.5 mm sieve to remove attached sediment, dried for 48 hours at 60 ^{o}C, and weighed (following methods employed by Sunders *et al.* 2006, Howard & Rafferty 2004, Boyer *et al.* 2001). Sections of live *S. foliosa* shoots were also removed from each sample and stored at -17 ^{o}C until they could be processed for tissue nitrogen (N) content analyses.

Acetylene reduction assays

Intact plant samples (sediment cores containing plant roots to a depth of 6 cm, with attached culm, stem and shoots) were extruded into 125 ml flasks (Figure II-1). Plant stems and shoots protruded from the flasks while roots and attached sediments were sealed inside using rubber stoppers and gas-tight tape. During assays, the flasks were wrapped in aluminum foil (to prevent artificial temperature increases upon exposure to

sun). Into the headspace of each flask, 15 ml of acetylene gas was subsequently injected. Sediment cores used in acetylene reduction assays did not exceed 2 cm diameter, so acetylene and headspace gas could diffuse to their interior within the time period of the assay. All assay flasks were incubated *in situ* by being placed into a tub of water, which was replenished hourly to maintain temperatures below 25 ^{o}C, and were positioned on the sediment in the upper marsh of Kendall Frost Reserve. Subsampling of the headspace in each flask was conducted upon initiation of the assay (injection of acetylene), and after 2 and 3.5 hours by withdrawing 2.5 ml of gas from the flask and storing it in N_2-flushed Vacutainers (Becton-Dickinson). These samples were analyzed on an FID-equipped gas chromatograph in peak height mode under conditions described by Capone and Montoya (2001). Although *S. foliosa* samples produced detectable acetylene reduction activity after only 2 hours, *Salicornia* samples did not show activity until 3.5 hours. For studies involving *Salicornia*-associated acetylene reduction activity, these later subsamples (at 3.5 hours) were used for analyses in this study.

S. foliosa manipulations (shading, rinsed roots, and sediment sectioning)

The triplicate *S. foliosa* samples collected in April (2005) were used to address: 1) whether nitrogen fixation rates differed for samples of vegetated sediments versus rinsed *S. foliosa* roots and 2) whether nitrogen fixation rates were affected by shading of plant shoots. One of each set of triplicate samples was exposed to sunlight during an *in situ* assay. For comparison to the first sample (containing an *S. foliosa* plant and sediment), the second sample of each block was rinsed free of sediment prior to having

its roots sealed in the 125 ml flask and was used to measure rates of acetylene reduction directly associated with plant roots. To compare acetylene reduction rates in sediments vegetated by light-exposed plants with those in sediments vegetated by shaded plants, the third sample in each group was left intact but shaded by being placed in a large wire cone (approximately 4 feet high) that had been wrapped in shade cloth to achieve approximately 95 percent light reduction. For all of the triplicate samples, the attached plant stem and shoots protruded from the assay flasks without being severed from the roots The shoots of plants used in such rinsed treatments were exposed to sunlight during the assay, but the roots were in foil-wrapped flasks. All assay flasks employed in this study (containing roots and sediment) were wrapped in foil so that nearly no light reached sediments regardless of the light treatment applied to *S. foliosa* plants, but plant shoots and leaves rose out of the flasks so that light levels reaching the plants could be manipulated independently of those affecting sediments within the flasks.

To compare nitrogen fixation rates in surface versus subsurface sediment depths, the additional vegetated cores sampled in April (one from each block) were sectioned into 0-1 cm and 4-5 cm depth intervals, and sediment (but not plant material) from each section was sealed in a 50-ml flask and processed similarly to the 125-ml flasks. Sediment sections were not shaded during acetylene reduction assays.

Nitrogen fixation in *Salicornia spp.* marsh

To measure nitrogen fixation activity in *Salicornia*-vegetated sediments, a total of eight blocks (10 m X 5 m) were interspersed (separated by approximately 20 m) throughout the Kendall Frost Reserve at elevations in which *S. virginica* and *S. bigelovii* occur (1.7-1.8 m above MLLW). These species co-occur throughout much of the salt marsh, and both species were present in each block. In May 2005, an experiment was conducted in order to: (1) compare rates of nitrogen fixation in intact cores of *Salicornia virginica* during day and night-time *in situ* incubations and (2) to test how nitrogen fixation rates varied under different conditions of oxygen exposure. For this experiment, triplicate vegetated sediment cores (approximately 2 cm diameter, 6 cm deep) were collected from each of 4 experimental blocks during the morning and again in the evening. Each of the triplicate samples received a different headspace oxygen concentration (aerobic, anaerobic, and anaerobic with 5 ml of air). For anaerobic assays, the headspace inside these flasks was flushed for 2 minutes with nitrogen gas prior to initiation of the acetylene reduction assay (described above). Anaerobic samples that had an additional 5 ml of air injected in the flasks prior to assay initiation were used to determine nitrogen fixation rates under microaerobic conditions. Acetylene reduction assays were then conducted as described above.

In a subsequent experiment (May 2005) designed to compare nitrogen fixation rates in sediments vegetated by different *Salicornia* species, paired samples of the two *Salicornia spp.* were extracted from each of 8 blocks. These samples were assayed *in situ* (as described above) during daytime incubations with exposure to natural light levels and

were aerobic. For each of these samples, individual plant heights were recorded. During August (2005), sediment cores containing *S. virginica* plants were sampled along with *S. virginica* shoots (clipped free of roots) from each of the 8 blocks, for comparison of acetylene reduction rates associated with sedimentary (rhizospheric) and epiphytic microbes in day-time and night-time assays. Plant shoots (without roots) were assayed in the same manner as vegetated sediments (described above), except plant shoots were entirely enclosed within the sealed flasks. All samples assayed in August were aerobic.

In each *Salicornia* block, the percent cover by each plant species was determined within randomly-positioned plots (0.5 m x 0.5 m). Sediment cores were also taken for determination of porewater ammonium (3.5 cm^2 x 6 cm), and analyses of sediment grain size and bulk organic matter content (18.02 cm^2 x 6 cm) and were processed according to methods described below. In August 2005, above- and below-ground biomasses of *S. virginica* samples used in acetylene reduction assays were measured by separating these portions at the surface of sediment cores in which they were collected and determining weights of plant matter dried overnight at 60^oC.

Laboratory analyses

Plant shoots were frozen at -17 ^oC until they could be processed for tissue nitrogen content. To remove epiphytes, shoots were rubbed with 5% hydrochloric acid and rinsed with distilled water prior to drying overnight at 60 ^oC. Samples were then ground to a fine powder and analyzed for carbon and nitrogen content (by % weight)

using a CHN elemental analyzer (Costech 4010, 0.5% precision). Porewater was extracted from sediment cores (2 cm diameter, 6 cm deep) via centrifugation on all sampling dates. Porewater was filtered with 0.45 μm filters (Acrodisc) and ammonium concentration was determined by colorimetric techniques following Solorzano (1969). Porewater nitrite levels were also measured following the protocol of Strickland and Parsons (1968). To determine the percentage of combustible organic matter content in sediments, sediment cores were homogenized and passed through a 2-mm sieve to remove large plant material. These sediments were combusted at 300^{o}C for at least 4 hours and organic matter content was calculated by mass difference. Grain size percentages (% sand, % clay) were determined, using a homogenized subsample of these sediments, from the dry mass of sediments separated by a 63-μm sieve following digestion with hydrogen peroxide to remove organic matter.

Statistical analyses

Nitrogen fixation rates associated with different plant species, shading treatments, and sediment intervals were compared using t-tests or one-way ANOVAs. Comparisons of nitrogen fixation rates for samples collected from the same experimental block were conducted using paired t-tests. Percentage composition data (organic matter, grain size, tissue nitrogen and carbon data) were arcsine-square root transformed prior to analyses. In cases where data did not meet assumptions of normality even after transformation, non-parametric tests, including the Wilcoxon test, were used to compare means. Relationships between nitrogen fixation rates and plant or environmental parameters were

analyzed and described, where appropriate, using linear regression models. All statistical analyses were performed with JMP 4.0 software.

Results

Nitrogen fixation rates associated with different plant species

Average day-time rates of nitrogen fixation measured in samples with *S. foliosa* in April were more than twice those detected in samples with *S. bigelovii* or *S. virginica* taken in early May (Figure II-2A). *S. virginica*-vegetated sediments were also assayed in August, with daytime nitrogen fixation rates averaging 26 $\pm$ 8.4 μmol C_2H_4 m^{-2} h^{-1}, but these activity rates were not significantly different from those determined in May (paired t-test, t_7=0.48, p=0.32). In night-time assays, average rates of nitrogen fixation in *S. foliosa* and *S. virginica*-vegetated sediments did not differ (Figure II-1B and II-1C, Wilcoxon, z=0.69, p=0.46).

In a day-time comparison of nitrogen fixation rates in sediments vegetated by *Salicornia virginica* and *Salicornia bigelovii*, which occupy a common elevation in Kendall Frost, no differences between nitrogen fixation rates were found (paired t-test, t_{14}=0.436, p=0.67).

Diel patterns of nitrogen fixation

Samples of *S. foliosa*-vegetated sediments that were collected and assayed during the daytime exhibited average rates of nitrogen fixation (62 $\pm$ 23 μmol C_2H_4 m^{-2} h^{-1}) that

were more than twice those of samples collected in the evening and assayed at night (21 ± 15 μmol C_2H_4 m^{-2} h^{-1}, Figure II-2B). These differences were not statistically significant however (paired t-test, t_{11}=1.28, p=0.11), likely due to the high variability among nitrogen fixation rates within each treatment.

Among *S. virginica*-vegetated samples collected in May, nitrogen fixation rates were higher during the night than during the day based upon measurements taken at 3.5 hours (paired t-test, t_{10}=6.51, p<0.01, Figure II-1C). Average daytime nitrogen fixation rates were 3.8± 0.5 μmol C_2H_4 m^{-2} h^{-1} while night-time rates were 6.5 ± 3.3 μmol C_2H_4 m^{-2} h^{-1}. As one night-time sample showed very high nitrogen fixation activity (38μmol C_2H_4 m^{-2} h^{-1}), there was large variance among samples assayed at night compared to those assayed during the day (Figure II-1C). This diel pattern was opposite to that observed in samples of *S. foliosa* (Figure II-1B). Nitrogen fixation rates in *S. virginica*-vegetated sediments did not differ across a gradient of three oxygen treatments during either day ($F_{2,8}$=1.56, p=0.27) or night assays ($F_{2,10}$=0.67, p=0.54). A few months later (in August), no differences were found between day-time (26 ± 8.4 μmol C_2H_4 m^{-2} h^{-1}) and night-time (21 ± 7.1 μmol C_2H_4 m^{-2} h^{-1}) nitrogen fixation rates measured in intact *S. virginica*-vegetated cores (p= 0.88) or between day-time (4.8 ± 1.6 nmol C_2H_4 $g^{-1}h^{-1}$) or night-time (5.4 ± 2.0 nmol C_2H_4 $g^{-1}h^{-1}$) epiphyte samples (plant shoots only) (p= 0.89).

Characterizing sites of nitrogen fixation

Nitrogen fixation activity (equivalent to 1.4 ± 0.63 μmol C_2H_4 m^{-2} h^{-1}) was detected only in top (0-1 cm) sections of *S. foliosa*-vegetated sediment cores and not in deeper (4-5 cm) sections, although variability was high, such that differences between these intervals were not significant (p=0.32).

Nitrogen fixation rates of intact *S. foliosa*-vegetated sediment cores were significantly higher than those of plants with rinsed roots regardless of whether plants with sediments were assayed in light (Wilcoxon test, z= -1.98, p= 0.04) or shade (z= 2.41, p= 0.01).The highest nitrogen fixation activity rate in *S. foliosa*-vegetated sediment was 282 μmol C_2H_4 m^{-2} h^{-1}. Twelve samples of rinsed roots were assayed, but only one produced detectable nitrogen fixation activity (119 μmol C_2H_4 m^{-2} h^{-1}).

In August, nitrogen fixation rates in *S. virginica*-vegetated sediment cores did not exceed those of *S. virginica* epiphytes during either day-time (p=0.79) or night-time assays (p=0.56), when the data were normalized to plant biomass. Nitrogen fixation rates for intact cores were equivalent to an average of 4.2 ± 1.3 nmol C_2H_4 g^{-1} h^{-1} and 4.8 ± 1.9 C_2H_4 g^{-1} h^{-1} during the day and night, respectively, while those for epiphytes alone were 4.8 ± 1.6 C_2H_4 g^{-1} h^{-1} (day) and 5.4 ± 2.0 C_2H_4 g^{-1} h^{-1} (night).

No significant effect of *S. foliosa* plant shading on nitrogen fixation rates in vegetated sediments was observed (p=0.99). Average nitrogen fixation rates (with standard error) in sediment cores containing shaded plants were 68 ± 27 μmol C_2H_4 m^{-2} h^{-}

[1] and those in cores with *S. foliosa* plants exposed to full sunlight were 62 $\pm$ 23 μmol C_2H_4 m^{-2} h^{-1}.

Relationships between nitrogen fixation, plant parameters, and abiotic factors

Spartina foliosa

Nitrogen fixation rates did not vary with *S. foliosa* plant height (of individual plants (p=0.91) or average heights within plots (p=0.14), total plant biomass (p=0.55), leaf tissue N content (p=0.41), or plant density (p=0.58). Of six plant samples randomly selected for tissue N content analyses, highest nitrogen fixation rates were found in samples with intermediate values of leaf N content. Nitrogen fixation rates were also not clearly related to porewater ammonium values, although the concentrations (5- 37 μM) measured in all *S. foliosa* blocks of Kendall Frost were well below reported inhibition thresholds (Carpenter *et al.* 1978, Teal *et al*. 1979).

***Salicornia* spp.**

Nitrogen fixation rates of sediment samples containing *S. bigelovii* were positively related to porewater nitrite levels (r^2=0.89, p<0.01) and to the percentage of mud in sediments (r^2=0.79, p=0.04). There was a negative trend between the percentage of nitrogen in *S. virginica* shoots and porewater ammonium concentrations (r^2=0.54, p=0.06). No such relationships were found for samples of *S. bigelovii.*

During August, nitrogen fixation rates were positively related to below-ground biomass of *S. virginica* plants (Figure II-2). On this date, there no relationship between nitrogen fixation rates in *S. virginica*-vegetated sediments and porewater ammonium levels ($r^2 = 0.44$, $p = 0.15$). The percentage of tissue nitrogen in *S. virginica* shoots was not related to either nitrogen fixation rates or porewater ammonium. There was a strong negative relationship in August between nitrogen fixation (acetylene reduction) rates and *S. virginica* height in daytime assays (Figure II-3A), but this relationship was not found in samples assayed at night. A non-linear relationship, with lowest nitrogen fixation rates occurring in samples with nearly the highest above-ground *S. virginica* biomass, also existed (Figure II-3B, see discussion).

Discussion

Nitrogen fixation in *Spartina foliosa* and *Salicornia* zones

Nitrogen fixation rates in the Kendall Frost Reserve have been shown to be substantial but dynamic in space and time (Figure II-1). In sediments vegetated by *S. foliosa* and *S. virginica*, rates as high as 282 μmol C_2H_4 m^{-2} h^{-1} (11 g N m^{-2} y^{-1}) and 76 μmol C_2H_4 m^{-2} h^{-1} (3.1 g N m^{-2} y^{-1}) respectively were observed, although average rates were lower (Figure II-1). Notable differences were found between rates associated with different plant species (Figure II-1A) as well as between day and night rates (Figure II-1B and 1c), although oxygen levels did not affect the magnitude of nitrogen fixation activities. The results of this study suggest that the performance of nitrogen fixation is partitioned both in space and time, such that higher daytime rates may occur in *S. foliosa*-

vegetated zones of the salt marsh, while nighttime activities may be greater in *Salicornia* spp. zones. Further, these opposite diel patterns of nitrogen fixation indicate that the nature and extent of interactions between *Spartina* or *Salicornia* and nitrogen fixers may differ substantially.

This is the first study to suggest that *S. foliosa,* which has been considered an inferior competitor for exogenous nutrients compared to *S. bigelovii* (Boyer and Zedler 1999), may rely more heavily on nitrogen fixation as a nutrient source than *Salicornia* species. *Salicornia* plants, on the other hand, may rely more on exogenous, recycled nutrients as suggested by both the lower day-time nitrogen fixation rates than those associated with *Spartina* plants (Figure II-1A) and the negative trend between *Salicornia virginica* shoot tissue nitrogen content and porewater ammonium (discussed above), which was consistent with uptake of porewater nutrients by that species.

Nitrogen fixation rates may vary between sediments vegetated by different plant species due to edaphic factors that vary between elevational zones occupied by each plant species. These factors include porewater nutrient concentrations, salinity or evaporation rates and temperature. Alternatively, particular plant species differentially affect nitrogen fixers in salt marsh sediments, either via production of distinct oxygenation patterns or exudation of specific organic substances. In this study, the two *Salicornia* species that occupied a common zone did not differ in nitrogen fixation rates (Figure II-1A), so environmental parameters rather than plant-species effects may have driven patterns in

nitrogen fixation. In particular, porewater ammonium concentrations were well below inhibitory thresholds (Carpenter *et al.* 1978, Teal *et al.* 1979) in *Spartina* zones where day-time nitrogen fixation rates were highest (Figure II-1A), but were possibly inhibitory in *Salicornia* zones where concentrations were significantly higher ($F_{3, 29}$=12.98, $p<0.01$) and exceeded 100 μM.

Diel patterns of nitrogen fixation

The opposite diel patterns of nitrogen fixation observed in *S. foliosa*- and *S. virginica*-vegetated sediments suggest that the active nitrogen fixers interacting with these plant species differ substantially. Different types of nitrogen fixers are thought to be active during the day than at night in salt marsh sediments (Currin 1996). High daytime nitrogen fixation rates measured in *S. foliosa*-vegetated sediments (Figure II-1A,B) are possibly performed by autotrophic cyanobacteria with oxygen tolerance or heterocysts or by plant-associated heterotrophic bacteria residing in plant rhizospheres that benefit from plant-derived photosynthetic products (i.e. labile carbon). As daytime nitrogen fixation rates associated with *S. foliosa* were greater than those for *Salicornia* spp. (Figure II-1A), there may be some degree of stimulation as a result of the specific influence of *Spartina* on microbes, but nitrogen fixation could also be higher as a result of favorable abiotic conditions, as discussed above. High night-time activity associated with *Salicornia* (Figure II-1C) might be attributed to non-heterocystous cyanobacteria that can be dominant seasonally among microphytobenthic communities (Currin 1996). A seasonal change in active nitrogen fixers could also explain the lack of a strong diel pattern in

nitrogen fixation of *S. virginica*-vegetated sediments during August compared to that found in May. Active nitrogen fixers were not identified in the present study but can be characterized via reverse-transcripted PCR targeting *nif*H genes (Brown *et al.* 2003). Nonetheless, the result that *Salicornia*- and *Spartina*-vegetated sediments showed opposite temporal patterns of nitrogen fixation activity suggests that different plant species can engage quite distinct nitrogen-fixing microbial communities.

Microhabitats for nitrogen fixation (sediments, epiphytes, roots)

Surface sediments as well as plant surfaces were active sites of nitrogen fixation. In sectioned cores from *S. foliosa* zones, nitrogen fixation was found only in surface sediments, while none was found in 4-5 centimeter depths. Nitrogen fixation activity was detected for only one rinsed *S. foliosa* roots and was less than half the rate of the most active sediments on the same date. These results suggest that nitrogen fixers associated with this species may not reside within plant tissues, as with other *Spartina* species (*S. alterniflora:* Whiting *et al.*1986; Gandy & Yoch 1988; *S. maritima:* Nielsen *et al.* 2001), but rather in surrounding sediments. However, disruption of biogeochemical gradients in rhizosphere sediments may have affected the activity of nitrogen fixers in subsurface sediment intervals and on rinsed roots, underestimating the rates of nitrogen fixation in these samples. Nonetheless, the failure of shading *S. foliosa* plants to affect nitrogen fixation activities in sediments is consistent with greatest activity being due to epibenthic nitrogen fixers rather than those in plant rhizospheres. In contrast, rapid responses of nitrogen fixers to changes in the plants' photosynthetic status have been observed for *S.*

alterniflora (Whiting *et al.* 1986). The photosynthetic status of *S. foliosa* samples was not quantified in this study. While rhizospheric bacteria have not been entirely ruled out as important players, these results contribute new evidence for the ability of surface-dwelling microbes to dominate nitrogen fixation activity in the immediate vicinity of a *Spartina* plant (as in Jones 1974).

In measurements of epiphytic nitrogen fixation rates associated with *S. virginica*, the only plant species for which such measurements were made, rates were comparable to those in intact sediment cores. In Atlantic coast marshes, epiphytic rates were similarly found to be substantial but were only approximately half the nitrogen fixation rates found in *S. alterniflora* rhizospheres (Currin & Paerl 1998). Epiphytic nitrogen fixation is not considered to be a direct source of nutrients to plants, although it can be of substantial importance as a nitrogen source in wetland food webs (Currin & Paerl 1998).

Nitrogen fixation and plant parameters

The positive relationship between belowground biomass of *S. virginica* and nitrogen fixation rates in August (Figure II-3) was consistent with that found in San Diego Bay in *S. foliosa*-vegetated cores (Zalejko 1989) as well as other wetland studies (Welsh 2000; McGlathery *et al.* 1998; Hanson 1983) and indicates nitrogen fixation may be stimulated by plant roots (i.e. via oxygenation of rhizospheres or release of labile carbon). Few other strong relationships were found between plant parameters and nitrogen fixation in this study, possibly because data were collected at only one site and

several plant properties did not vary substantially across salt marsh zones. Nitrogen fixation rates, on the other hand, were highly variable and possibly governed by patchy but highly active cyanobacteria on sediment surfaces rather than in rhizospheres, confounding relationships between rhizospheric nitrogen fixation rates and plant parameters.

The negative relationship between nitrogen fixation rates and *S. virginica* height (Figure II-4A) and the decline in nitrogen fixation rates with increasing plant biomass (the left portion of Figure II-4B) in August was likely due to positive correlation of these two *S. virginica* parameters with environmental factors that inhibited nitrogen fixation. In fact, porewater ammonium concentrations showed positive trends with both *S. virginica* height (r^2=0.57, p=0.08) and above- ground biomass (r^2=0.52, p=0.10) while a negative trend was found between ammonium and nitrogen fixation (r^2=0.44, p=0.15). Although previous reports of relationships between *Salicornia virginica* and nitrogen fixation are not known, positive relationships between *S. virginica* characteristics (biomass, number of branches, branch tissue nitrogen concentration) and sediment nutrient levels have been reported previously (Boyer *et al.* 2001). Contrary to the case with plant height, samples with the highest above-ground biomass showed a secondary increase in nitrogen fixation that is consistent with plant stimulation of nitrogen fixation found in several other studies (Hanson 1977; Boyle & Patriquin 1981; Hanson 1983). While plants are often reported to stimulate nitrogen fixation via release of organic exudates from roots, above-ground plant

biomass may also benefit nitrogen-fixing communities via shading and retention of vital moisture in arid Mediterranean marshes.

Significance of nitrogen fixation in southern Californian marshes

Annual average rates of nitrogen fixation in *S. foliosa* habitat found in the present study (equivalent to 5.1$\pm$ 0.56 g N m^{-2} y^{-1}) are of the same order as those reported for a mature *S. alterniflora* marsh on the Atlantic coast (6.1 $\pm$ 0.5 g N m^{-2} y^{-1}, Tyler *et al.* 2003) but are higher than those reported for *S. foliosa* in San Diego Bay, the only other known nitrogen fixation data for southern California marshes, by more than an order of magnitude (Langis *et al.* 1991). Several methodological differences might contribute to the disparities between the results of this study and those of Langis *et al.* (1991). Specifically, this study employed shallower sediment cores but also used intact plant samples while Langis *et al.* (1991) clipped plants at the sediment surface. Langis *et al.* also incubated samples at relatively low light levels (10.5 μmol m^{-2} s^{-1}) rather than in natural sunlight, as done in this study at Kendall Frost Marsh. These nitrogen fixation measurements have also been taken in a different bay than those of Langis *et al.* and therefore many site-specific factors could differ between these locations.

Using literature-based estimates of aboveground net primary productivity for *S. foliosa* in a nearby salt marsh (Winfield 1980), along with average tissue nitrogen content of *S. foliosa* shoots obtained in this study, one may estimate that nitrogen fixation rates can meet between 36 and 92 percent of annual nitrogen demands. This estimate is based

upon the average daytime and average nighttime fixation rates for *S. foliosa*-vegetated sediments in this study, which had large standard errors due to high variability among nitrogen fixation rates. Similar calculations for *S. virginica*-vegetated sediments suggest that nitrogen fixation could provide between 32 and over 100 percent of nitrogen demanded by the plant, although the majority of this would be fulfilled by night-time activity, and is likely uncoupled with plant nitrogen demands. These calculations do not include the nitrogen fixation performed by epiphytes or that in sediments deeper than 6 cm and therefore underestimate nitrogen fixation associated with both plant species As productivity of salt marsh plants in southern California exhibits high interannual variability (Zedler *et al.* 1992), assessments of the significance of nitrogen fixation rates in this study could be improved if derived from more recent or contemporaneous productivity measurements. Nitrogen fixation rates may also show seasonal variability, which might not be accurately reflected in nitrogen fixation rates calculated from one or two sampling dates and was not addressed in this study. The actual contribution of nitrogen fixation to plant nitrogen demands will depend on several factors including the extent to which fixed nitrogen is actually being assimilated by the plant. Reliance of vascular plant species on fixed nitrogen has not been demonstrated in this study but can be determined through use of $^{15}N_2$ enrichment experiments to trace fixed nitrogen into plant tissues (as in Jones 1974).

Nonetheless, this calculation shows that the magnitude of nitrogen fixation is high enough that it could be an important nutrient source to primary producers and consumers

in southern California and in Mediterranean marshes in general. In southern California, where the need for effective salt marsh restoration is especially pertinent to the survival of endangered bird and plant species, nitrogen fixation may warrant consideration as an important function affecting the development of a healthy ecosystem. As *Spartina* and *Salicornia* species are common in marine wetlands worldwide (i.e. U.S. Atlantic and Gulf coasts, Mediterranean, South African coasts), the different ways that these plants engage nitrogen-fixing microbes require further study to advance understanding of nutrient dynamics in wetlands globally. Further study of interactions between habitat-forming plants and the microbial communities with which they coexist can improve ecosystem-based views of wetland health and function, enabling greater understanding of the ramifications of biodiversity loss and degradation that increasingly threaten coastal habitats.

Conclusions

Nitrogen fixation activity associated with vascular plants of Kendall Frost Marsh Reserve was high but dynamic across marsh elevations and time. Sediments vegetated by two dominant plants, *S. foliosa* and *S. virginica,* showed opposite diel patterns, with higher daytime nitrogen fixation rates found in *S. foliosa*-vegetated sediments than in those vegetated by *S. virginica*. Nitrogen fixation activity was highest in *S. foliosa*-vegetated sediments, although little activity was found in rinsed *S. foliosa* roots. Surface sediments also supported greater rates of nitrogen fixation than subsurface (4-5 cm) intervals, suggesting patchy but active epibenthic cyanobacteria may be major nitrogen fixers in this system. Nitrogen fixation by sedimentary bacteria could provide substantial

portions of *S. foliosa* nitrogen demands, though *S. virginica* is less likely to benefit as greatly from this nutrient source as it does from exogenous nitrogen, with implications for restoration and conservation strategies for these habitat-forming wetland plant species.

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Figures

Figure II-1: Example of acetylene reduction assay set up with an intact S. foliosa plant. The roots of *S. foliosa* and attached sediments were sealed in an air-tight flask into which acetylene gas was injected to initiate the assay. Flasks were also wrapped with foil and all samples were incubated in situ as described above (see Methods). Similar set ups were employed for *S. virginica* and *S. bigelovii*

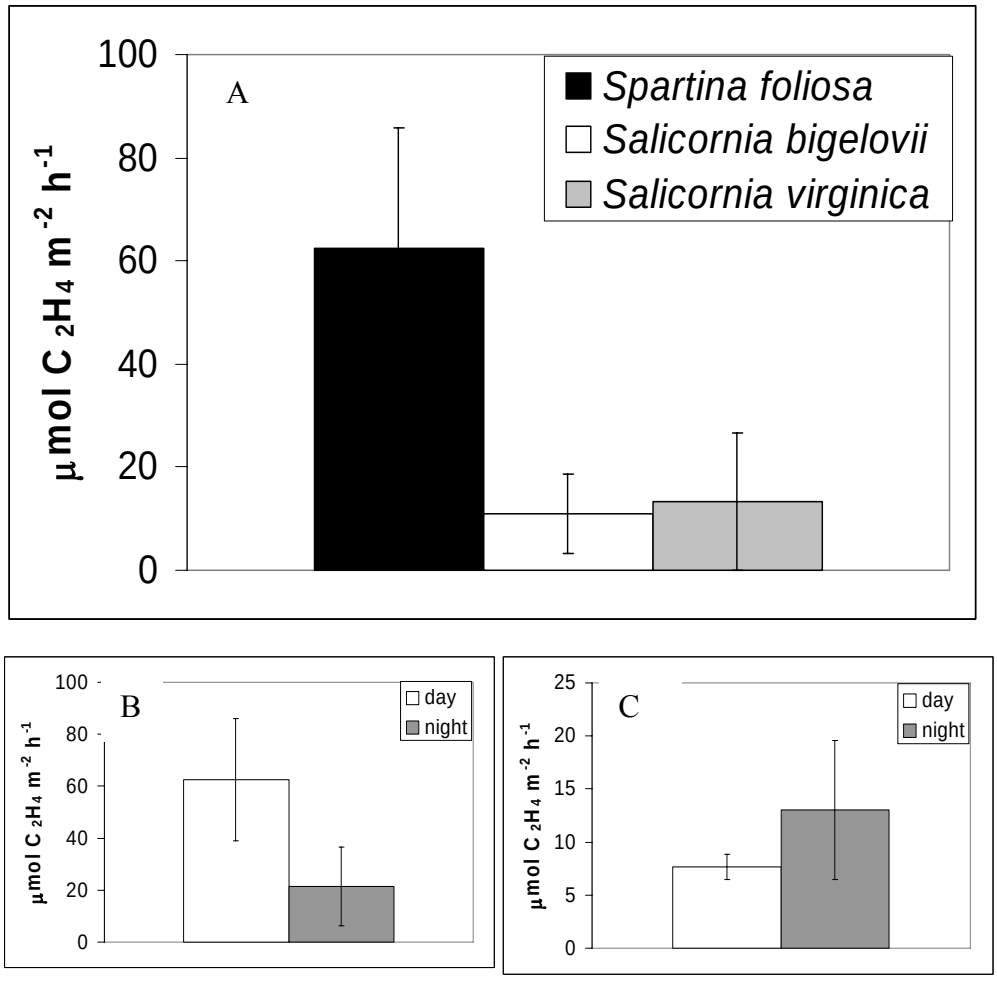

Figure II-2: (A) Average day-time nitrogen fixation (acetylene reduction) rates and standard error bars in sediments vegetated by *3 plant species* in Kendall Frost with 2 hour exposure to full sunlight (Kruskal Wallace, χ^2=6.88, p=0.03) (B) Nitrogen fixation (acetylene reduction) rates (and one standard error) of *S. foliosa*-vegetated sediments during the day and at night (paired t-test, t_{11}=1.28, p=0.11) (C) Nitrogen fixation (acetylene reduction) rates and standard error measured in *S. virginica* samples in May 2005 during daytime and night-time assays after 3.5 hours (paired t-test, t_{10}=6.51, p<0.01)

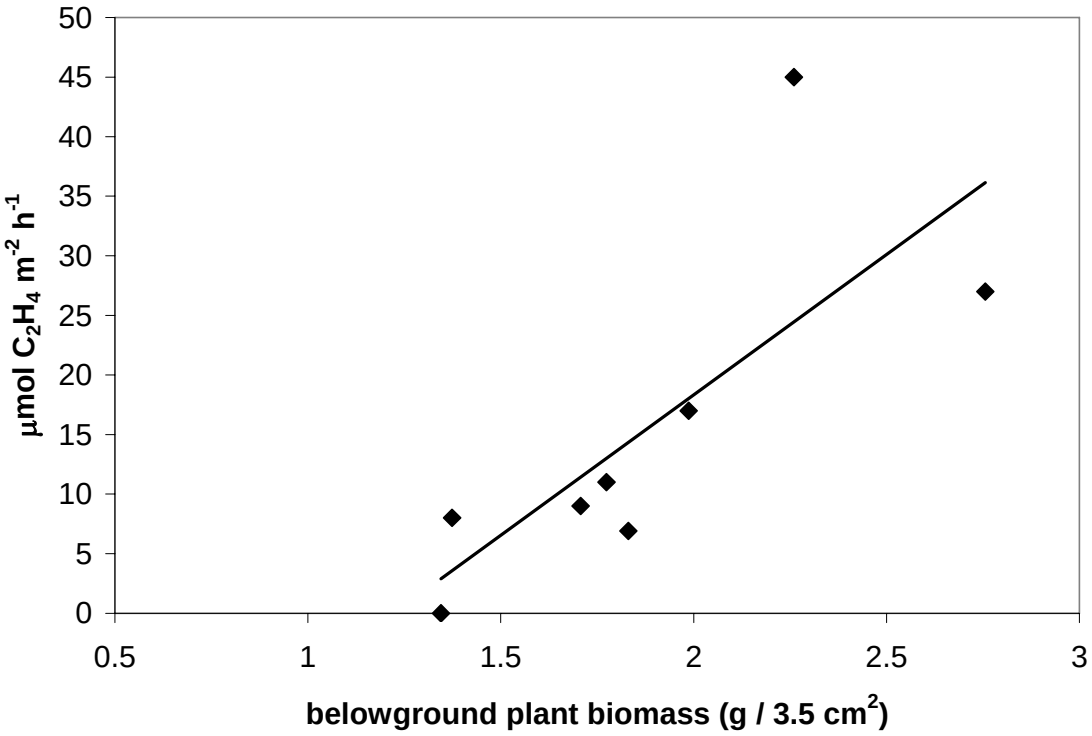

Figure II-3: The relationship between nitrogen fixation (acetylene reduction) rates and belowground biomass of *S. virginica* in August ($r^2 = 0.58$, $p = 0.03$)

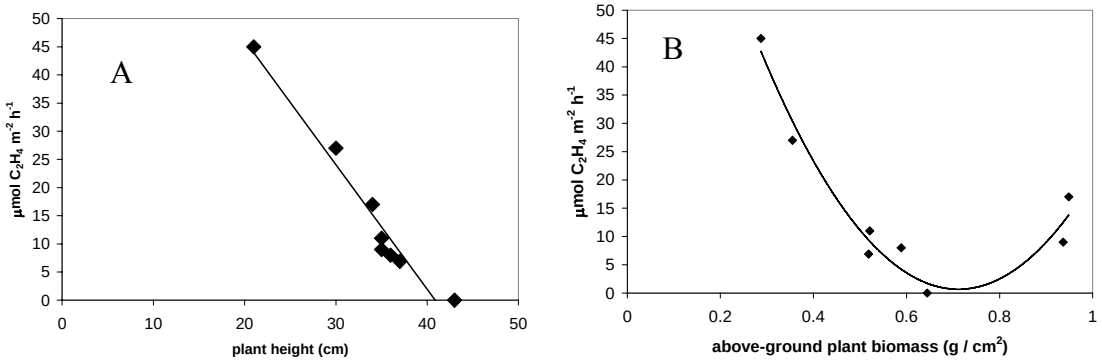

Figure II-4: (A) The relationship between nitrogen fixation (acetylene reduction) rates and *S. virginica* plant height in August ($r^2 = 0.95$, $p < 0.01$); (B) The relationship between above-ground biomass of *S. virginica* and nitrogen fixation rates in August ($p < 0.01$, $r^2 = 0.95$)

CHAPTER III[2]

DIVERSITY AND FUNCTIONAL RESPONSES OF NITROGEN-FIXING MICROBES TO THREE WETLAND INVASIONS

Abstract

Impacts of invasive species on microbial components of wetland ecosystems can reveal insights regarding functional consequences of biological invasions. Nitrogen fixation (acetylene reduction) rates and diversity of nitrogen fixers, determined by genetic fingerprinting (T-RFLP) of the *nif*H gene, were compared between native and invaded sediments in three systems. Variable responses of nitrogen fixing microbes to invasion by an invasive mussel, *Musculista senhousia*, and mangrove, *Avicennia marina*, in Kendall Frost- Northern Wildlife Preserve (Mission Bay) and salt cedar, Tamarisk (*Tamarix* spp.) in Tijuana Estuary suggest microbes respond to both species- and site-specific influences. Structurally similar invaders (the mangrove and salt cedar) produced different effects on activity and diversity of nitrogen fixers, reflecting distinct environmental contexts. Despite relative robustness of microbial community composition, subtle differences in total diversity or activity of nitrogen fixers reveal that microbes are not immune to

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impacts of biological invasions, and that functional redundancy of microbial diversity is limited, with significant consequences for functional dynamics of wetlands.

Introduction

In recent decades, the human-mediated introduction of invasive species into coastal ecosystems has dramatically transformed both the structure and function of their biological communities (Wallentinus and Nyberg 2007, Crooks and Ruiz 2001, Bertness 1984). The magnitude of this threat to biological diversity has been recognized mostly via examination of invasive species' impacts on macroscopic components of ecosystems, such as plant and animal communities. Yet, in addition to alterations of native plant and animal community composition, non-native species modify less visible, functional aspects of the ecosystems they invade (Tyler and Grosholz 2007).

Invasive species occupy diverse niches within coastal ecosystems. In wetlands, invasive species range from conspicuous habitat-generating vascular plants, such as the *Spartina* hybrid that transforms mudflats to densely vegetated marsh (Brusati and Grosholz 2006, Levin et al. 2006), to the less evident mussel *M. senhousia* that carpets seagrass beds with byssus cocoons (Crooks 1998). Although their specific effects may vary, many invasive species modify key environmental factors of the benthos (Wallentinus and Nyberg 2007, Crooks 2002, Bertness 1984) such as organic matter content, sediment grain size (Crooks 1998), light levels (Strong et al. 2006), and nutrients

(Larned 2003) which can have functional implications for affected ecosystems (Levin et al. 2006, Allen 1998).

Microbially-mediated nitrogen dynamics are of particular importance to biogeochemical functions in marine ecosystems, such as coastal marine wetlands, where many primary producers are nitrogen-limited (Valiela and Teal 1974, Covin and Zedler 1988, Boyer and Zedler 1998). Key nitrogen transformations by bacteria affect the balance and availability of nutrients to the rest of the ecosystem. However, despite the key roles microorganisms play in biogeochemistry, few studies have focused on impacts of these invasions on microbial communities (Ehrenfield 2006), particularly in coastal marine environments. Nonetheless, microbes potentially offer indications of the ecosystem-level implications of habitat changes induced by invasive species (Gribsholt and Kristensen 2002, Chishoulm and Moulin 2003, Hawkes et al. 2005).

Nitrogen-fixing bacteria constitute one key functional group of microorganisms relevant to the function of wetland ecosystems. Nitrogen fixation in the benthos underlies the high productivity of these ecosystems by offsetting nitrogen losses to denitrification (Capone 1988). The function of nitrogen fixation is mediated by diverse microorganisms including autotrophic cyanobacteria in mats on wetland sediments (Zehr et al. 1995) and plant surfaces (Currin and Paerl 1998) as well as heterotrophic sulfate reducers that intimately engage roots of cordgrasses (Brown et al. 2003, Lovell 2002, Whiting et al.

1986), seagrasses (Welsh 2000), mangroves (Holguin et al. 2001), and other salt marsh plants (Bagwell et al. 2001) by providing fixed nitrogen in exchange for organic carbon.

Specific predictions can be made about the relationships of invasive species to nitrogen-fixing bacteria in wetlands. Some nitrogen fixers are known to be autotrophic as well as oxygen sensitive and thus invasive trees which reduce light levels or oxygenate rhizosphere sediments may negatively affect them. In other cases, invasive species can stimulate nitrogen fixation by providing carbon sources for heterotrophic nitrogen fixers that reside in wetland sediments. At least one conspicuous algal invader, *Caulerpa taxifolia*, is known to overtake coastal ecosystems via stimulation of nitrogen fixation by this mechanism (Chisholm and Moulin 2003). Invasive animals, such as mussels, can also increase carbon availability to benthic nitrogen-fixing bacteria via biodeposition (Crooks 1998, Reusch et al. 1994) and production of fecal matter (Bartoli et al. 2001). Further, invasive species may offer new surfaces (niches) upon which nitrogen fixing bacteria may grow. In their native ecosystems, mangroves harbor nitrogen fixers on pneumatophores (aerial roots) and shoots (Holguin et al. 2001) and could introduce new niches for nitrogen fixers in the systems they invade. Changes in the nitrogen-fixing microbial community may therefore not only be a consequence but also a mechanism by which invasions occur.

Invasive species have the potential to influence both the function and diversity of nitrogen-fixing microbes. Perturbation of microbial functions may occur independently or

in conjunction with shifts in microbial community composition, functional group and overall diversity. As patterns of microbial diversity are poorly understood (Fitter 2005, Torsvik and Ovreas 2002), studies of biological invasions potentially offer valuable insights to biotic and abiotic controls on microbial communities. In particular, the response of a key microbial functional group (diazotrophs) to invasions can reveal the role of diversity in conferring stability or resilience to an ecosystem function (nitrogen fixation).

This study addresses the following questions regarding effects of invasive species on nitrogen fixing microbes: (1) Do nitrogen fixation rates in invaded sediments differ from un-invaded sediments? (2) Does the diversity of nitrogen-fixing microbes in invaded sediments differ from un-invaded sediments? (3) Is there a relationship between nitrogen-fixer diversity (*nif*H T-RFs) and function (nitrogen fixation rates) in invaded ecosystems?

The impact of three invasive species (one mussel and two trees) on epibenthic nitrogen fixers were studied via mensurative experiments in southern Californian wetlands. The effect of an invasive mussel, *Musculista senhousia,* on nitrogen fixers in sediments of *Zostera marina* beds and of an invasive mangrove, *Avicennia marina,* in *Sarcocornia pacifica* (previously known as *Salicornia virginica*) marsh were studied in the Kendall Frost-Northern Wildlife Preserve (KF-NWP, Mission Bay). The effect of the salt cedar, Tamarisk (*Tamarix spp.*), on benthic nitrogen fixers was also examined in a *Sarcocornia pacifica*-dominated marsh of Tijuana Estuary. This range of systems enabled

comparison of whether the invasive mussel and the mangrove within the same wetland (KF-NWP) produced more similar effects on nitrogen-fixing microbes than two invasive trees, mangrove and tamarisk, invading different wetlands (KF-NWP and Tijuana Estuary).

Methods

Study sites

Northern Wildlife Preserve and Kendall Frost Marsh Reserve

The Northern Wildlife Preserve of the city of San Diego (CA) in Mission Bay includes 25 acres of coastal wetland habitats including *Spartina foliosa*-vegetated salt marsh, unvegetated mudflat, and *Zostera marina* seagrass beds and is conjoined with the University of California Kendall Frost Marsh Reserve (32^{o} 47'35' N, 117^{o} 13'00" W). The latter consists of 16 acres of salt marsh dominated by *Sarcocornia pacifica* and *Salicornia bigelovii* in upper elevations and *S. foliosa* at low elevations. Together, these protected areas constitute the last remnants of a wetland that once spanned the more than half of Mission Bay, prior to its transformation in the late 1940s to a recreational water park (City of San Diego, Parks and Recreation).

Two non-native species that have invaded different parts of the Mission Bay wetland are the focus of this study. First, the asian mussel, *Musculista senhousia*, has become extensively established within tidal flats and *Z. marina* beds of the Northern Wildlife Preserve. The mussel was first reported in Mission Bay in the 1960s and, reaching typical densities of 5,000 to 10,000 individuals m^{-2} (Morton 1974, Crooks and

Kim 1999), is a dominant species in the inter- and subtidal benthos of the bay (Crooks 1996, Crooks 1998). Secondly, the mangrove, *A. marina* has invaded upper salt marsh zones dominated by *Sarcocornia pacifica* in the Kendall Frost Reserve. This species was intentionally introduced to Mission Bay but was removed in the early 1980s in an effort to conserve native salt marsh habitat. In 2006, a resurgence of *A. marina* in the Kendall Frost Reserve was identified and is currently the focus of renewed removal efforts (Kay ESA abstract).

Tijuana Estuary National Estuarine Research Reserve

The Tijuana Estuary National Estuarine Research Reserve is located just to the north of the U.S.-Mexico border in Imperial Beach, California (32^{o} 34' N, 117^{o} 7' W). The reserve encompasses wetland, riparian, and upland transition ecosystems including 176 acres (71.2 hectares) of salt marsh (Zedler et al. 1992). Four species and 3 hybrids of Tamarisk have become established in salt marsh habitats along brackish streams (Whitcraft et al. 2007). Native to Eurasia and Africa, at least 7 species have become established in the U.S. since the early 1800s (Baum 1978, Di Tomaso 1998). Known to occupy at least 1.5 million acres of riparian and freshwater wetlands in the western U.S. (Steinquest 2000), the first report of this invader in salt marshes was in Tijuana Estuary, where it dramatically transforms salt marsh habitat structure by towering several meters above native vegetation (Whitcraft et al. 2007). Ideal germination conditions were thought to have been established by severe flood events in the 1980s that deposited sediments and lowered salinity in several portions of the estuary (Whitcraft et al. 2007).

Field Sampling and Experimental Design

Mussel (*Musculista senhousia)* invasion of seagrass (*Zostera marina)*

The effects of *M. senhousia* invasion on nitrogen fixation rates in *Z. marina*-vegetated sediments were addressed in a mensurative and a manipulative experiment.

Experiment A: M. senhousia-invaded versus uninvaded sediments (Mensurative comparison)

Nitrogen fixation (acetylene reduction) rates were compared in 6 paired sediment cores (4.8 cm diameter, 6 cm deep), sampled within intertidal *Z. marina* beds in the Northern Wildlife Preserve of Mission Bay during September 2004 that either contained or lacked visible *M. senhousia* byssus cocoons. These structures are formed by and encompass *M. senhousia* individuals. The paired sediment samples were extracted from within 1 m of each other, while sample pairs were randomly positioned along an approximately 30 m long transect, running parallel to the shoreline, 2 meters below the upper edge of the *Zostera marina* zone. Sample pairs were separated by at least 5 m intervals. Sediment cores were sealed and transported on ice to the laboratory where they were employed in acetylene reduction assays. In both experiments (A and B), seagrass biomass and mussel density (number of mussels found in sediment core samples) was measured in order to test relationships of these biotic factors with nitrogen fixation rates.

Experiment B: Mussel cocoons: impacts on activity and diversity of N fixers (Manipulative experiment)

To address mechanisms by which mussel presence, via the presence of byssus cocoons, might affect on nitrogen fixation rates, a second manipulative experiment was performed. This experiment tested the null hypothesis that removal of *M. senhousia* cocoons would not affect nitrogen fixation rates in vegetated sediments of *Z. marina*. In addition, the diversity of nitrogen-fixing bacteria was also compared in byssal cocoon material versus underlying *Z. marina* rhizosphere sediments.

Twelve pairs of *M. senhousia*- invaded sediment samples vegetated by *Z. marina* were collected in December 2004 from random coordinates along the same 30 m transect employed in Experiment A. Also, a third sediment core was taken at each coordinate for determination of porewater ammonium concentrations. These samples were transported on ice to the laboratory for manipulation and acetylene reduction assays. All sediment cores collected on this date contained visible mussel cocoons. Using forceps, all visible cocoons were carefully removed from one of the randomly-assigned cores in each pair. In each of the cores from which cocoons were *not* removed, forceps were used to mimic disturbance associated with mussel removal. Sediment cores, otherwise unperturbed, were extruded into 125 ml flasks and processed in acetylene reduction assays as described above. Plant and mussel biomass (dry weight) and mussel densities were determined for each sample.

Diversity of nitrogen fixers was also determined in a randomly selected samples of vegetated sediment or byssus cocoon material (7 sediment and 3 cocoon samples)

taken from the same transect in KF-NWP in December 2004. The 3 cocoon samples were paired with underlying sediment samples. All samples were kept on ice, returned to the laboratory and frozen at -80 ^{o}C until DNA extraction was performed.

Tamarisk invasion of *Sarcocornia pacifica*

Comparisons of nitrogen fixation rates in sediments invaded by the salt cedar Tamarisk with those vegetated by native salt marsh vegetation were conducted in a mensurative experiment following a randomized block design. Eight pairs of sediment cores (2.1 cm diameter, approximately 5 cm deep) were taken from salt marsh sediments in Tijuana Estuary during January 2004 and were extracted either immediately beneath native plants (*Juncus sp*. or *Sarcocornia pacifica*) or under canopies of Tamarisk plants (as detailed by Whitcraft 2007, p.80-89). Samples within each pair were located within 2 m of each other, and were used for determination of nitrogen fixation rates via acetylene reduction. Experimental blocks were separated by at least 15 m.

To compare the diversity of nitrogen fixers in Tamarisk-invaded and native salt marsh sediments, sediment cores (2 cm diameter, 1 cm deep) were sampled immediately adjacent to each of the sediment samples taken for determination of nitrogen fixation rates. These were placed on ice until returned to the laboratory where they were frozen at -80 ^{o}C until analyzed for diversity of nitrogen fixer diversity via TRFLP analyses.

Environmental data regarding abiotic sediment properties, light reduction by plants, and belowground plant biomass in the experimental blocks employed in this study were obtained via collaboration with Dr. Christine Whitcraft and Dr. Jeff Crooks (Whitcraft 2007). The next nearest date on which environmental data (sediment temperature, grain size, and belowground biomass) were collected was February 2004. In addition, the percentage of light reduction by plant cover, sediment water content, redox, and chlorophyll a content (a proxy for microalgal biomass) were also determined in September 2003 (Whitcraft 2007, p.80-89). Data from both dates were analyzed for relationships to nitrogen fixation rates and diversity of nitrogen fixing bacteria.

Mangrove *(A. marina)* invasion of *Sarcocornia pacifica* salt marsh

The effect of mangrove invasion on nitrogen fixation in *Sarcocornia pacifica*-dominated salt marsh was assessed during June 2006 in the Kendall Frost Reserve of Mission Bay, California. Sediment cores (4.8 cm diameter, 6 cm deep) were collected for acetylene reduction assays from 5 blocks established according to the distribution of the 5 total mangrove patches in the salt marsh. In each block, two sediment cores were taken beneath mangrove canopies (one immediately adjacent to a root), while a third core was taken adjacent to the base of the native, *Sarcocornia pacifica,* no more than 2 meters away. Two samples were taken from mangrove sediments in each block in anticipation of high variance in nitrogen fixation rates in invaded microhabitats. Immediately adjacent to each core extracted for determination of nitrogen fixation rates, 3 smaller cores (1.3 cm diameter, up to 5 cm deep) were sampled to measure nitrogen fixer diversity. To compare

the diversity of nitrogen fixers not only between native and invaded sediments but also between surface and subsurface sediments, these sediment cores were sectioned into 0-1 and 4-5 cm depth intervals prior to T-RFLP analysis.

For blocks in which mangrove-invaded sediments were sampled, environmental data were collected via collaboration with Dr. Amanda Demopoulos (USGS), including sediment redox, porewater salinity, pneumatophore densities, and percentage of light reduction by mangrove canopies. Porewater salinity was measured by analyzing water passed through disposable syringes containing Whatman filters onto a hand-held refractometer. Light reduction was determined by measuring light levels above and below mangrove canopies (at the sediment surface) with a light meter (Apogee Instruments)**.**

Acetylene Reduction Assays

In experiments with *M. senhousia* (1a and 1b), vegetated sediment cores were extruded into 125 ml flasks and sealed with rubber stoppers and electrical tape. To initiate assays, 15 ml of acetylene gas was injected into the headspace of each flask for just over 10% concentration (v/v). Flasks containing sediment samples from Mission Bay were placed in an incubator and kept at 22 ^{o}C with 12 h of exposure to light and 12 h of dark. Sub-sampling of the headspace in each flask was conducted upon initiation of the assay (injection of acetylene), and after 12 (light) and 24 hours (light plus dark) by withdrawing 2.5 ml of gas from the flask and storing it in N_2-flushed Vacutainers (Becton-Dickinson).

For samples from the mangrove-invaded marsh of Mission Bay, nitrogen fixation rates were determined with *in situ* incubations, in order to determine more natural activity levels. Samples were incubated in the marsh, in a tub of water that was flushed periodically to maintain temperatures below 22 ^{o}C. In this experiment, headspace samples were collected after only 2 hours, in attempt to more accurately reflect *in situ* nitrogenase activity, by allowing less time for changes in the microbial populations contained in each sample. Assays were conducted during the afternoon and samples were exposed to indirect natural sunlight.

In Tijuana Estuary, acetylene reduction procedures followed those used in studies of *M. senhousia* (detailed above), as logistical challenges prohibited *in situ* incubations. However, headspace gas was subsampled after only 2 hours following acetylene injection. All gas samples were analyzed on an FID-equipped gas chromatograph in peak height mode under conditions described by Capone and Montoya 2001.

Laboratory Analyses

After acetylene reduction assays were completed, plant material was separated from the sediment samples and collected into pre-weighed tins. The plant material (larger than 100 μm) was rinsed with water, dried overnight in a 60 ^{o}C oven and final weights were measured to determine plant biomass contained in each sample.

Pore water was extracted from sediment via centrifugation from sediment cores and ammonium levels were determined following standard methods (Strickland and Parsons 1968). These factors were examined for relationships with nitrogen fixation rates via linear regression analyses performed with JMP 4.0 software.

Terminal Restriction Fragment Length Polymorphism with *nif*H

In all experiments, the diversity of nitrogen fixers in these samples was examined via T-RFLP (terminal restriction fragment length polymorphism) with *nif*H primers, which were conserved throughout *nifH* genes in clusters I, II, III, and IV. (Zehr and McReynolds 1989, Zani et al. 2000). DNA was extracted from 0.25 g of cocoon material (experiment 1 only) or sediment (all experiments) using a Soil DNA Extraction kit according to the manufacturer's protocol (Mo Bio Laboratories). Amplification of the *nifH* gene was performed via nested PCR with FAM labeled inner primer (nifH1) following the protocol of Zani 2000. The PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit (TaKaRa) and digested at 37^{o} for 6 hours with the restriction enzyme *Msp* I. Fluorescence signals from terminal restriction fragments (T-RFs) were analyzed on a MegaBace 500 genetic analyzer. These data were obtained via collaboration with Dr. Pei-Yuan Qian at the Coastal Marine Laboratory of the Hong Kong University of Science and Technology.

Statistical analyses

Nitrogen fixation (acetylene reduction) rates and total community diversity of nitrogen-fixers, in terms of total average number of terminal restriction fragments, were compared between native and invaded sediments via paired-t tests, with significant one-tailed p values reported. In one case, tranformations could not produced normality among data distributions, and the non parametric signed-rank test was applied. For samples from mangrove-invaded marsh of the KF-NWP, diversity was compared via a two-way nested ANOVA (with depth as a factor nested within plant species). Data for the paired mangrove samples in each block were averaged prior to comparison with samples of *Sarcocornia* habitats. JMP 4.0 software was employed for all univariate statistical analyses.

For diversity analyses, T-RFLP profiles for each sample were transformed into binary character tables representing presence or absence of T-RFs of varied length, using the Genetic Profiler package (Amersham Biosciences). The total number of T-RFs observed in each sample was used as an indication of species richness of *nifH* gene. Richness (Margalef d), and the Shannon index for diversity (H') were determined from T-RFLP profiles of nitrogen fixing communities in each invaded and native habitat via Primer 4.0 software. Margalef's index (d) includes abundance in determinations of diversity, while the Shannon index (H') accounts for evenness plus richness.

The composition of nitrogen-fixing communities was examined, using the presence and absence of particular peaks within each sample, through Multidimensional Scaling

(MDS) analyses, performed with Primer 4.0 software. The Sorenson coefficient was used to calculate similarity matrices, and stress values below 0.20 generated an interpretable MDS pattern (Clarke and Warwick 1994). Through MDS, the similarity of T-RF composition is represented by spatial proximity of samples (shown as points) in 2-dimensional space. Dissimilarities between different sample treatments (native or invasive species, sediment depth) were tested for significance via ANOSIM, employing a Bonferroni correction in cases of multiple comparisons.

Relationships between environmental factors and the structure of nitrogen fixing microbial assemblages were examined via BIOENV analyses, using Primer 4.0 software, which test correlations for all possible combinations of environmental factors with community fingerprints. Through this technique, a fixed similarity matrix based on T-RF profiles is compared to several dissimilarity matrices calculated from multivariate environmental data using Spearman rank correlations. Results indicate the set of environmental variables which produce highest correlations with community fingerprints (Clarke and Gorley 2001). Environmental data were not available for each sample from which T-RF profiles were constructed, but the most complete possible subset was employed. For *M. senhousia*-invaded sediments, T-RF patterns were tested for relationships to plant biomass, porewater ammonium, nitrogen fixation (acetylene reduction) rates, mussel density, mussel biomass, and average mussel length (n=5) using BIOENV. In Mangrove-invaded sediments, T-RF patterns were tested for multivariate relationships to nitrogen fixation (acetylene reduction) rates, sediment redox, porewater

salinity, pneumatophore density, or percentage of light reduction (n=5). In Tijuana Estuary, T-RF profiles of sediments from both Tamarisk-invaded and native habitats (collected in 1/04) were tested via BIOENV for relationships to light reduction by plant canopies, sediment temperature, water content, redox, Chl a, and nitrogen fixation (acetylene reduction) rates (n=4) as determined in Fall 2003 when these data were most complete. Environmental data were log-transformed or arcsin-square root transformed (if percentages) prior to incorporation in BIOENV analyses.

Results

Musculista senhousia invasion of seagrass (*Zostera marina)*

Nitrogen-fixation rates measured in the mensurative experiment (A) were qualitatively higher in sediments containing mussels and their surrounding cocoons than in samples without *M. senhousia* (29 ± 11 μmol C_2H_4 m^{-2} h^{-1}, 11 ± 2.8 μmol C_2H_4 m^{-2} h^{-1}) although variances were unequal between the treatments and the difference was not significant (signed rank, $T_5 = 6.50$, $p=0.12$, one-way $p=0.06$). The trend of higher nitrogen fixation rates in the presence of mussels was hypothesized to be due to accumulation of organic matter associated with the mussel cocoons that could stimulate heterotrophic nitrogen fixation. This hypothesis was tested in experiment B via removal of mussels and their cocoons from samples. However, short-term rates of fixation were not significantly affected by the experimental removal of mussels from samples of *Z. marina*-vegetated sediment ($t_{11} = -0.57$, $p=0.58$).

A trend of greater nitrogen-fixer diversity, as determined by total average number of terminal restriction fragments (T-RFs), richness, and Shannon diversity, was found in seagrass-vegetated sediments (34 ± 2.1) than in samples of mussel cocoons (26 ± 3.0) (paired t-test, t_2=3.58, p=0.06; t_2=-3.54, p=0.07; t_2=-3.26, p=0.08, respectively) (Table III-1). However, the composition of nitrogen-fixing communities, as revealed by TRF profiles, did not significantly differ between sediments and mussel byssus cocoons (R=-0.005, p=0.45 ANOSIM). The average similarity among sediment sample TRF composition was 69 percent, while that among mussel cocoon material was 60 percent. The dissimilarity between these sample types however was only 33 percent.

No significant linear relationship was found between nitrogen fixer activity and diversity in *Z. marina*-vegetated sediments invaded by *M. senhousia*, but highest activity rates were found in samples with lowest nitrogen fixer diversity (Figure III-1A).

Relationships of microbes and mussels to environmental factors

There was a positive relationship between acetylene reduction rates and the average length of live mussels within each sediment sample assayed in experiment A (r^2=0.54, p=0.02) (Figure III-2). Plant biomass was also positively related to the number of mussels contained in each sample (r^2=0.70, p<0.01) and was higher in samples containing mussels than in those without them (t_3=-4.86, p=0.02).

Among all environmental data, nitrogen fixation (acetylene reduction) rates were most strongly correlated to nitrogen fixing community T-RF profiles in *M. senhousia*-invaded sediments (BIOENV ρ=0.419), followed by the combination of nitrogen fixation (acetylene reduction) rates and mussel density (ρ=0.371).

Nitrogen fixation rates were not related to plant biomass in either samples from which mussels had been removed (r^2=0.23, p=0.11) nor in samples containing mussels (r^2=0.16, p=0.19). There was no also significant relationship nitrogen fixation rates and porewater ammonium in samples with mussels or without mussels (r^2=0.14, p=0.78, r^2=0.34, p=0.52, respectively). Further, nitrogen fixation rates were not related to mussel biomass (p=0.13, r^2=0.21) or the number of mussels in each sample (r^2=0.18, p=0.17). Plant biomass did not differ in samples with mussels versus those from which mussels were removed (t_{11}= ⁻1.44, p=0.18).

Tamarisk invasion of *Sarcocornia pacifica*

Nitrogen fixation (acetylene reduction) rates of native vegetated sediments did not significantly differ from those of sediments invaded by Tamarisk (t_4=1.11, p=0.33). Nitrogen fixation rates in Tamarisk-invaded sediments were 6.1 $\pm$ 1.5 μmol C_2H_4 m^{-2} h^{-1} while those of native, *S. pacifica*-vegetated sediments were 3.1 $\pm$ 1.9 μmol C_2H_4 m^{-2} h^{-1}.

There was a trend of lower diversity among nitrogen fixers in sediments invaded by Tamarisk (21 $\pm$ 3.9 T-RFs) than in sediments vegetated by native plants (37 $\pm$9.9 TR-

Fs) (t_7=-1.58, p=0.08). The total average number of terminal restriction fragments (T-RFs) in native and tamarisk-vegetated sediments was 38 ± 10 and 21 ± 4, respectively. However, richness (t_7=-1.60, p=0.15) and Shannon diversity (t_7=-1.67, p=0.14) did not significantly differ between invaded and native sediments (Table III-1). The community composition of nitrogen fixers, reflected in terminal restriction fragment profiles, was heterogeneous, with only 30% similarity among *Sarcocornia pacifica*-vegetated sediments and 24% similarity among Tamarisk-vegetated sediments (SIMPER). Dissimilarity between community composition of nitrogen fixers in sediments occupied by either the native *Sarcocornia pacifica* or Tamarisk was 74% (SIMPER) but was not significant (R=0.009, p=0.37 ANOSIM).

A significant negative relationship was found between nitrogen fixation activity (in terms of acetylene reduction rates) and diversity of nitrogen fixers (total number of nifH TRFs), in combined native and invaded sediments (Figure III-1B). Most of the samples with the highest activity, and lowest diversity, were Tamarisk- invaded sediments.

Relationships of microbes and Tamarisk to environmental factors

Nitrogen fixation rates (in Feb. 04) were highest in plots with intermediate levels of light reduction by plant canopies, and although these latter data were only measured during the previous fall (in Sept. 2003), this non-linear relationship was significant

(Figure III-3). Light reduction by Tamarisk canopies did not differ from that by native plant canopies (t_5=-0.29, p=0.79).

Nitrogen fixation rates and diversity (T-RFs) showed trends towards a positive relationship with sediment temperature (Figure III-4A and B, respectively). However, diversity was only related to temperatures measured in Fall 2003 (excluding one outlier in native sediments,) when temperatures in Tamarisk invaded plots were lower than in uninvaded plots (t_3=3.47, p=0.04,) and were not related to contemporaneous temperatures (Winter 2003, r^2=0.13, p=0.29).

Diversity of nitrogen fixers (T-RFs) showed a non-linear trend with belowground plant biomass, such that plots with intermediate levels of biomass contained highest diversity, with Tamarisk-invaded sediments comprising the upper end of the curve (highest biomass, low diversity) (Figure III-5).

The T-RF profiles of nitrogen fixing microbes was most strongly correlated with the combination of temperature and benthic chlorophyll a concentrations, among all environmental data (from Winter 2003) (BIOENV ρ=0.636). The combination of these factors and sediment redox was the next strongest correlate with microbial community profiles (BIOENV ρ=0.616).

Mangrove invasion of *Sarcocornia pacifica*

Average nitrogen fixation rates of sediments inhabited by the invasive mangrove, *A. marina* were lower than those of native *Sarcocornia pacifica*-vegetated sediments (paired t-test, t_4=2.29, p=0.04). Nitrogen fixation was also much more variable and had higher maximum activity (81 μmol C_2H_4 m^{-2} h^{-1}) in native salt marsh sediments than in sediments sampled in mangrove-invaded habitats (11 μmol C_2H_4 m^{-2} h^{-1}) (Figure III-6). This variability was largely driven by one sample of *Sarcocornia pacifica*- vegetated sediment that had very high nitrogen fixation activity (81 μmol C_2H_4 m^{-2} h^{-1}) compared to the average activity in sediments among native vegetation (18 ± 15 μmol C_2H_4 m^{-2} h^{-1}). The block from which this sample was extracted contained very sulfidic sediment mixed with a white fibrous film that may have indicated the presence sulfur-oxidizing microbes. In this block, the sediment core extracted from beneath the mangrove, also displayed relatively high activity (11 μmol C_2H_4 m^{-2} h^{-1}).

The total average diversity of nitrogen-fixing microbes in *Sarcocornia pacifica*-vegetated sediments (number of T-RFs) did not differ from that of mangrove-invaded sediments at either 0-1 cm or 4-5 cm sediment depth intervals (t_4=1.23, p=0.28 for 0-1 cm and t_4=1.12, p=0.32 for 4-5 cm). The total average diversity of nitrogen-fixing microbes also did not differ between surface and subsurface sediment intervals of samples from native and invaded habitats (t_{14}=1.12, p=0.28). Richness, by Margalef's index and Shannon diversity did not differ between invaded and native sediments at either 0 to1 cm or 4 to 5 cm depths (Table III-1).

The composition of nitrogen fixing communities in native, *S. pacifica* habitat did not significantly differ from that of invaded, mangrove habitat (R=-0.05, p=1.0, ANOSIM). Nitrogen-fixing communities in mangrove- invaded sediments and those in *S. pacifica*-vegetated sediments were 44% similar within groups, while the two groups showed 59% dissimilarity in nitrogen-fixing community composition. No distinctions were found for the community composition of surface and subsurface samples collected in this study (R=0.08, p=0.10, ANOSIM, with Bonferroni-corrected α=0.025). Highest nitrogen fixation rates were found in sediments with intermediate nitrogen-fixer diversity (Figure III-1C).

Relationships of microbes and mangrove to environmental factors

The activity of nitrogen fixing microbes in mangrove-invaded plots showed negative relationships with sediment redox values (Figure III-7). Redox was also the environmental factor most strongly correlated to nitrogen-fixing community structure (T-RF profiles) among mangrove-invaded plots (ρ=0.685). The combination of nitrogen fixation (acetylene reduction) rates, redox, and percentage of light reduction by mangrove canopies was the next strongest correlate to nitrogen fixer community profiles (ρ=0.673).

Discussion

Understanding consequences of disturbances such as biological invasions for microbial diversity and function constitutes an important challenge in ecology (Fitter 2005, Torsvik and Ovreas 2002). In the three wetland systems examined, invasive species

(one mussel and two trees) were found to have variable effects, suggesting species- and site-specific influences on nitrogen-fixing microbial communities. Compared to sediments with native wetland plants, nitrogen fixation rates were marginally higher in *M. senhousia*-invaded sediments. However, nitrogen fixation rates were lower in mangrove-invaded sediments in Mission Bay, but they did not vary in Tamarisk invaded-sediments of Tijuana Estuary, compared to those of native salt marsh. Despite the lack of a general response, relationships of nitrogen fixers to key environmental factors enable discussion of potential mechanisms by which invasive species impact nitrogen fixation in each system.

Qualitative enhancement of nitrogen fixation rates in sediments with *M. senhousia*, compared to native *Z. marina*- vegetated sediments (experiment A), and correlations of T-RF profiles with *M. senhousia* density suggest that this invader affects structure and function of microbial communities. Impacts of the mussel on nitrogen fixation rates were possibly underestimated by the long periods and laboratory incubations of acetylene reduction assays. Nonetheless, observed patterns in nitrogen fixation rates are consistent with indirect stimulation of microbes by *M. senhousia* via increases in sedimentary organic matter and fine-grained sediments, providing more surface area for microbial colonization and activity (Crook and Khim 1999). Low densities of this mussel have been found to directly promote seagrass growth by such enhancement of organic matter (Reusch and Williams 1998) but these results suggest indirect stimulation of nitrogen fixation rates in *Z. marina* rhizospheres may also be

involved. The Manila clam, *Tapes philippinarum,* in the Adriatic Sea was similarly found to increase bacterial growth in sediments by production of faeces and psuedofaeces (Bartoli et al. 2001). However, manipulative experiments in Mission Bay have demonstrated that physical effects alone of the *M. senhousia* mat structures alter hydrodynamic properties of the benthos by which fine-grained sediments and organic matter are deposited (Crooks and Khim 1999). Greater structural effects of cocoons formed by larger mussels may thus drive the positive relationship observed between nitrogen fixation rates and lengths of live *M. senhousia* individuals in assayed sediments (Figure III-2).

In the mangrove-invaded salt marsh of KF-NWP, high spatial variability in nitrogen fixation rates contributed to patterns of lower activity in invaded versus native sediments. This variation likely reflects the patchy distribution of active diazotrophs in sediment and the environmental factors controlling them. As a single block with high nitrogen fixation rates drove the difference between invaded and native salt marsh sediments, rather than consistent disparities between these sample types, abiotic environmental controls seem to have dominated biotic influences of the invasive mangrove on nitrogen-fixing bacteria. In KF-NWP, nitrogen fixation rates were negatively related to redox (Figure III-7), which was also the strongest environmental factor correlating with the nitrogen-fixing community structure. Oxygen is known to inhibit nitrogenase activities (Capone 1988) and may also affect microbial community structure. Redox did not differ between surface sediments of *A. marina*-invaded and

native plots (A. Demopolous, unpublished data), but enhanced sulfide at sediment depths of 30 cm has been found to result from deforestation of mangroves in their native habitats (Sjoling et al. 2005); invasive mangroves may likewise influence conditions at deeper sediment depths.

In Tijuana Estuary, the lack of substantial differences in diazotroph activities of Tamarisk-invaded versus native sediments may have been due to overall lower nitrogen fixation rates compared to those from the mussel and mangrove systems in Mission Bay. Although this difference between wetlands may have been influenced by differences in incubation conditions for acetylene reduction assays (which were *in situ* for Mission Bay samples only), Tijuana Estuary more frequently suffers from sewage-based pollution that would be likely to increase exogenous nitrogen inputs (Zedler 1992) and inhibit nitrogen fixation rates (Zhaoyong et al. 2006). The positive relationship of bulk belowground plant biomass with nitrogen fixation (Figure III-5), possibly reflects benefit of heterotrophic microbes from plant root exudates (as in Whiting et al. 1986, Livingstone and Patriquin 1980). The pattern of highest nitrogen fixation rates at intermediate light levels (Figure III-3) implies a contribution of autotrophs to nitrogen fixation. Although activity rates were measured roughly 4 months following collection of light data, light patterns were consistent across seasons (Whitcraft 2007, p.80-89) and are thus relevant to nitrogen fixer activity and diversity. Mechanisms for reduced activity at highest light levels are unknown, but differences between plots in chlorophyll a concentrations (which were maximal in the plot with highest nitrogen fixation), temperature, or redox, may be

involved; the combination of these factors was most highly correlated to community structure of nitrogen fixers. Similarity of key environmental factors, except for temperature, between Tamarisk-invaded and native plots likely accounts for the similarity in their nitrogen fixation rates, and is consistent with previous work that found stronger environmental impacts of Tamarisk at lower marsh zones in Tijuana Estuary (Whitcraft 2007, p.80-89).

Nitrogen-fixing bacteria were relatively resistant to changes in community composition based on comparisons in invaded and native sediments in all 3 systems. Neither the diversity nor composition of nitrogen fixers differed between invaded and uninvaded sediments in Mangrove-invaded salt marsh. No compositional differences were observed between the Tamarisk-invaded and native marsh sediments or between invasive *M. senhousia* cocoons and underlying seagrass-vegetated sediment. The abundance of nitrogen fixers was not measured in this study and likely accounts for differences in microbial activity despite similar community composition. Invasive plants increased nitrification rates in California grasslands largely by increasing the abundance of ammonia-oxidizing bacteria, although community composition of this functional group was also altered (Hawkes et al. 2005).

Trends of lower diversity among nitrogen-fixing microbes (in terms of number of T-RFs) in Tamarisk invaded sediments of Tijuana Estuary and mussel cocoons in Mission Bay, compared to native sediments, suggest microbial diversity is not immune to

biological invasions. Reduced diversity in the Tamarisk-invaded sediments may involve effects of the invasive tree on sediment temperature, to which diversity was positively related (Figure III-4B) ; temperature has been found to be reduced in Tamarisk invaded portions of high salt marsh habitats (Whitcraft 2007, p.80-89). Trends of lower diversity among *M. senhousia* cocoons than in sediments were consistent across diversity metrics (Table III-1), despite being based on few samples (n=3), and may reflect greater niche diversity in sediments than in bysuss cocoons.

Subtle changes in the diversity of a microbial functional group such as nitrogen fixers may have substantial consequences for ecosystems. First, loss of diversity in nitrogen-fixing microbes may negatively affect temporal stability (functional redundancy) of nitrogen fixation (Tilman 1999), potentially uncoupling this nutrient source daily, seasonally, or on intermediate time scales from the demands of wetland primary producers and consumers. Although rates did not differ between native and invaded sediments on a single date in Tamarisk-invaded marshes, future studies can address this hypothesis by examining impacts of invasions over time. Secondly, nitrogen fixing microbes may differ from each other in terms of the other functions they perform within an ecosystem, as s autotrophic ones contribute to primary production while heterotrophic diazotrophs (i.e. sulfate-reducers) play key roles as decomposers. Diversity that is of little consequence to the function of nitrogen fixation may be significant to other biogeochemical (carbon or sulfur) cycles, reflecting the hypothesis, posed for salt

marsh plant communities, that diversity is required for maximal performance of multiple functions (Zedler et al. 2001).

Relationships between diversity and functional attributes of the nitrogen fixing community are just beginning to be unveiled. Maximal nitrogen fixation activity was observed in samples with minimal or intermediate diversity levels (Figure III-1), implying that a few dominant nitrogen fixers may be most active in these communities. This pattern counters notions of complementarity (as in Mc Kane et al. 2002), that would predict maximal activity at intermediate or high diversity. However, it is consistent with production patterns among salt marsh vascular plants which a few species dominate.

Despite their robust diversity and composition, differences in the activity rates of nitrogen fixers between native and invaded sediments in Mission Bay (with *M. senhousia* and *A. marina*) suggests that microbially-mediated functions are not immune to disturbance via invasion. This result reveals limits in the extent to which diversity confers functional redundancy among microbial communities, and highlights disconnects between the genetic potential and actual expression of nitrogen fixing genes in disturbed environments. Additional examination of nifH gene expression via reverse transcriptase PCR of nifH (as in Zani et al. 2000, Brown et al. 2003) and relative abundance via QPCR (Zehr et al. 2007) may further elucidate relationships between genetic diversity and function of nitrogen fixing microbes.

Impacts of the trees, *A. marina* and *Tamarisk* spp., on nitrogen fixers were not more similar to each other than to those produced by the invasive mussel, *M. senhousia*. Differences in physical habitat modifications, despite structural similarity of invaders, may be important for determining functional consequences of invasions. Of the two invasive trees, *A. marina* had reduced nitrogen fixation rates beneath its canopies, while *Tamarisk* spp. did not, possibly because benthic light availability was reduced by the former (A. Demopolous unpublished data) but not the latter tree species (Whitcraft 2007, p.80-89). Declines in autotrophic nitrogen fixation associated with such light reductions likely contributed to disparities between invaded and native salt marsh of KF-NWP. Species-specific differences in secondary chemistry of plants can also affect microbial community composition and associated ecosystem processes (Ehrenfield 2006).

Microbial response to invasions, though variable among systems, holds promise for mechanistically understanding functional changes induced by biological invasions. Despite environment- and species-specific effects of invasive species on nitrogen fixation, indirect habitat modification seems likely to be a common means by which invasions influence microbial community structure and function in wetland ecosystems. Specifically, nitrogen fixation may be particularly affected by modification of redox, physical substrate properties (byssus cocoon vs. sediment), or light availability, while relatively robust community structures may integrate ecological factors over longer time scales. Nitrogen fixation functions differed most between native and invaded habitats in systems where rates were higher, possibly reflecting influences of other anthropogenic

disturbances that varied between systems (nitrogen loading in Tijuana Estuary). Manipulative experimentation, including removal of invasive species, and application of tools that enable simultaneous examination of microbial diversity, function, and abundance will further reveal the roles microbes play in ecosystem response to disturbance.

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Figures

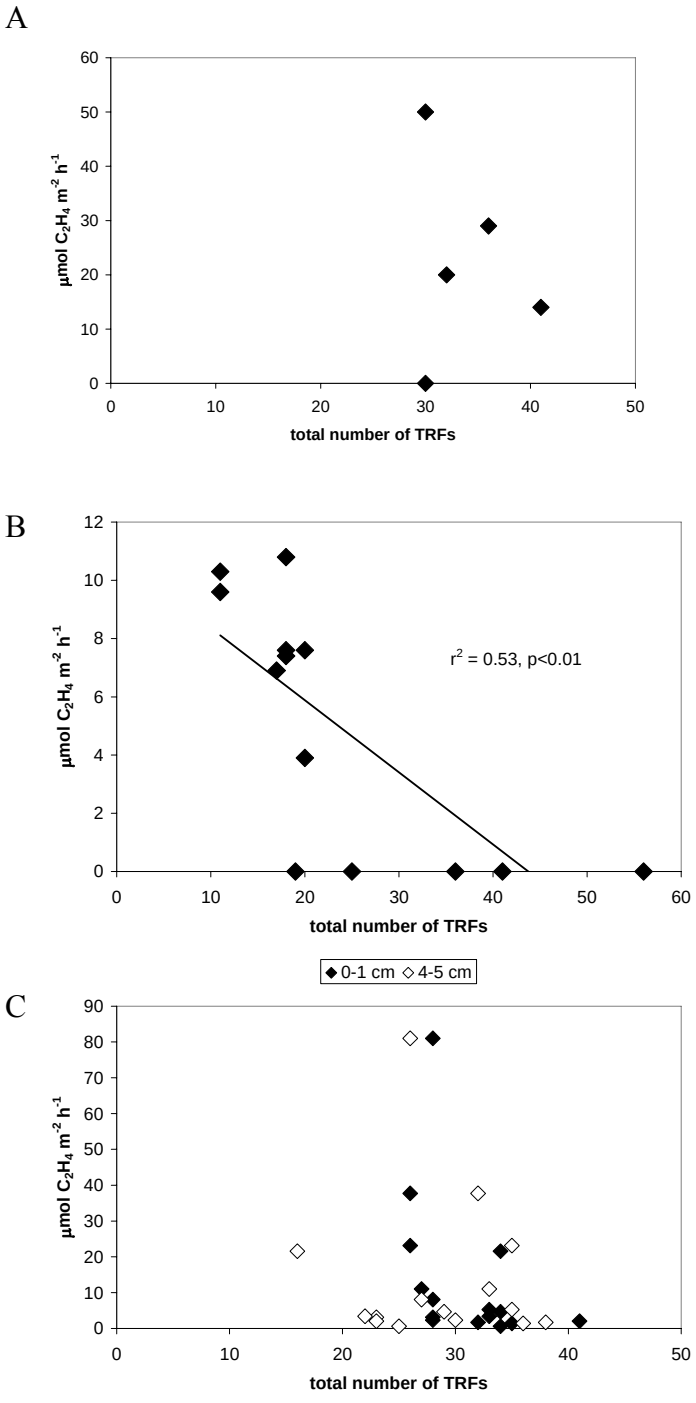


Figure III-1: The relationship between activity (micromole C_2H_4 m^{-2} h^{-1}) and diversity (total number of TRFs) of nitrogen fixing microbes in ecosystems invaded by a) *M. senhousia*, b) Tamarix spp. and c) *A. marina*

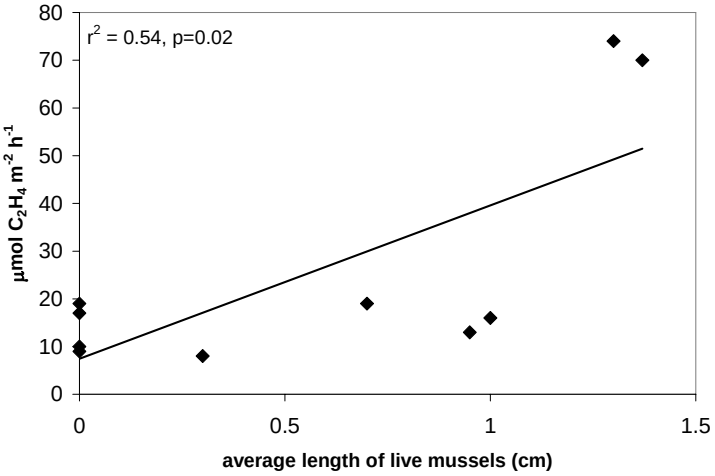


Figure III-2: The relationship between nitrogen fixation (acetylene reduction) rates and length of live *M. senshousia* mussel in the assayed sample (containing *Z. marina* vegetated sediment and mussels with byssus cocoons)

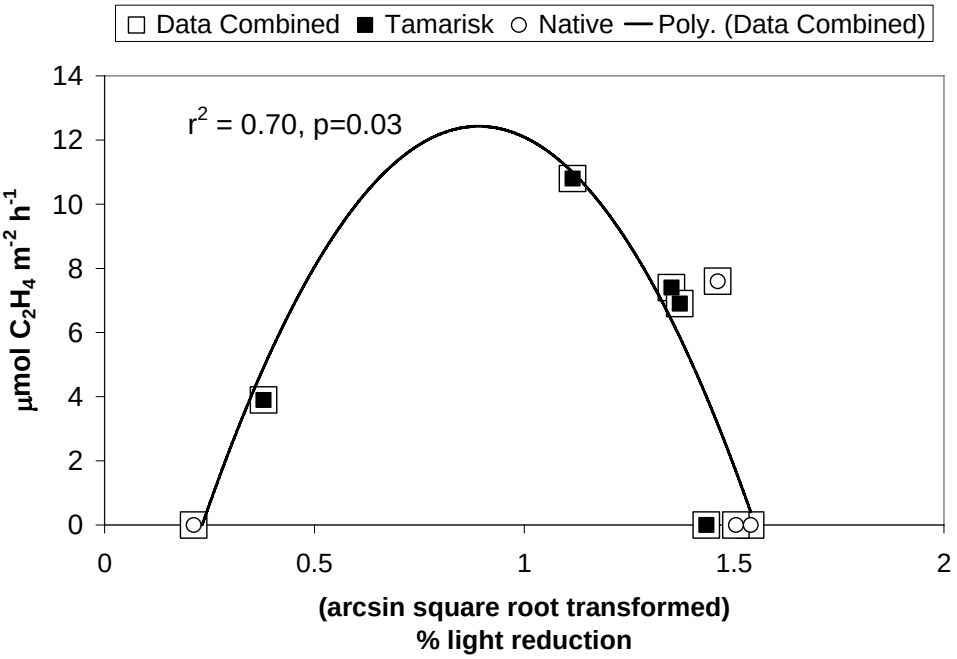
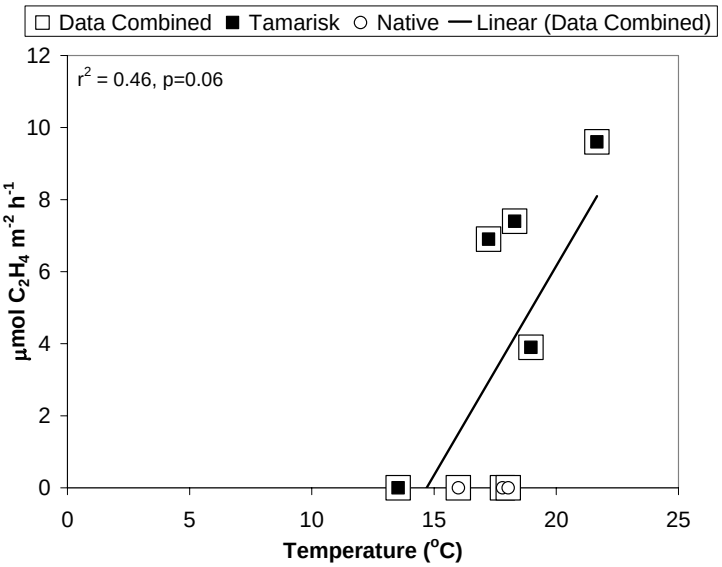


Figure III-3: The relationship between light reduction by plants (during Fall) and nitrogen fixation (acetylene reduction) rates (in Winter 2003)

A



B

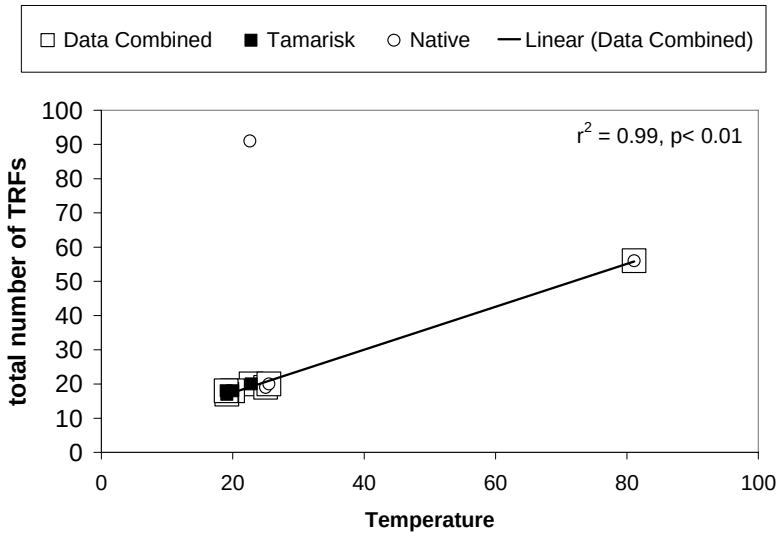


Figure III-4: (A) The relationship between activity of nitrogen fixers (acetylene reduction rates) and temperature of sediment of Tijuana Estuary salt marsh (Winter 2004) (B) Relationship between diversity (total number of TRFs) of nitrogen fixers in Winter 2003 and sediment temperature (determined in Fall 2003) in the Tijuana Estuary salt marsh
(excluding one outlier)

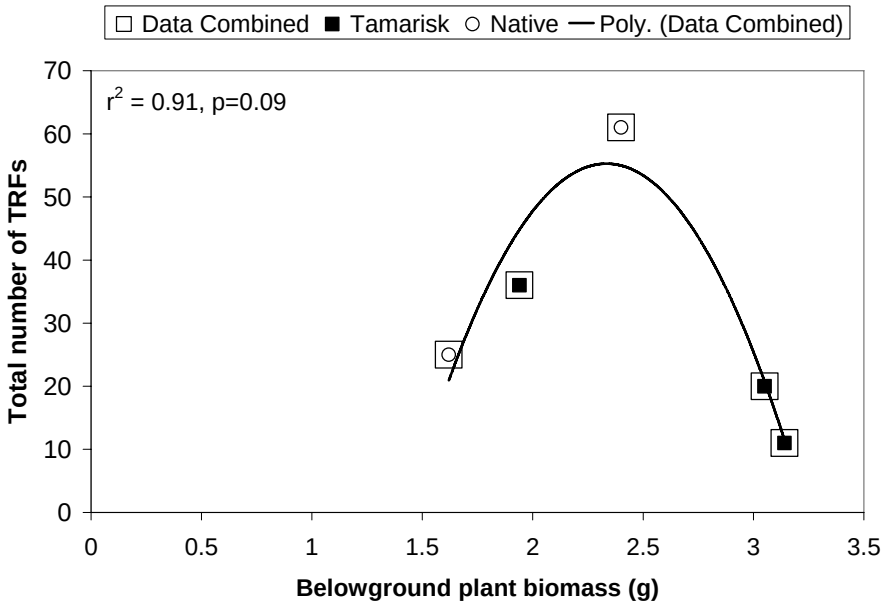


Figure III-5: Relationships of belowground plant biomass with diversity (total number of TRFs) of nitrogen fixers in Tamarisk-invaded salt marsh of Tijuana Estuary

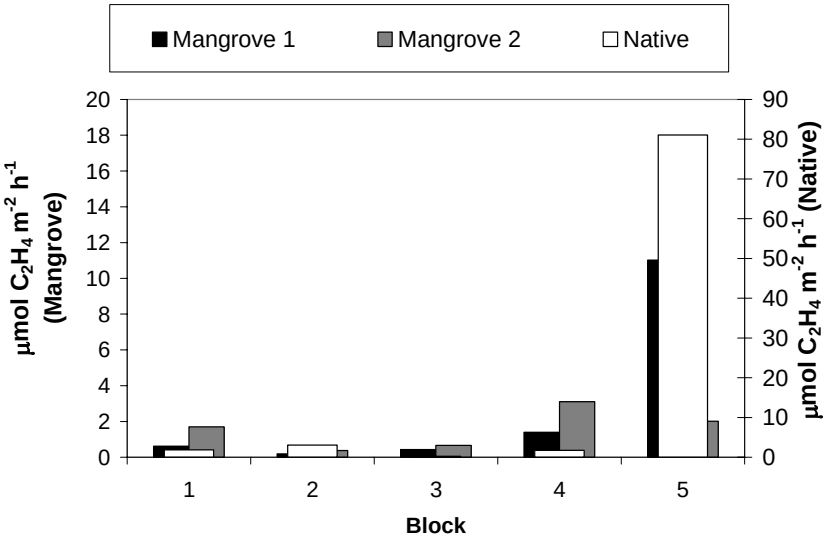


Figure III-6: Nitrogen fixation (acetylene reduction) rates of sediments in 5 blocks of paired mangrove (*A. marina*) plots and adjacent (single) plots with native *S. pacifica* vegetation in Mission Bay

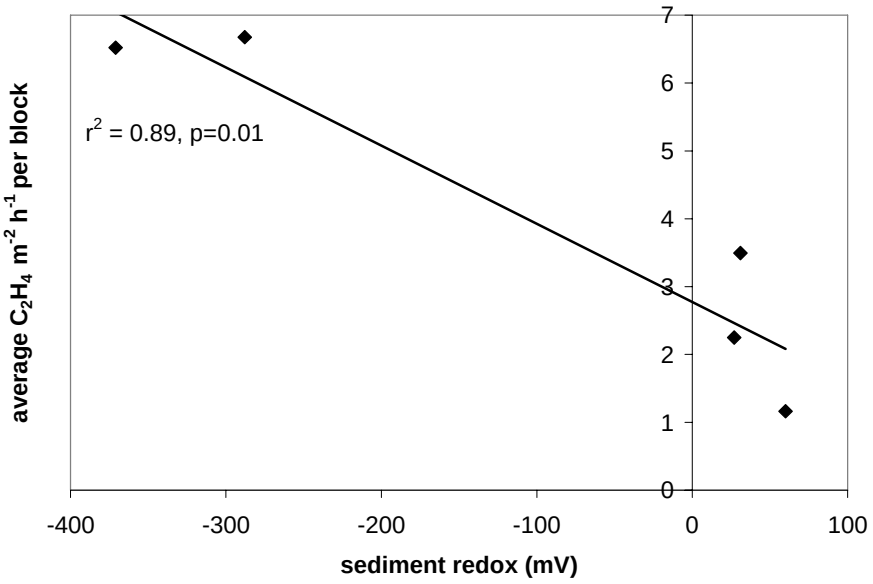


Figure III-7: The relationship between nitrogen fixation (acetylene reduction) rates and sediment redox in mangrove-invaded sediments of Mission Bay

Tables

Table III-1: Diversity and evenness indices for nitrogen fixing communities in habitats of the Kendall Frost-Northern Wildlife Preserve (KF-NWP) and Tijuana Estuary (TJE) based on T-RFLP profiles (averages with standard errors) * indicates trends of difference between habitats ($p < 0.08$)

Habitat	Richness (Margalef,d)	Shannon (log e, H')	Number of T-RFs
Native-NWP	9.3 ± 1.5*	3.51 ± 0.04*	26 ± 3.0*
Musculista-NWP	7.7 ± 3.0*	3.26 ± 0.11*	34 ± 2.1*
Native- TJE	9.84 ± 1.95	3.40 ± 0.25	37 ±9.9*
Tamarisk-TJE	6.58 ± 0.86	2.96 ± 0.17	21 ± 3.9*
Native- KF (0-1 cm)	8.39 ± 0.36	3.37 ± 0.05	29 ± 1.7
Mangrove-KF (0-1 cm)	8.18 ± 0.42	3.46 ± 0.06	32 ± 1.4
Native- KF (4-5 cm)	7.68 ± 0.74	3.23 ± 0.14	26.2 ± 3.4
Mangrove-KF (4-5 cm)	8.49 ± 0.50	3.38 ± 0.12	29.9 ± 1.7

CHAPTER IV[3]

SUCCESSION AND PLANTS DRIVE DIVERSITY-FUNCTION RELATIONSHIPS FOR NITROGEN-FIXING MICROBES IN WETLAND ECOSYSTEMS

Abstract

Relationships between microbial diversity and function may depend on ecosystem successional stages as well as inter-specific (across guild) interactions. We addressed the hypothesis that relationships between diazotroph diversity and nitrogen fixation vary with successional stage and season in coastal wetlands. Vascular plants were also hypothesized to influence the diversity and function of nitrogen fixers. Diazotroph diversity and function (nitrogen fixation) were compared biannually for 2 years, via genetic fingerprinting (T-RFLP with *nif*H) and acetylene reduction, in an early- and late successional *S. foliosa* marsh in Tijuana Estuary (CA). Nitrogen fixation rates were higher in the early successional marsh during Fall months of both years, possibly due to consistent differences in composition of diazotroph communities between marshes. However, diazotroph diversity was temporally dynamic, particularly in the early successional marsh, where significant reductions among rhizosphere sediments (4-5 cm deep) paralleled declines in aboveground biomass and shoot nitrogen content of *S. foliosa*. Complementarity, or enhancement of nitrogen fixation in microbial assemblages with greater diazotroph diversity, was observed only in the early succession marsh on one

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date among plant rhizospheres, suggesting that diversity-function relationships may be dynamic and context-specific. Nonetheless, positive relationships between diazotroph diversity in rhizosphere sediments and *S. foliosa* height and biomass suggest a ecosystem-level significance of microbial diversity, beyond that of the biogeochemical functions they mediate, via inter-specific interactions with vascular plants.

Introduction

Amidst rapid and widespread biodiversity losses, few consistent paradigms are emerging regarding consequent shifts in ecosystem functions (Cardinale et al. 2000, Baldanavera et al. 2007, Stachowicz et al. 2007). In studies of diversity-function relationships, ecologists have only recently begun to consider the significance of environmental contexts (Cardinale and Palmer 2002, Cardinale et al. 2000) and inter-specific interactions (Reynolds et al. 2003, Stachowicz et al. 2007) or to question applicability of current theories to microbes (Smith 2007).

Relationships between biodiversity and ecosystem functions can be organized into spatial and temporal categories. Positive effects of diversity on an ecosystem function (i.e. productivity) at one point in time have frequently been attributed to complementarity of resource use or niches among species (Loreau et al. 2000). Across time, diversity has been frequently hypothesized to maintain stability of ecosystem function against fluctuation, as a result of unique responses of different species to environmental change (the “insurance hypothesis”). These spatial and temporal phenomena require distinction

from sampling or "portfolio" effects, in which higher performing species are more likely to be found in more diverse communities (reviewed in Loreau et al. 2000, Stachowicz et al. 2007).

Diversity-function relationships may differ among distinct ecosystems on comparable spatial and temporal scales. Few cases have assessed diversity across realistic environmental gradients (Worm et al. 2006) or described diversity-function relationships under different disturbance regimes (Cardinale and Palmer 2002). Yet, relationships of biological diversity to ecosystem functions, particularly those linking plant and microbial communities, can depend strongly on environmental conditions (i.e. nutrient availability) (Zak et al. 2003). Inspection of the simultaneous responses of diversity and functional attributes of an ecosystem to disturbance can better constrain the environmental dependence of diversity-function relationships and ways they vary during succession.

Broader understanding of functional attributes of biodiversity may also require explicit consideration of roles of interspecific, micro-macrobe interactions. Ecosystem functions, including production, elemental cycling, trophic dynamics, and invasion resistance, are aggregate processes that result from interactions across microscopic and macroscopic biological assemblages. For example, plant interactions with symbiotic mycorrhizae have been found to change the form and strength of relationships between primary production and plant diversity (Klironomos et al. 2000). Plant-microbe

interactions may similarly affect diversity and biogeochemical functions of microorganisms (Gutknecht et al. 2006).

Diverse microbial communities may yield valuable insights regarding biodiversity-function relationships (Torsvik and Ovreas 2002) through their capacity for mediating ecosystem functional response to disturbance. Microbial diversity may particularly drive ecosystem function via influences upon vascular plants in disturbed and developing ecosystems. Positive interactions, such as nutritional mutualisms, between microbes and plants have been specifically hypothesized to predominate during early stages of ecosystem succession (Reynolds et al. 2003).

In coastal wetland ecosystems, which are frequently nitrogen-limited (i.e. Covin & Zedler 1988), nitrogen fixing microbes transform dinitrogen gas into biologically available forms through mutualistic associations with cordgrass rhizospheres. Diazotrophs (nitrogen fixers) can offer a rapid and significant source of nitrogen to cordgrass plants that is particularly important in developing or restored wetland ecosystems (Whiting et al. 1986, Tyler et al. 2003). However, these relationships may vary seasonally, matching plant growth patterns and associated demands for nitrogen (Whiting et al. 1986). Epibenthic diazotrophs like cyanobacteria may reside on surface sediments where they contribute to marsh stabilization through mat formation as well as productivity via photosynthesis and trophic support, particularly in developing ecosystems (Moseman et al. 2004). Thus nitrogen fixation rates may reflect broader

ecosystem-level functions of diazotrophs. In wetlands, the high "microdiversity" of diazotroph assemblages has been specifically hypothesized to confer stability to the function nitrogen fixation, although no known studies have tested this hypothesis (Bagwell & Lovell 2000).

This study addressed, for nitrogen fixing microbes in salt marsh sediments, the role of ecosystem successional stage and plant-microbe interactions as drivers of diversity- function relationships. We hypothesize that diversity- function relationships among diazotrophic communities vary between early and late successional wetlands. Positive interactions between microbes and plants were also expected to predominate in an early successional marsh stages. A mensurative comparison of nitrogen fixation rates and diazotroph diversity in an early and a late-successional salt marsh addressed the following questions: (1) Do the function (nitrogen fixation rates) and/or diversity of diazotrophs differ between early and advanced successional marshes? (2) Are the functions (nitrogen fixation rates) or diversity of (diazotroph) microbial assemblages in epibenthic (0-1 cm) or rhizospheric (4-5 cm deep) marsh sediments related to structural properties (height, biomass) of vascular plants, and do they vary across seasons (periods of plant growth or senescence)? (3) What are relationships between the diversity of nitrogen fixing microbes and the function of nitrogen fixation? (Functional redundancy, composition effects, and complementarity are specifically addressed.)

Methods

Nitrogen fixation rates and diazotroph diversity were compared between an early successional (5-6 year restored) and late successional (natural) *S. foliosa* salt marsh in Tijuana Estuary, San Diego, California on a biannual basis for two years. Although the lack of additional marsh replicates limits extrapolation of results beyond these two marshes (Hurlbert 1984), prior studies have shown these two marshes to be highly characteristic of early and late successional wetland ecosystems based on structural and functional analyses of animal and plant communities (Moseman et al. 2004, Zedler et al. 1992). Nitrogen fixation and diazotroph diversity (and plant and environmental factors) were measured in Fall and Winter of each year to contrast dynamics of plant-microbe interactions during periods of plant growth and senescence respectively. Marsh access was not permitted during nesting season of the endangered Lightfooted Clapper Rail in Spring and Summer months.

Sampling site description

The Tijuana River National Estuarine Research Reserve, in San Diego County (CA) consists of 200 ha of intertidal salt marsh dominated by Pacific cordgrass (*Spartina foliosa*) and pickleweed (*Salicornia (now Sarcocornia) virginica*) (Zedler et al. 1992). Sampling was conducted in the restored Friendship Marsh (32° 34'N, 117° 7'W), in the southwestern region of the reserve, that was opened to tidal inundation via excavation of historic fill material in February 2000. The 20 acre marsh consists of a lower zone dominated by *S. foliosa* and a higher zone by *S. virginica*. The nearest adjacent late

successional *S. foliosa* marsh, Oneonta Slough (32° 86' N, 117° 25' W), is thought to have formed in response to drowning of the Tijuana Estuary River Valley approximately 15,000 years ago (Zedler et al. 1992). A creek fed predominately by tidal influx runs through the middle of this marsh, with *S. foliosa* dominating in regions closest to the creek and *S. virginica* found in higher zones. This marsh has been cited as "naturally functioning" reference for other marshes in the region due to adequate cordgrass habitat for sustaining Clapper Rails and other higher trophic level species (Zedler et al. 1992).

Field collections

Sediment cores (2.1 cm, 6 cm deep) containing *Spartina foliosa* plants were collected for acetylene reduction assays (to measure nitrogen fixation), using corers constructed from disposable 30 ml syringes, on a biannual basis from the two salt marshes (described above) in Tijuana Estuary between February 2005 and September 2006. (Incubation temperatures ranged from 22-25 ^{o}C across winter and fall dates.) In Winter 2005, one vegetated core (2.1 cm diameter, 6 cm depth) was extracted at 12 equally spaced points (approximately 20 meters apart) along a transect within *Spartina foliosa* beds in each marsh. On subsequent dates, only 10 samples were collected per marsh in this manner, except during Winter 2006 when only 5 samples were collected from the early succession marsh due to rain.

For analyses of diazotroph diversity, 12 smaller sediment plugs (1.2 cm diameter, 6 cm deep) were extracted immediately adjacent to the root of *S. foliosa* plants within

0.25 m of those that were collected for acetylene reduction assays in Winter 2005. (On subsequent dates, the number of replicates was reduced to 10.) These were frozen at $^{-}80^{o}$C and sectioned (0-1 and 4-5 cm depth intervals) prior to analyses.

To characterize environmental factors in each marsh, one large sediment core (4.8 cm diameter, 6 cm deep) was extracted for determination of bulk sediment parameters (% organic matter, grain size), and an additional unvegetated sediment core (2.1 cm, approximately 6cm deep) was extracted for porewater ammonium and nitrite analyses, within 0.25 m of the vegetated sediment samples. These samples were collected on all dates except the first sampling period (Winter 2005). Plant properties (average height, density) were measured within plots (0.5m X 0.5m) positioned at sampling locations on each transect. In Fall 2006 only, a light meter (Apogee instruments) was obtained for determination of percentage of light reduction by *S. foliosa* canopies.

Terminal Restriction Fragment Length Polymorphism with *nif*H

Microbial diversity was determined via the genetic fingerprinting technique of T-RFLP (terminal restriction fragment length polymorphism) with *nif*H DNA (coding nitrogenase reductase) extracted from environmental sediment samples. The *nif*H primers are conserved throughout *nifH* genes in clusters I, II, III, and IV (Zehr & McReynolds 1989, Zani et al. 2000). DNA was extracted from 0.25 g sediment using a Soil DNA Extraction kit according to the manufacturer's protocol (Mo Bio Laboratories). Amplification of the *nifH* gene was performed via nested PCR with FAM labeled inner

primer (nifH1) following the protocol of Zani 2000. The PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit (TaKaRa) and digested at 37^{o} C for 6 hours with the restriction enzyme *Msp* I. Fluorescence signals from terminal restriction fragments (T-RFs) were analyzed on a MegaBace 500 genetic analyzer.

Acetylene reduction

To assess nitrogen fixation rates using acetylene reduction assays (Moseman 2007), intact plant samples (sediment cores containing plant roots to a depth of 6 cm, with attached stem and shoots) were extruded into 125 ml flasks. These flasks were sealed with rubber stoppers and gas-tight tape, and wrapped in aluminum foil (to prevent artificial temperature increases upon exposure to sun). Into the headspace of each flask, 15 ml of acetylene gas was subsequently injected. Sub-sampling of the headspace in each flask was conducted upon assay initiation (injection of acetylene), and after 2 and 4 hours by withdrawing 2.5 ml of gas from the flask and storing it in N_2-flushed Vacutainers (Becton-Dickinson). In 2005, additional time samples were taken at 6 and 20 hours to test for lags in activity (observed in winter). These samples were analyzed on an FID-equipped gas chromatograph in peak height mode under conditions described by Capone & Montoya (2001).

Laboratory Analyses

To measure biomass, plants were separated from root material, rinsed free of sediment and dried at 60 ^{o}C. Samples were then weighed to determine aboveground

biomass. Belowground biomass was determined by rinsing roots and plant material to remove sediment using a 2mm sieve, drying it overnight at 60 ^{o}C and weighing it. *S. foliosa* tissue nitrogen content was determined by rinsing live shoots, clipped from plants following acetylene reduction assays, with 5% hydrochloric acid and distilled water prior to drying for approximately 24 hours at 60 ^{o}C. Samples were then ground to a fine powder and analyzed for carbon and nitrogen content (by % weight) using a CHN elemental analyzer (Costec 4110). Porewater was extracted from sediment cores (2 cm diameter, 6 centimeter depth) through centrifugation, filtered with 0.45μm filters (Acrodisc), and analyzed for ammonium concentration via colorimetric techniques (Solorzano 1969). Porewater nitrite analyses followed protocol of Strickland and Parsons (1968). Combustible organic matter content and grain size percentages of sediments were determined via methods previously described (Moseman et al. 2004).

Statistical Analyses

Differences in nitrogen fixation rates between marshes and seasons were examined using a one-way ANOVA or t-tests. In the winter of 2006, when unequal numbers of samples were collected between marshes, differences in nitrogen fixation rates and environmental factors were tested using a Welch ANOVA (for unequal variances) or nonparametric 2 sample tests. Tests for normality were applied to all data and if normality was not established, then non-parametric tests were performed. Percentage composition data (organic matter, grain size data) were arcsine-square root transformed prior to statistical analysis. Relationships between nitrogen fixation rates and

plant or environmental parameters and porewater nutrients were analyzed using linear regression models. Both linear and quadratic regression analyses were applied to test relationships of diazotroph diversity to nitrogen fixation rates (specifically complementarity).

Patterns of diazotroph diversity were compared between early and late succession marshes, seasons, and sediment depths (0-1 cm vs. 4-5 cm) via t-tests, ANOVAs, and paired t-tests, respectively. Diversity, or richness, was measured as the total average number of restriction fragments (T-RFs). Shannon (H') and Margalef's d indices were also calculated from T-RFLP profiles among marshes, seasons, and sediment depths within marshes using DIVERSE analyses with Primer 4.0 software. Relationships between diazotroph richness (number of T-RFs) and plant and environmental factors were explored with linear regression analyses in JMP 4.0.

To assess mechanistic hypotheses for diversity-function relationships, including functional redundancy and composition effects, diazotroph community composition (profiles of T-RF identity) was compared between marshes and seasons at each sediment depths using nonmetric Multidimensional scaling (MDS). Percent similarities and dissimilarities among treatments were studied via SIMPER, while tests for significance of differences were performed with two factor ANOSIM tests (with marsh and season or season and sediment depth as the specified factors). For multiple comparisons, significance levels of alpha were Bonferroni-adjusted. Multivariate analyses, MDS,

ANOSIM, SIMPER, and BIOENV, were performed with Primer 5.0 software (Clarke & Warwick 2001). To test stability-diversity relationships, spatial and temporal stability in nitrogen fixation rates were determined via calculation of coefficients of variation (CV), where CV=100 X (standard deviation/mean), following Tilman (1999).

Results and Discussion

Function and diversity of diazotrophs across marsh successional stage and season

Nitrogen fixation rates were significantly higher during Fall 2005 in the early-successional (5 year old) than in the late- successional marsh (t_{18}=-2.67, p=0.02), and a similar trend persisted the following Fall (t_{17}=-1.57, one-way p=0.07; Figure IV-1). In the early successional marsh, nitrogen fixation rates were significantly lower during winter months of both years than during the fall ($F_{3,31}$=6.04, p=0.002). In the late successional marsh, a trend of higher activity in fall than winter was found only during 2005 (t_{20}= ˉ2.011, p=0.06).

Seasonal changes in nitrogen fixation rates broadly reflected temporal patterns of plant growth in each marsh. *S. foliosa* height and aboveground biomass increased significantly from winter to fall of each year in the early successional marsh but remained significantly lower than in its late successional counterpart (Table IV-1). In the late successional marsh, *S. foliosa* biomass (aboveground: t_{19}= ˉ2.57, p=0.02, total: t_{19}=ˉ2.45, p=0.02) and height (t_{20}=-3.55, p<0.01) were significantly greater in the Fall than the Winter during 2005 but not 2006.

Greater nitrogen fixation rates in the early succession marsh than its late succession counterpart (during Fall seasons) are consistent with higher nitrogen demands and smaller pools of reduced nitrogen that are typical of developing or disturbed marsh ecosystems (Tyler et al. 2003, Piehler et al. 1998, Currin et al. 1996). Lower porewater ammonium levels in the restored marsh during growing seasons (Table IV-1) as well as persistent disparities in *S. foliosa* biomass and height (Table IV-1) are also consistent with less developed nitrogen reserves compared to a natural marsh ecosystem (Langis et al. 1991, Covin & Zedler 1988). In addition, inhibition of the nitrogenase enzyme by ammonium (Yoch & Whiting 1986) or decreased light availability due to more developed plant cover may have contributed to its lower nitrogen fixation rates in the late successional marsh.

Significant temporal dynamics in diazotroph diversity (measured as total number of T-RFs) were also observed (Figure IV-2), particularly in the early succession marsh but not over seasonal cycles. Despite comparable annual nitrogen fixation rates, total average diversity (number of T-RFs) of diazotrophs in the early-successional marsh among rhizosphere sediments decreased significantly from 2005 to 2006 (4-5 cm: $F_{3,33}$=3.88, p=0.02), with similar trends of decline in surface sediments (0-1 cm: $F_{3,34}$=2.14, p=0.11; Figure IV-2). There were no seasonal or interannual differences in diazotroph diversity in the late-successional marsh (4-5 cm: $F_{3,40}$=1.55, p=0.21, 0-1cm: $F_{3,38}$=0.84, p=0.48, Figure IV-2).

The maintenance of high fixation rates in the early succession marsh over time (between Fall 2005 and Fall 2006, Figure IV-1) despite dramatic declines in diversity (Figure IV-2) suggests that shifts in composition of microbial communities had more functional significance than those in diversity. Composition of diazotroph communities (who fixes nitrogen at a given place and time) was assessed via comparison of the identity of T-RF profiles. Compositional disparities among diazotroph communities reflected differences in nitrogen fixation functions between marshes during Fall 2005 and Fall 2006 (2005: Global $R=0.931$, $p<0.01$, 2006: Global $R=0.901$, $p<0.01$, Figure IV-3). Significant compositional differences were also found between seasons and sediment depths (Table IV-4).

Strong functional roles of community composition have been frequently observed among macroscopic species in other systems (Bruno et al. 2006, Wedin & Tilman 1990, Vitousek et al. 1987). However, species composition is linked to diversity via sampling effects (the increased probability of including a functionally dominant species in assemblages of higher diversity). To distinguish roles of microbial community composition from sampling effects, in linking diversity to function, combinations of techniques that evaluate the relative abundance of nitrogen fixers (Q-PCR with *nif*H) and identify active nitrogen fixers (i.e. reverse transcriptase PCR) may be useful.

Surface (0-1 cm) and subsurface sediments (4-5 cm) consistently differed in diazotroph (T-RF) composition within the late succession marsh (Table IV-4B), but in the early succession marsh, distinctions between surface and subsurface asssemblages

were observed only during 2005 (Table IV-4B), prior to significant diversity declines (Figure IV-2). Niche differentiation between surface and subsurface communities likely requires time for establishment in early succession marshes. The persistence of greater *S. foliosa* biomass, height, and light reduction in the late succession marsh by Fall 2006 (Table IV-1) suggests there was a general lack of structural development of both plant and microbial assemblages within the early succession marsh.

Relationships of function and diversity of diazotrophs to vascular plant structures

Reflecting similar temporal changes in nitrogen fixation rates, both height and biomass of *S. foliosa* in the early succession marsh showed pronounced increases from winter to fall in 2005 (height: t_9=6.80, p<0.01; biomass: t_9=6.16, p<0.01) but not in 2006 (height: t_4=1.99, one-way p=0.06; biomass: t_4=0.39, p=0.72). In contrast, no such relationships were found in the late succession marsh between nitrogen fixation rates and plant properties.

Although temporal dynamics of microbial functions and community structures may simply reflect abiotic differences in environments between seasons, parallel declines after Fall 2005 in microbial diversity of *S. foliosa* rhizospheres and *S. foliosa* aboveground biomass and tissue nitrogen content in the early succession marsh (Figure IV-2, IV-4B) did not show simple seasonal variability and further support a plant-microbe link. Temporal changes in diversity of rhizosphere diazotrophs also paralleled dynamics of sediment organic content (Figure IV-4A), which decreased from 2005 to

2006 but increased from winter to fall of 2006. Notably, in the late succession marsh, where rhizosphere diazotroph diversity was maintained over time, an increase in nitrogen content of *S. foliosa* shoots from Fall 2005 to Fall 2006 (t_7=10.17, $p<0.01$) countered the change observed in the early successional marsh during this period. These dynamics suggest that the diversity of diazotrophs in subsurface sediments may decrease as properties of salt marsh plants decline in response to disturbance.

Within each season, relationships between nitrogen fixation rates or diazotroph diversity and vascular plant properties of the marshes in Tijuana Estuary were variable (Tables IV-2,3). Some initial positive relationships were found between nitrogen fixation rates and plant biomass or height in both marshes (Figure IV-5A and B). Significant positive relationships were observed more frequently between diversity of diazotrophs in rhizosphere sediments (4-5 cm) and *S. foliosa* height and biomass in the early- compared to the late successional marsh (Table IV-3). These results are consistent with ecological theories that predict maximal facilitation under conditions of environmental stress (Bruno et al. 2003). Positive interactions between nitrogen fixing microbes (activity rates or diversity) and salt marsh plants may also have been weaker in the late succession marsh as a result of higher porewater nitrogen reserves or lower light availability (Tables IV-2, IV-3).

Spatial differences in diazotroph composition (Table IV-4B) and diversity (Figure IV-2) between surface and rhizosphere sediments further suggest an influence of plants

on microbial communities. Positive relationships between diazotroph diversity in *S. foliosa* rhizospheres and *S. foliosa* structural properties in the early successional marsh (Table IV-3) were not observed for diazotrophs in surface sediments. These results are consistent with previous descriptions of mutualistic interactions between salt marsh plants and rhizosphere diazotrophs, including observations of highest nitrogen fixation rates in seasons of plant growth (Wolfenden & Jones 1987) and tight coupling between plants and microbes via carbon and nitrogen exchanges (Bergholz et al. 2001, Whiting et al. 1986). Positive relationships between plant structural properties (height, biomass) and diazotroph diversity (Figure IV-5A,B) in the early succession marsh are particularly significant in southern California, where marsh restorations are increasingly required and tall *S. foliosa* canopies are critical for provision of nesting habitat for the federally endangered Light-footed California Clapper Rail (Zedler 1992).

Relatively few studies have tested effects of plants on diazotroph community structures. Experimental clipping of *S. alterniflora* shoots did not affect diazotroph communities as determined by denaturing gradient gel electrophoresis (Piceno & Lovell 2000). However, trends of higher nitrogen fixation rates in surface sediments of a *S. foliosa* marsh of Mission Bay (CA) were found where plants were repeatedly clipped (S. Moseman, unpublished data). Also, diazotroph communities were found to differ when compared between rhizospheres of field- collected and lab-cultured salt marsh plants (Chelius & Lepo 1999). Further, nitrogen- fixing microbes have been known to show plant-host specificity (Bagwell et al. 2001), suggesting major differences in plant communities can alter composition of microbial assemblages.

Diversity- function relationships among nitrogen fixing microbes

Diversity-function relationships were separately examined across marshes of different successional stages and seasons to test their context dependence. As distinct diversity patterns were observed in surface and rhizosphere (subsurface) sediment communities, relationships between diversity and function were also analyzed separately according to sediment depth (0-1 vs 4-5 cm).

Diversity-function relationships varied over time (both season and year) and space (between marshes) (Figure IV-6), highlighting a key role of environmental context. Major ecosystem functions often display pronounced seasonal variation (nitrogen fixation, primary production) (this study, Boyer et al. 2001), and when functional variation is more pronounced than that of the diversity to which it is related, diversity-function relationships may not remain consistent over time.

Functional redundancy

The hypothesis that microbial diversity confers functional redundancy was not directly applicable in this study due to temporal dynamics of diazotroph diversity.

Nonetheless, the ability for distinct diazotrophs to maintain function (nitrogen fixation rates) over time was evaluated between fall seasons in the early successional marsh (because nitrogen fixation rates were comparable on these dates) by testing for temporal

shifts in community composition (Figure IV-7). Significant compositional shifts were observed within both surface (ANOSIM Global $R=0.473$, $p<0.01$) and subsurface sediments in Fall 2005 and 2006 (ANOSIM Global $R=0.42$, $p=0.01$, Figure IV-7), suggesting that distinct microbes may maintain nitrogen fixation at different times in a given environment. Similar patterns were observed in the late successional marsh (data not shown). These results offer some support the insurance hypothesis, specifically posed for wetland diazotrophs (Bagwell & Lovell 2000).

Complementarity

Few strong linear relationships (and no significant non-linear relationships) were observed between diazotroph diversity and nitrogen fixation rates in either marsh (Figure IV- 6A, B). Nonetheless, in Fall 2005, some support for complementarity was observed in the early succession marsh (Figure IV-6B), as highest nitrogen fixation rates were measured in sediments with the greatest diversity of rhizosphere diazotrophs. This relationship was largely driven by a single point (representing the sample with the highest diazotroph diversity in this study), and complementarity was not observed on subsequent dates. Absence of a similar relationship in surface diazotroph assemblages suggests that subsurface communities may have played a more prominent role in actively fixing nitrogen than those in surface sediments during this period of plant growth, as observed in seagrass beds (McGlathery et al. 1998). The observation of a link between diversity and function only in the early succession marsh may also support suggestions that disturbance may increase the importance of diversity, as observed in stream mesocosms

(Cardinale & Palmer 2002). For microbial communities, in which small-scale spatial differentiation may have larger-scale biogeochemical consequence, particular attention must be paid to identifying hot spots of activity and context-specificity of diversity-function relationships.

Diversity-stability relationships

No strong evidence was found to link diversity of nitrogen fixing microbes to temporal stability of nitrogen fixation rates. Temporal variability in microbial composition and diversity may be a unique challenge for relating microbial diversity to ecosystem function. In this study, diversity-stability relationships could only be analyzed on the temporal scales in which diversity itself did not vary (Table IV-5, Figure IV-2). On annual time scales, greater temporal stability in nitrogen fixation rates was observed in the marsh with greater total diazotroph diversity (Table IV-5), although the identity of the marsh with greater diversity varied. Moreover, differences in average diversity (sum of surface and subsurface sediment diversity) between late and early succession marshes were minor or not observed (Table IV-5 rows 1, Figure IV-2) in contrast to large distinctions in magnitude and stability of nitrogen fixation rates (Table IV-5, rows 2 and 3, Figure IV-1).

Broader functional consequences of microbial diversity

The functional significance of diazotroph diversity for plant-associated ecosystem functions, not just nitrogen fixation, was a novel finding. Positive links between

rhizosphere diazotrophs and aboveground *S. foliosa* biomass (Figure IV-5B, Table IV-3) or *S. foliosa* height (Figure IV-5A, Table IV-3) are not known to have been previously described. Diazotroph diversity may have broad functional consequences in wetland ecosystems for habitat provision, through such relationships to plant height, as well as for primary production, stabilization, and water purification function of wetlands that can be attributed to plant biomass (Levin et al. 2001). The positive relationships between diazotroph diversity and *S. foliosa* height were specific to a single date when environmental conditions were most similar across marshes (Fall 2005), perhaps resulting in common observation of this diversity- function relationship in both systems (Figure IV- 5A). Mechanisms underlying these relationships may involve complementarity between nitrogen fixation rates and diazotroph diversity, also observed in Fall 2005 (Figure IV-6B), or longer-term links between nitrogen fixation and plant structure. Diazotroph diversity and plant height or biomass may also have covaried with another environmental factor although none of the ones examined in this study (ammonium, sediment organic content, grain size) fit that pattern. These relationships between diazotroph diversity and plant-related functions combine the ecological concept that the functional significance of biodiversity reflects interspecific (plant-microbe) interactions (Klironomos et al. 2000) with the possibility that diversity of a given taxon or guild may affect multiple ecosystem functions (Zedler et al. 2001, reviewed in Stachowitz et al. 2007) and transition theories of diversity-function relationships into microbial realms.

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Chapter IV, in full, has been submitted for publication and thus may appear in Aquatic Microbial Ecology, as: Moseman, Serena M.; Johnson, Rebecca; Zhang, Rui; and Pei Yuan Qian. *submitted.* Succession and plants drive diversity- function relationships for nitrogen-fixing microbes in wetland ecosystems.

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Figures

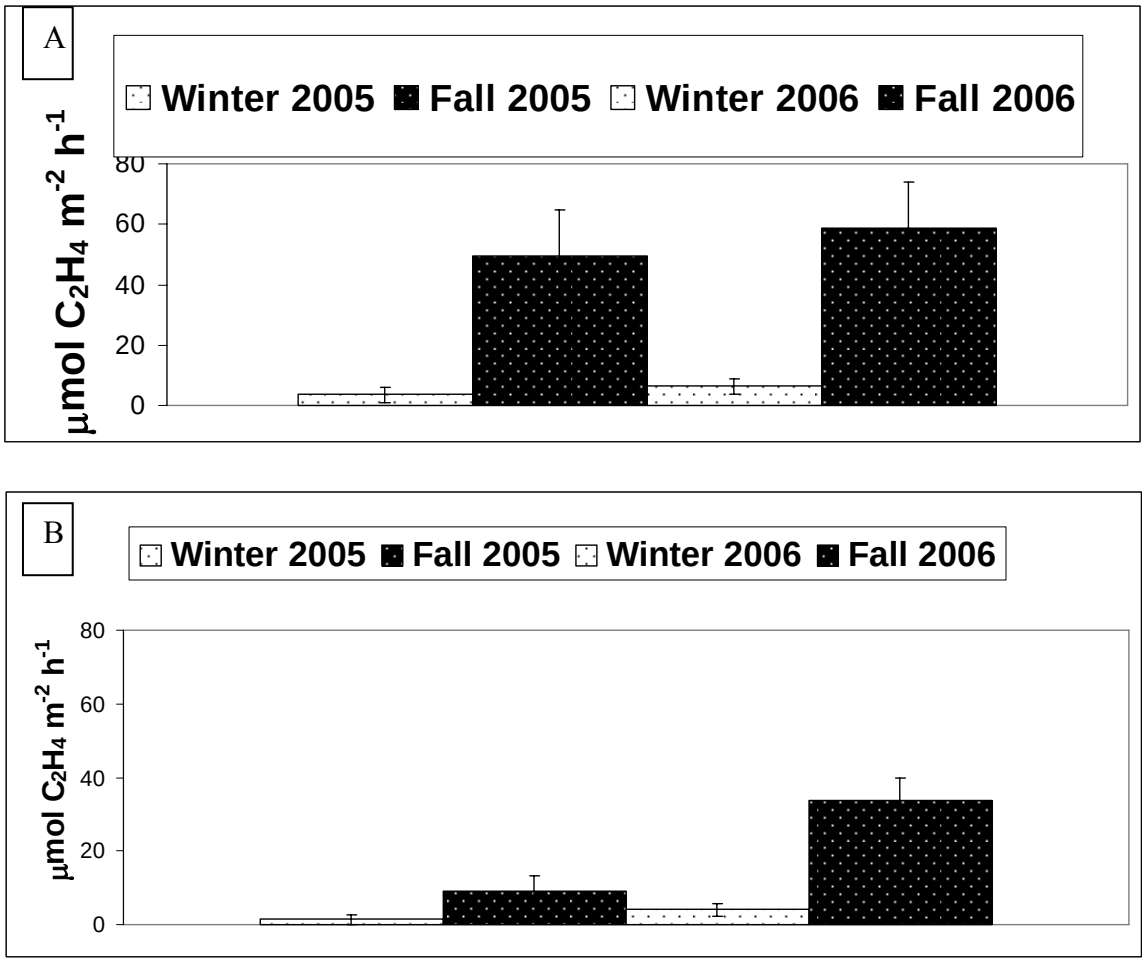

Figure IV-1: Average nitrogen fixation (acetylene reduction) rates of *Spartina foliosa*-vegetated sediment cores (± 1 standard error) from (A) the early succession (Friendship) marsh and (B) late succession marsh (Oneonta Slough) of Tijuana Estuary in 2005-2006

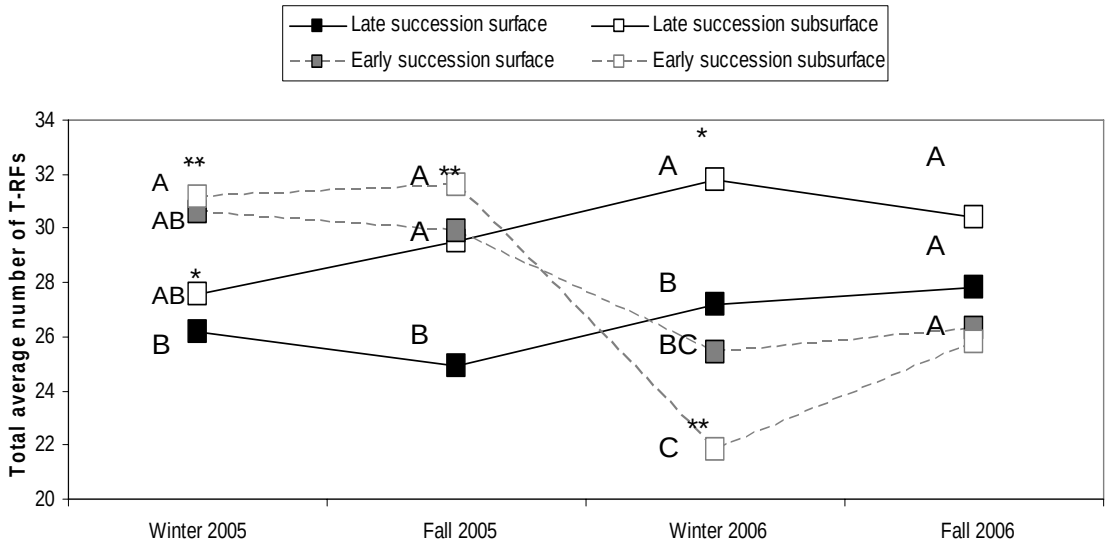

Figure IV-2: Temporal patterns of average diazotroph richness (with standard error bars) in surface and subsurface sediments of the natural and restored marshes in Tijuana Estuary between 2005 and 2006; Letters indicate significant differences within a season between marshes and depths; stars denote significant temporal differences within marshes (*= Late succession, **= Early succession)

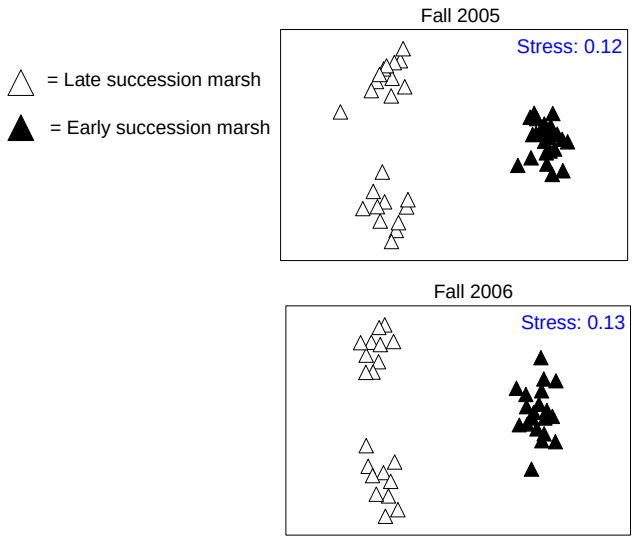

Figure IV-3: Multidimensional scaling plot representing differences in rhizosphere (4-5 cm deep) diazotroph community composition between marshes in Fall 2006 (surface communities are not shown but displayed similar patterns)

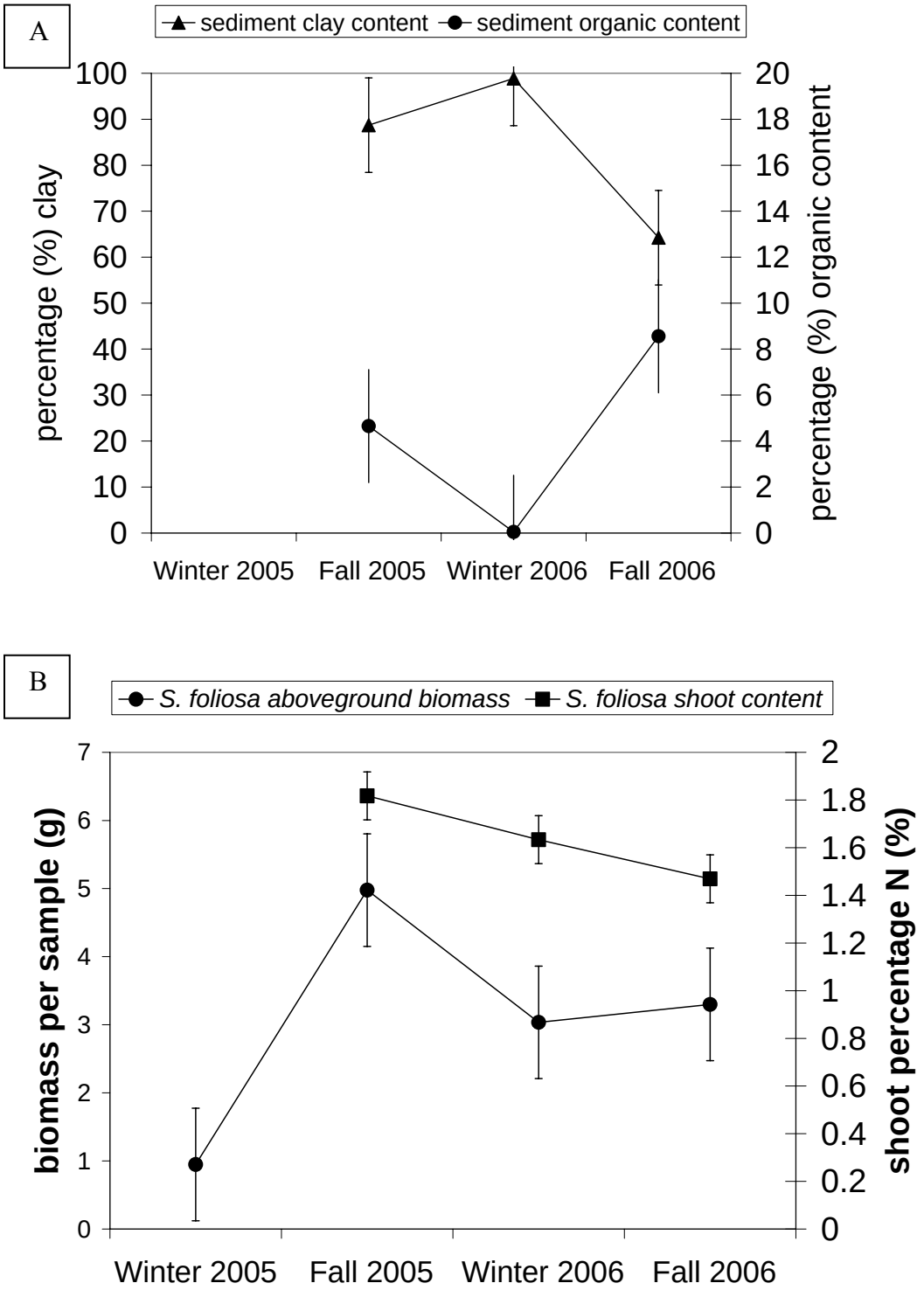

Figure IV-4: Temporal changes in selected (A) sedimentary and (B) *Spartina foliosa* factors of the restored Friendship Marsh in Tijuana Estuary between 2005 and 2006 (averages are plotted with standard error bars)

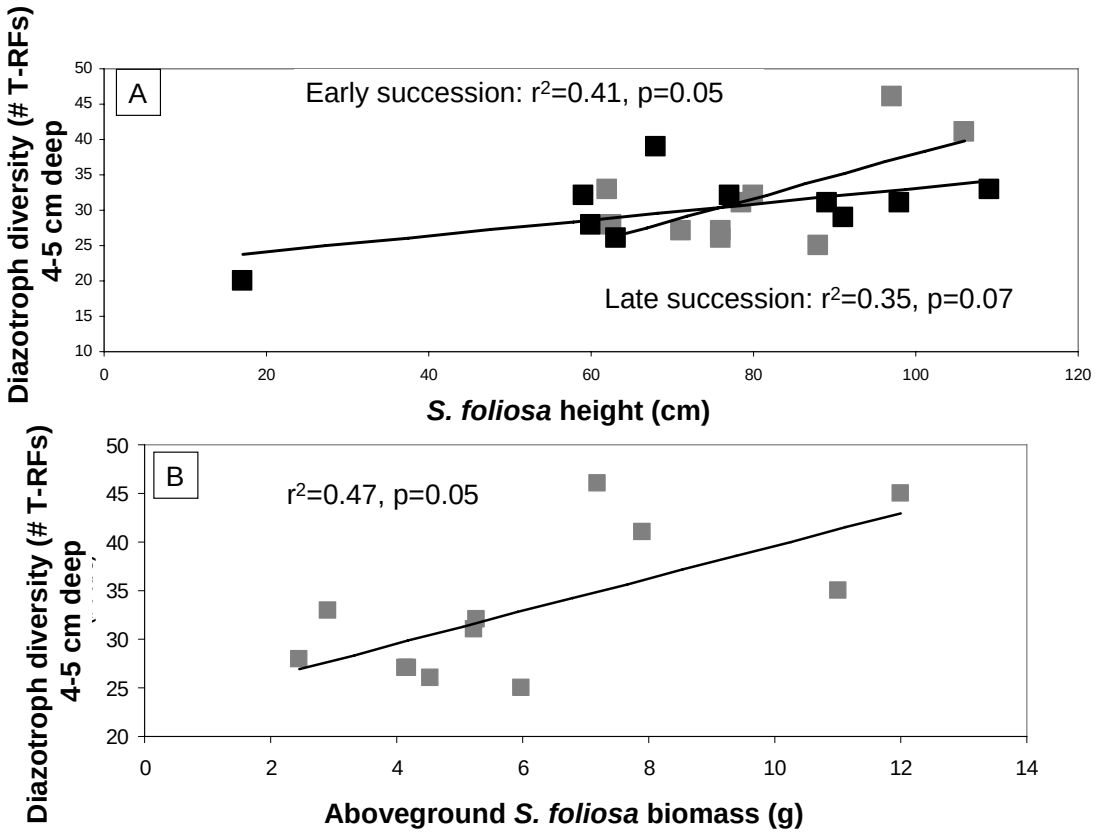

Figure IV-5: Relationships during Fall 2005 between rhizosphere (4-5 cm) diazotroph diversity and (A) *S. foliosa* height in the early (grey squares) and late (black squares) successional marshes (B) aboveground *S. foliosa* biomass in the early succession marsh (grey squares) only

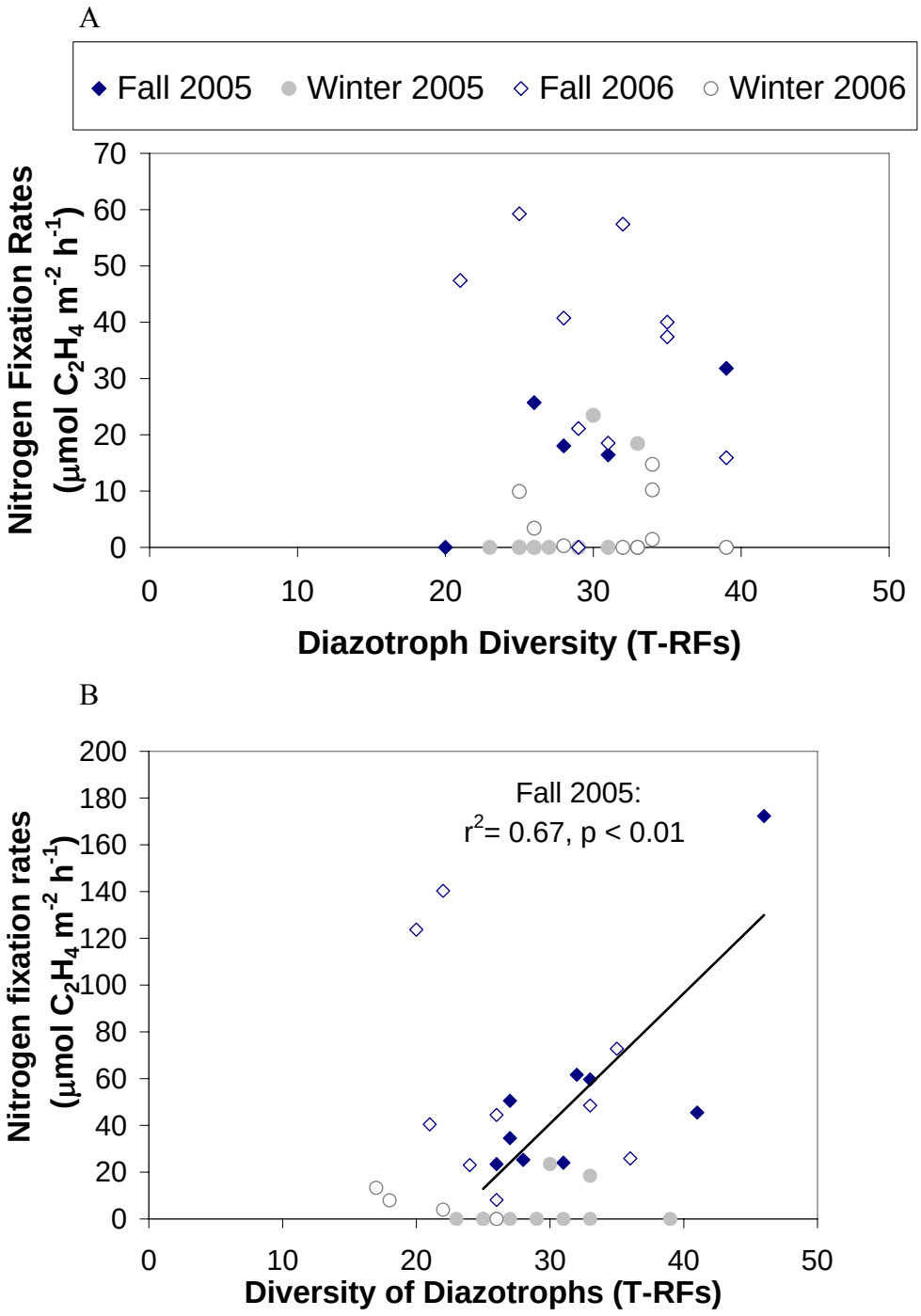

Figure IV-6: Relationships between diazotroph function (nitrogen fixation rates) and diversity in rhizosphere (4-5 cm deep) sediments of (A) the late succession marsh and (B) the early succession marsh of Tijuana Estuary during each of the four dates in this study

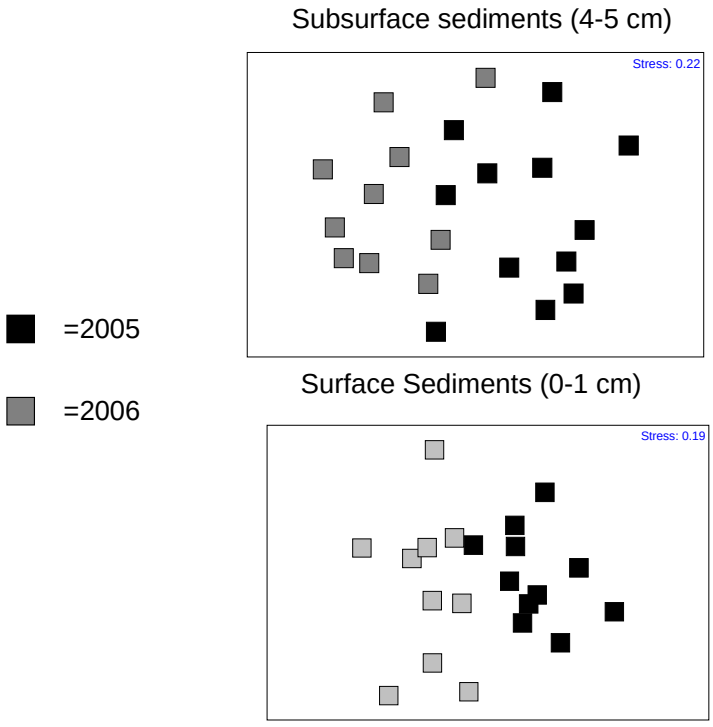

Figure IV- 7: Multidimensional scaling plot representing compositional differences in surface and subsurface diazotroph communities of the early succession marsh between Fall 2005 and Fall 2006

Tables

Table IV- 1: Comparison of plant and abiotic factors of the early ("E") and late succession ("L") marshes during this study; Statistical results are shown only for factors that differed significantly (----- = no significant difference, ^{T}= trend)

	Winter 2005	Fall 2005	Winter 2006	Fall 2006
S. foliosa height	L>E t_{22}=2.091, p=0.05	----	L>E S=22, p=0.05	L>E^{T} t_{18}=1.96, p=0.06
S. foliosa aboveground biomass	L>E t_{22}=2.107, p=0.05	----	----	L>E t_{12}=3.98, p<0.01
S. foliosa belowground biomass	----	----	----	----
S. foliosa shoot N content	n/a	----	----	----
S. foliosa density	n/a	----	----	L<E t_{15}=-4.90, p<0.01
Sediment organic content	n/a	L>E	L>E (Welch F_1=57.55, p<0.01)	----
Sediment clay content	n/a	----	----	----
Porewater salinity	n/a	n/a	n/a	L<E^{T} t_9=-1.84, p=0.10
Porewater ammonium	n/a	L>E ^{T} t_{16}=-1.915, p=0.07	----	L>E t_{11}=-1.37, p=0.05
Porewater nitrite	n/a	----	----	----

Table IV-2: Relationships of nitrogen fixation rates (dependent variable) with environmental factors (independent variable) in the early and late succession wetlands (correlation coefficients and p-values are provided for notable relationships only, "----" = relationship not significant, ^{T}= trend)

Early succession wetland					**Late succession wetland**				
	Winter 2005	Fall 2005	Winter 2006	Fall 2006	***S. foliosa* properties**	Winter 2005	Fall 2005	Winter 2006	Fall 2006
Height	…..	…..	- ^{T} r^2=0.89, P=0.06	…..	Height	+ *r^2=0.47, p<0.01*	…..	…..	…..
Total Biomass	…..	…..	…..	…..	Total Biomass	+ *r^2=0.54, p<0.01*	…..	…..	…..
Above-ground Biomass	…..	…..	…..	…..	Above-ground Biomass	…..	…..	…..	…..
Below-ground Biomass	+ *r^2=0.35, p=0.04*	…..	…..	…..	Below-ground Biomass	…..	…..	…..	…..
Shoot N content	…..	…..	…..	…..	Shoot N content	…..	…..	- r^2=0.54, p=0.02	…..
Shoot C content	…..	…..	…..	…..	Shoot C content	…..	…..	- ^{T} r^2=0.57, p=0.08	…..
Density	n/a	n/a	…..	…..	Density	n/a	…..	…..	…..
Sediment organic content	n/a		…..	…..	Sediment organic content	n/a	∪ *r^2=0.65, p=0.02*	…..	…..
Sediment clay composition	n/a	+ *r^2=0.56, p=0.02*	…..	…..	Sediment clay composition	n/a	…..	…..	…..
Salinity	n/a	…..	…..	…..	Salinity	n/a	…..	…..	- r^2=0.51, p=0.03
Porewater NH_4^+	n/a	…..	…..	…..	NH_4^+	n/a	…..	…..	- r^2=0.38, p=0.06

Table IV-3: Relationships (linear regressions) of diazotroph diversity (dependent variable) at sediment depths (in cm) indicated inside parentheses with environmental factors (independent variables) in an early and late successional wetlands (correlation coefficients and p-values are provided for notable relationships only, "….." = relationship not significant, ^{T}= trend); porewater ammonium was not significantly related to diazotroph diversity and thus is not displayed

	Early successional wetland				Late successional wetland			
***S.foliosa* properties**	Winter 2005	Fall 2005	Winter 2006	Fall 2006	Winter 2005	Fall 2005	Winter 2006	Fall 2006
Height	**…..**	+ (4-5) r^2=0.41, p=0.05	…..	…..	…..	+ (4-5) ^{T} r^2=0.35, p=0.07	…..	**…..**
Total Biomass	**…..**	+ (4-5) r^2=0.39, p=0.05	…..	…..	…..	…..	…..	**…..**
Aboveground Biomass	**…..**	+ (4-5) r^2=0.41, p=0.05	+ (4-5) r^2=0.94, p=0.03	…..	…..	…..	…..	**…..**
Belowground Biomass	- (0-1) r^2=0.37, p=0.03	…..	…..	…..	…..	…..	…..	- (4-5) ^{T} r^2=0.3, p=0.1
Shoot N content	n/a	…..	…..	…..	n/a	+ (4-5) ^{T} r^2=0.41, p=0.06	- (0-1) ^{T} r^2=0.36, p=0.09	**…..**
Shoot C content	n/a	…..	- (4-5) r^2=0.89, p=0.05	…..	n/a	…..	…..	**…..**
Density	n/a	…..	…..	…..	n/a	…..	…..	∩ (4-5) r^2=0.61, p=0.04
Sediment org. content	n/a	…..	…..	…..	n/a	…..	…..	**…..**
Sediment clay composition	n/a	- (0-1) ^{T} r^2=0.36, p=0.06	…..	+ (4-5) r^2=0.56, p=0.03	n/a	+ (4-5) r^2=0.48, p=0.03	…..	**…..**
Salinity	n/a	…..	…..	∩ (0-1) r^2=0.77, p=0.01	n/a	…..	…..	+ (0-1) r^2=0.49, p=0.02 + (4-5) ^{T} r^2=0.36, p=0.07
Porewater NO_2^-				∩ (4-5) r^2=0.49, p=0.10			- (4-5) r^2=0.45, p=0.07	

Table IV-4: Differences in diazotroph community composition (based on T-RF profiles) between: (A) the early and late succession marshes or fall versus winter seasons in Tijuana Estuary, based on two-way ANOSIM tests (averaging across season or marsh, respectively) (B) surface versus subsurface sediment depths during each season in both marshes, based on one way ANOSIM tests in Primer 4.0 (*= only 5 replicates)

Table 4A	Difference between marshes	Difference between season
2005 surface	Global R=0.566, p<0.01	Global R=0.497, p<0.01
2005 subsurface	Global R= 0.541, p< 0.01	Global R=0.388, p<0.01
2006 surface	Global R=0.354, p<0.01	Global R=0.413, p<0.01
2006 subsurface	Global R=0.442, p<0.01	Global R=0.324, p<0.01

Table 4B	Winter 2005	Fall 2005	Winter 2006	Fall 2006
Early succession marsh	Global R=0.151 p=0.004	Global R=0.189 p=0.008	*Global R=-0.136 p=90	Global R=-0.029 p=0.61
Late succession marsh	Global R=0.906 p=0.001	Global R=0.955 p=0.001	Global R=1.0 p=0.001	Global R=0.98 p=0.001

Table IV-5: Summary of average annual diazotroph diversity (# T-RFs), average annual nitrogen fixation rates and coefficients of variation in the early and late-successional stage marshes of Tijuana Estuary between 2005 and 2006 (Coefficients of variation, CV= 100 X (standard deviation/ mean))

	2005		2006	
	Early succession marsh	Late succession marsh	Early succession marsh	Late succession marsh
Total average diversity (± CV)	31 (0.18)	27 (0.19)	25 (0.22)	29 (0.15)
Average annual nitrogen fixation rates	24	4.9	42	20
CV of annual nitrogen fixation rates	160	420	106	49

CHAPTER V[4]

WETLAND RESPONSE TO SEDIMENTATION AND NITROGEN LOADING: DIVERSIFICATION AND FUNCTIONAL DECLINE OF NITROGEN-FIXING MICROBES

Abstract

Ecosystem responses to multiple anthropogenic impacts may be influenced by microbial communities through their biogeochemical functions and interactions with higher organisms. Anthropogenic inputs of nutrients and sediment often simultaneously impact coastal ecosystems such as wetlands, which are historically nitrogen limited. In surface sediments and rhizospheres (roots and surrounding sediments) of dominant wetland plants, diazotrophic microbes fix nitrogen and constitute key components of macrofaunal diets. The hypothesis that diazotroph diversity in wetlands sustains nitrogen fixation amidst independent and combined effects of sediment and nitrogen (ammonium nitrate) loading was tested via field additions of these variables in *Spartina foliosa*-vegetated and unvegetated marsh zones at Tijuana Estuary (CA). Responses of diazotrophs were assessed after 2 and 17 days using acetylene reduction assays and genetic fingerprinting (terminal restriction fragment length polymorphism, T-RFLP) with the *nif*H gene that codes for dinitrogenase reductase. Ammonium nitrate loading (independent of sediment) increased the diversity (number of T-RFs) and evenness (relative T-RF fluorescence) of diazotrophs in surface sediments and significantly

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reduced nitrogen fixation rates within 17 days, but diazotroph composition (T-RF profiles) and rhizosphere diazotroph diversity were not affected. In the presence of sediment additions (1 cm deep layers), porewater ammonium concentrations were significantly higer and lasted longer throughout the 17 day experiment, suggesting that recovery of nitrogen fixation functions may be delayed by a combination of sediment and nutrient impacts. Nutrient loadings similarly inhibited nitrogen fixation in the laboratory among sediments (containing *S. foliosa)* in which sulfate reducing bacteria were found to perform about 70 % of the diazotroph activity through chemical inhibition. Fates of newly fixed nitrogen in wetland ecosystems were characterized via *in situ* isotopic enrichment studies. Recently fixed nitrogen ($as^{15}N_2$) reached plant roots within 3 days and several animal consumers by 8 days in microcosms of intact marsh sediment. Long-term declines in nitrogen fixation rates, in response to increasingly frequent nutrient loading and sedimentation, may potentially alter nitrogen sources and mechanisms of niche partitioning among key plant species, with possibly significant consequences for animal consumers and wetland ecosystem function.

Introduction

Numerous anthropogenic impacts may profoundly alter ecosystem function by shifting community composition, biological diversity, and ecological interactions. Although multiple stresses naturally influence biological communities, anthropogenic impacts can increase the magnitude, duration, and complexity of the environmental changes that affect a variety of ecosystems (Halpern et al. 2008). Human- induced

disturbances to nutrient and sediment regimes threaten biological diversity on increasingly global scales as well as core ecosystem functions that maintain diversity. Predictions regarding the consequences of multiple human impacts require a fundamental understanding of mechanisms that underlie ecosystem function and the simultaneous and synergistic ways that they may interact (Breitburg et al. 1999).

Wetland ecosystems at the land-sea interface, such as temperate coastal salt marshes, are maintained by processes that naturally introduce sediments and nutrients which are both essential building blocks of these ecosystems. Riverine inputs of sediment maintain wetland elevation against sea level rise. Nutrient inputs, particularly of nitrogen, from riverine and marine sources and nitrogen fixation (the bacterial conversion of N_2 into biologically available NH_3) support high wetland productivity (Valiela 1983, Boyer et al. 2001, Traut 2005, Scott et al. 2007). Availability of nitrogen, which is often the limiting nutrient in wetlands, exerts a major influence on plant community structures and ecosystem productivity and sustains secondary functions that depend upon the presence and structure of vascular plants.

In coastal zones worldwide, strong anthropogenic alteration of sediment and nutrient delivery affects biological diversity and ecosystem function, particularly in estuaries and wetlands (Howarth et al. 2000, Thrush et al. 2004, Deegan et al. 2007). Although sediment and nitrogen are both key components of wetlands, the magnitude and frequency of sedimentation and nutrient loading are increasing far beyond historical

ranges 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). Coastal eutrophication, the increase in organic matter in an environment that results from nitrogen addition via fertilizer and atmospheric sources (Galloway et al. 1995), afflicts more than half of the estuaries in the U.S. (Howarth et al. 2000), leading to loss of vascular plants through algal blooms, smothering, and associated hypoxia (Herbert 1999 Nixon et al. 2001, Duarte 2002). Further, extreme climatic events such as hurricanes and other coastal storms may further exacerbate sediment and nutrient loads to coastal ecosystems such as wetlands (Paerl et al. 2002, Day et al. 2007).

The cycling of nitrogen, in all ecosystems, is driven by microorganisms (Howarth 1993, Ward 2005). Thus microbial communities, and their interactions with plants, may largely determine the effects of nutrient loading on nitrogen cycling at ecosystem scales. In coastal wetlands, nitrogen-fixing microbial communities reside in surface sediments (cyanobacterial mats) or on plants shoots (Currin and Paerl 1998), as well as in plant rhizospheres. Nitrogen fixers (diazotrophs) can rapidly channel reduced nitrogen to vascular plants via specific, mutualistic associations with roots (Whiting et al. 1986, Bagwell et al. 2000). However, the nitrogenase enzyme with which diazotrophs catalyze nitrogen fixation is known to be inhibited by ammonium (Yoch and Whiting 1986). Effects of nutrient loading on diazotrophs have not been constrained in the context of other simultaneous human impacts, but may significantly affect functional responses of

wetland ecosystems. Diazotrophs have been hypothesized be less competitive among microbial communities in the presence of high exogenous nitrogen availability (Kolb and Martin 1988, Piceno and Lovell 2000). Potential declines in nitrogen fixation, or shifts in diazotroph community structures in nutrient or sediment loading, may have consequences not only for vascular plants but also consumers in wetlands, as diazotrophs play key trophic roles particularly in developing marsh ecosystems (Moseman et al. 2004).

The structure of microbial communities may hold important consequences for maintenance of the biogeochemical functions that they perform (Tiedje et al. 1999, Smith 2007). Microbial diversity within a functional guild is hypothesized to confer functional redundancy against environmental perturbations (Bagwell and Lovell 2000). Although the diversity of diazotroph communities has thus far seemed resistant to effects of nitrogen loading in natural environments (Piceno and Lovell 2000), microbial communities (like their macroscopic counterparts) may be more susceptible to multiple environmental changes. For instance, chemical effects of anthropogenic nutrient additions on an ecosystem can be exacerbated by physical impacts from sedimentation such as smothering of the benthos (Thrush et al. 2004).

In Tijuana Estuary, a National Estuarine Research Reserve immediately north of the U.S. Mexico Border, 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 sewage when heavy rainfall exceeds the capacity of

treatment plants. Urban and agricultural runoff also brings sediment and nutrient loads into the estuary (King 2003). This setting is particularly relevant for studies of sedimentation impacts, as construction of the congressionally-mandated Triple Border Fence on the U.S.-Mexico border will require 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 ammonium nitrate loading, on nitrogen fixation in coastal wetland ecosystems. The following hypotheses were addressed:

(1) Nutrient loading decreases the diversity of diazotroph assemblages in both surface (0-1 cm) and rhizosphere (4-5 cm) sediments (via porewater mixing through sediment).

(2) Sediment loading also decreases diversity of diazotrophs in surface sediments (via physical smothering) but does not affect diversity in rhizospheres.

(3) Diversity among diazotrophic microbes does not confer functional redundancy (stability) to nitrogen fixation amidst impacts of sediment and nitrogen additions.

(a) Sediment and nutrient impacts decrease nitrogen fixation rates via smothering of marsh surfaces and increasing availability of exogenous nitrogen, respectively.

(b) Combined effects of these impacts were hypothesized to be greater than their individual influences on nitrogen fixation.

(c) Changes in nitrogen fixation occur independently of shifts in the diazotroph composition.

A manipulative field experiment was conducted that exposed *Spartina foliosa* plants and diazotrophic microbes in salt marsh sediments to one of four treatments: (1) sediment and nitrogen loading, (2) sediment loading only, (3) nitrogen loading only, and (4) control (no sediment or nitrogen). To assess the response of diazotrophs to these environmental changes, the diversity and activity of nitrogen fixing microbes were contrasted among the 4 treatments over a period of 17 days. The potential for plants to ameliorate impacts of nutrient additions on diazotrophs (i.e. by assimilating nutrients) was also investigated through examination of vascular plant properties among treatments over this time period. Diazotroph assemblages in surface sediments and rhizospheres may not only respond differently to sediment and nitrogen loading, but also, they have distinct roles for ecosystem function. Thus, diazotroph assemblage responses to treatments were measured (in terms of shifts in diversity and composition) in both of these micro-environments.

Potential consequences of changes in nitrogen fixation for primary and secondary production in wetland ecosystems were explored via isotopic enrichment experiments using $^{15}N_2$. These studies tested whether newly fixed nitrogen can be used as a nutrient source by *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. 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 studied.

Methods

Study area

The Tijuana River National Estuarine Research reserve, located immediately north of the U.S.-Mexico border (32°34'N, 117°7'W), includes salt marshes, tidal creeks, and upland-wetland transition areas (Kennish 2004). Sedimentation from the surrounding watershed originates from urbanized and destabilized hillsides (Zedler et al. 1992). 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). 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 y^{-1}(Cahoon et al. 1996). Average rates of 1.3 cm y^{-1} were measured in the restored Friendship marsh in the southern region of the estuary (Wallace et al. 2005).

The 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 1992). 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 and test the effects of a one-time sedimentation event and associated nitrogen loading on nitrogen fixation rates, the diversity of nitrogen fixers, and the health of *Spartina foliosa*, a manipulative experiment was conducted in the vegetated Friendship marsh during Fall (October –November) 2006 **(Experiment 1).** For this experiment, sediment was collected from a catchment basin adjacent to the Friendship marsh in Tijuana Estuary. These sediments, eroding from hillsides within the watershed of Tijuana Estuary, are typical of those that would flood into the estuary during heavy rains. The sediment was filtered through a 100-μm screen, homogenized by stirring, and applied to experimental plots (1 m^2) that were positioned at approximately 20-m intervals along a transect in the *S. foliosa* zone of the Friendship marsh.

A total of 10 experimental plots (Figure V-1) were subdivided (0.5 m X 0.5 m) into 4 compartments which 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 H_2O), (B) sediment addition in artificial seawater slurry, (C) addition of ammonium nitrate-enriched artificial seawater only, and (D) artificial seawater addition only (salinity= 40, comparable to flood waters). Garden lining (about 7 cm deep) was

used to divide experimental treatments as well as to surround the 1 m X 1 m grouped quadrat. This lining was positioned to protrude about 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. 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.

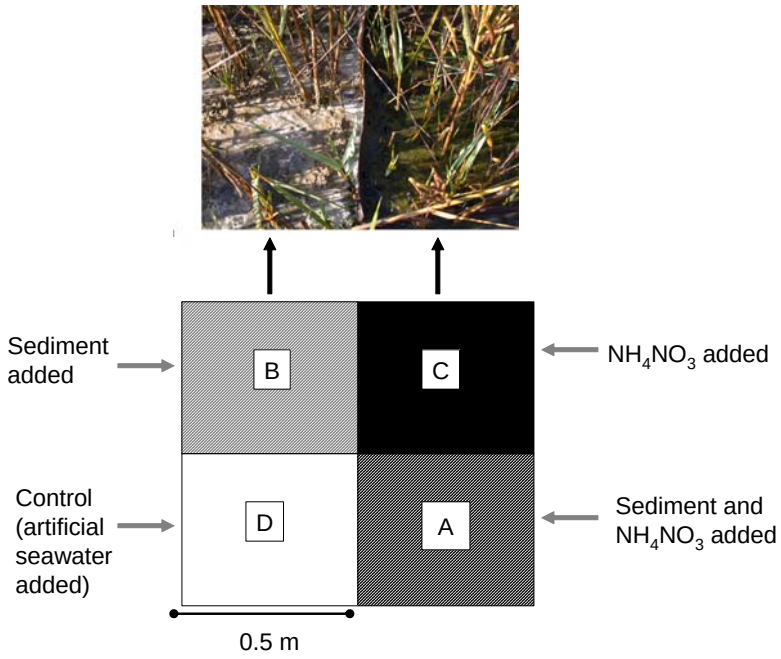

Figure V-1: Diagram of experimental treatments (0.25 m^2) applied during Experiments 1 and 2 in the Friendship marsh to 10 and 16 regularly spaced replicates in the *S. foliosa* (Experiment 1) and unvegetated zones (Experiment 2), respectively between late Fall and early winter 2006; the photograph above the diagram shows immediate visible difference in the benthic surface between plots to which sediment was added compared to those in which no sediment was added.

Nitrogen fixation rates, diazotroph diversity and *S. foliosa* plants (tissue N, biomass, height, density) were assessed immediately prior to experiment initiation, as well as 2 and

17 days 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 on all 3 sampling dates. For characterization of porewater ammonium concentrations and bulk sediment parameters (organic matter content and grain size), two additional sediment cores (6 cm deep, approx. 2.1 cm diameter) were collected. All sediment samples were stored on ice until they could be processed or transported to the laboratory. Porewater salinity was measured in each quadrat by analyzing filtered seawater, extruded from the top 2 cm of sediment, on a handheld refractometer. Light levels were also measured above and below plant canopies in each quadrat using a hand-held light meter (Apogee Instruments) to determine the percentage of benthic light reduction by *S. foliosa* in each quadrat. *S. foliosa* heights (average of 10 randomly selected shoots) and densities (total number of live shoots per quadrat) were recorded just prior to and following application of experimental treatments (2 days and 17 days later). 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).

The manipulative field experiment was subsequently repeated in the unvegetated zone at higher 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. Sediment samples were collected for determination of nitrogen

fixation rates and diazotroph diversity (0-1 and 4-5 cm) following the same procedures as in the vegetated zone (the latter was analyzed only for premanipulation conditions).

Acetylene reduction

Nitrogen fixation rates were determined on the same day of sample collection in 2 hour aerobic assays, using the acetylene reduction method (described in Moseman 2007), in flasks sealed with rubber stoppers such that plant shoots protruded from vessels, but roots and sediments were confined. Samples were assayed outdoors of laboratory facilities at Scripps Institution of Oceanography as soon as possible (within 3 hours) following their collection from the field and processing; evening assay times were employed for premanipulation and 17-day samples, while 2-day samples were assayed in the late afternoon. Incubation temperatures did not exceed 21 ^{o}C.

Laboratory inhibition of nitrogen fixation

To evaluate the relative contribution of sulfate reducing bacteria to nitrogen fixation **(Experiment 3)**, a total of 30 *S. foliosa* samples and attached sediments (2.1 cm, approx 5 cm deep) were taken from an approximately 1m^2 area near the mouth of the restored Friendship marsh. Core bottoms were sealed with plastic-wrapped foil and cores were stored on ice for transport. In the laboratory, bottoms of the cores were sealed with electrical tape and left overnight (dark conditions for 12 hours). To test parallel effects of nutrient additions on nitrogen fixation activities of these microbial assemblages, one of

the following 3 treatments (n=10) were applied via injection into the center of the vegetated sediment core on the following day:

(1) Artificial sea water added (12 ml per sample)

(2) 30g N L^{-1} ammonium nitrate added (12 ml per sample)

(3) Sodium molybdate added (20mM, 12 ml per sample)

Following injection of the treatment solutions, samples were immediately transferred to 125 ml flasks in which acetylene reduction assays were performed as described above. Samples were incubated with indirect exposure to natural sunlight in uncovered insulated plastic tubs. Temperatures did not exceed 22 ^{o}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 on plant uptake of fixed nitrogen **(Experiment 4)**, 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 from 3 blocks (approximately 20 m apart) within the Friendship Marsh. Each core was injected with one of the following: (I) 2 ml $^{15}N_2$-saturated artificial seawater (44.6 μM) + 2 ml NH_4NO_3 (30 g L^{-1}) (II) 2 ml $^{15}N_2$-saturated artificial seawater (44.6 μM) + 2 ml artificial seawater Concentrations of ammonium nitrate additions (30 g N m^{-2}) in treatment I were equivalent to those employed in Experiments 1 and 2.

The $^{15}N_2$-saturated seawater was prepared by sealing 4 ml of artificial seawater (salinity of 37, 20 ^{o}C) into gas tight glass vials (5.3 ml total volume, Becton Dickinson). 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) which was injected directly into the seawater. This gas was shaken and allowed to equilibrate for more than 48 hours 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 ½ gauge needles) about 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.

Figure V-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 about 30 cm high.

Immediately following injections, sediment and plant samples were sealed with closed Mylar caps with Parafilm and tape and then returned in their original positions in the field (Figure V-2).These chambers were intended to prevent loss of $^{15}N_2$ label but were applied to all cores, even those not receiving $^{15}N_2$, so that effects of the enclosure would apply equally across treatments. When necessary, plants were bent to fit in the caps, but none

were broken or clipped. Samples were retrieved after 3 and 8 days, respectively, to compare nitrogen pathways 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 ^{o}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 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) using a Micromass (Manchester, UK) Isoprime isotope ratio mass spectrometer (IRMS) for determination of δ^{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 sediments using the Power Soil DNA kit (Mo Bio Laboratories). The manufacturer's protocol was followed with minor alterations, including substitution of a vortex with a bead beater for purposes of cell lysis and increased centrifugation times (from 1 to 5 minutes). The *nif*H gene was amplified via nested PCR with degenerate primers (Zehr and McReynolds 1989). Primers for round 1 were *nif*H 3: 5'-ATR TTR TTN GCN GCR TA-3'and *nif*H 4: TTY TAY GGN AAR GGN GG-3'. For round 2, primers were *nif*H1: 5'- TGY GAY CCN AAR GCN GA-3'and *nif*H2: 5'- ADN GCC ATC ATY TCN CC-3'. The nested PCR was used as a means to improve PCR yields. The PCR was 50 μL in total volume and consisted of GoTaq (5 units per microL) and 5X Colorless Flexi Buffer

(Promega), 25 mM $MgCl_2$, 10mM DNTPs (Promega), 10μM of forward and reverse primers (IDT DNA), and nuclease-free water (Ambion) which had also been passed through 5 kB filters (Millipore Amicon Ultra) for additional purification. The conditions for round 1 PCR were 95°C for 3 min., followed by cycles of 95°C for 30s, 57°C for 30s, and 72°C for 45s (25 times for round 1, 30 times for round 2), then 72°C for 7 min, and 4.0°C until samples were removed from the thermocycler.

PCR products were digested, in 20 μL batches, with the Hae III restriction enzyme (4bp) for 6 hours at 37°C. Digested products were re-combined 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 UCSD Cancer Center Sequencing Facility using ABI GeneScan capillary electrophoresis. Data were analyzed using Peak Scanner Software v1.0 (Applied Biosystems).

Statistical analyses

The effects of sediment and nitrogen addition (experimental treatments) on nitrogen fixation rates, plant parameters (biomass, height, shoot N content), and environmental factors (porewater ammonium, sediment organic matter, and grain size) were compared across all dates using a two-factor repeated measures ANOVA. As time was found to be a significant factor in Experiment 1, comparisons were also drawn among treatments on a given date (premanipulation, 2 days later, and 17 days later) using one-way ANOVAs (followed by Tukey Kramer tests) when homogeneity of variance was

met among groups. In cases where homogeneity of variance could not be met (i.e. porewater ammonium), the nonparametric Welch's ANOVA test was used. Kruskal-Wallis tests, via Chi-square approximations in JMP 4.0, were employed when assumptions of normality failed but variances were equal. In the unvegetated zone (Experiment 2), day of experiment initiation was a significant factor affecting nitrogen fixation rates across all dates. Thus, experimental treatment was nested within day of experiment initiation in testing effects of these factors on nitrogen fixation rates.

As visual inspection of data repeatedly suggested a role of nitrogen alone in affecting nitrogen fixation rates and diazotroph diversity (Experiment 1), the effect of single treatments (nitrogen or sediment addition) on diazotroph or plant properties were tested via paired t-tests on each date. Paired tests were employed because samples were not distributed independently of one another throughout the study area, but rather were paired in plots to which treatments had been applied. Paired tests of treatments A and B (both receiving sediment) or treatments C and D (neither receiving sediment) were made to compare effects of nitrogen addition only on nitrogen fixation rates and diazotroph diversity. Bonferroni-corrections were applied to correct for repeated comparisons (significant adjusted $p=0.025$). Data were log-transformed prior to statistical analyses when needed to achieve normality.

Diazotroph diversity, or richness, was measured (in Experiment 1) as the total average number of *nif*H restriction fragments (T-RFs) and compared between dates and

treatments using ANOVAs or t-tests as described above. Pielou's evenness (J) was also calculated from the arcsin-square root transformed relative fluorescence of T-RFLP profiles (peak heights) in cases where significant diversity changes were found. Both diversity and evenness were calculated via DIVERSE analyses with Primer 4.0 software. Diazotroph community composition (profiles of T-RF identity) was compared between treatments using 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 (Clarke & Warwick 2001). These analyses were also conducted to compare diazotroph communities in sediment depths and across dates.

Effects of measured environmental (plant or sediment) factors on nitrogen fixation rates and diazotroph diversity were examined via linear or quadratic regression analyses, with Bonferroni corrections applied for multiple comparisons of nitrogen fixation rates, diazotroph diversity, 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

In the *S. foliosa* zone (Experiment 1), nitrogen additions (treatments A and C) increased porewater ammonium concentrations by roughly 8 times compared to premanipulation conditions after 2 days (Welsh ANOVA $F_{3,13}=5.73$, $p=0.01$), with

significantly greater ammonium in treatment A than C (Wilcoxon $\chi^2_4 = 4.29$, p=0.04, Table V-1A). After 17 days, porewater ammonium concentrations were significantly greater in plots of treatment A (sediment and nitrogen added, 350 μM) than all other treatments ($F_{3,31}$=8.58, p<0.01, about 50 μM, Table V-1A). Experimental sediment additions (in treatments A and B) decreased the clay content of surface (0-1 cm) sediments (relative to treatments C and D) ($F_{4,30}$= 3.57, p=0.02), but did not affect this factor in bulk sediments (0-6 cm deep) ($F_{4,21}$=1.00, p=0.43) (Table V-1). Combustible organic content of surface sediments was also decreased in plots receiving sediment additions ($F_{4,29}$=3.91, p=0.01, Table V-1), though bulk sediment organic composition (0-6 cm) was not affected ($F_{4,23}$=1.33, p=0.29).

Similar environmental effects of experiment manipulations were observed in the unvegetated zone (Experiment 2) where nitrogen additions (Treatment A and C) significantly increased porewater ammonium concentrations by almost an order of magnitude (1353 μM) compared to premanipulation levels (195 μM, Welch ANOVA $F_{3,32}$=20.4, p<0.01). Ammonium concentrations were also higher in treatment A (sediment and nitrogen added) than treatment C (nitrogen only) (t_{30}=2.96, p<0.01). As in the *S. foliosa* zone, experimental sediment additions did not affect bulk (0-6 cm) organic content ($F_{4,24}$=1.47, p=0.24) or grain size ($F_{3,8}$=0.79, p=0.53).

Responses of diazotrophs to sediment and nutrient loads

Diversity and functional responses of nitrogen-fixing microbes to changes in the sediment and nutrient properties of marsh sediments in Experiment 1 were not immediate. Two days following experimental manipulations, diazotroph diversity (total average number of T-RFs) did not differ among treatments (0-1cm: $F_{4,26}$=0.48, p=0.75; 4-4cm: $F_{3,28}$=0.13, p=0.94). Nitrogen fixation rates did not significantly differ between treatments after 2 days either ($F_{3, 26}$=1.20, p=0.33), although there were trends of lower rates in treatment A (sediment and nitrogen added; 3.4 $\pm$1.3 μmol C_2H_4 m^{-2} h^{-1}) than treatment B (sediment only; 7.7 $\pm$ 2.9 μmol C_2H_4 m^{-2} h^{-1}) (paired t_7=1.97, p=0.09, one-tailed p=0.04), and in treatment C (nitrogen added; 9.0 $\pm$ 3.5 μmol C_2H_4 m^{-2} h^{-1}) than in D (control; 10.5 $\pm$ 3.1 μmol C_2H_4 m^{-2} h^{-1}) (paired t_6=1.83, p=0.12, one-tailed p=0.06).

After 17 days, trends of higher diazotroph diversity (number of T-RFs) were found among surface sediments in plots with nitrogen additions (treatments A and C) than those without nitrogen additions (treatments B and D, t_{14}=2.11, p=0.05, Figure V-3A). Compared to premanipulation conditions, diazotroph diversity in surface sediments was only higher among plots of treatment A (receiving both sediment and nitrogen), with no significant change over this time period among other treatments (student's t=2.00, p=0.05, Figure V-3A). Shifts in diazotroph diversity among surface sediments of treatments A and C, compared to premanipulation conditions, were accompanied by

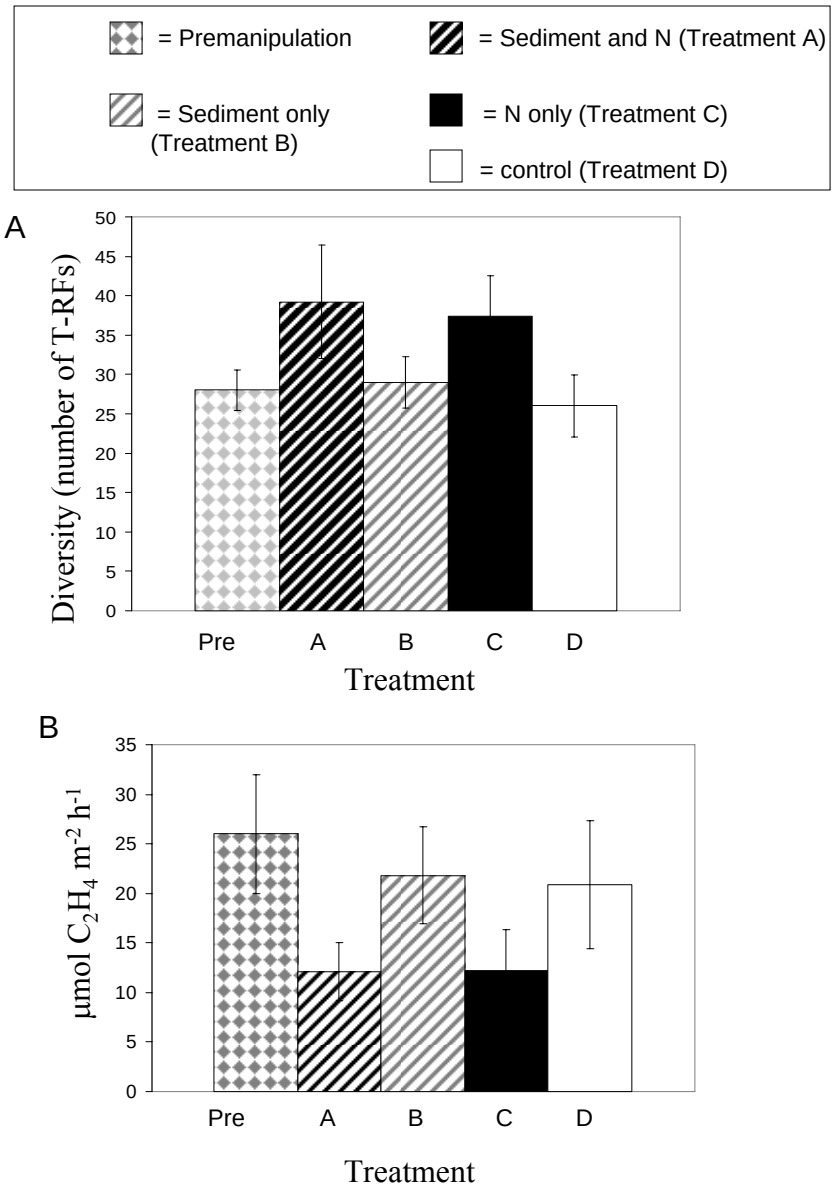

Figure V-3: Averages and standard error bars of (A) diversity of diazotrophs (determined as the number of terminal restriction fragments from analysis of the *nif*H gene) among treatments in the *S. foliosa* zone (Experiment 1) after 17 days; (B) nitrogen fixation (acetylene reduction) rates among treatments in the *S. foliosa* zone after 17 days (Experiment 1).

increases in the evenness of diazotroph communities, as reflected by relative fluorescence of each terminal restriction fragment (T-RF). In treatment A, evenness among T-RFs increased significantly within the first 2 days of the experiment from 0.71 $\pm$ 0.06 to 0.82 $\pm$ 0.09 (t_8=-3.13, p=0.01), but had declined to premanipulation levels (0.75 $\pm$ 0.04) by 17 days (t_5=-1.28, p=0.26). Evenness of diazotroph T-RFs in treatment C only increased significantly from 0.72 $\pm$ 0.09 to 0.85 $\pm$ 0.09 after 17 days (t_5=-6.63, p<0.01). Diversity of rhizosphere diazotrophs (4-5 cm) had not changed after 17 days among any treatments or compared to premanipulation conditions ($F_{4,53}$=0.82, p=0.51). Nitrogen fixation rates, after 17 days, were significantly lower in plots receiving nitrogen additions than those which had not, regardless of sediment treatment (A<B: paired-t, t_8=1.84, p=0.05, C<D: t_9=2.59, p=0.04) (Figure V-3B).

Responses of diazotroph diversity to sediment and nitrogen additions were not investigated in the unvegetated zone of the marsh (Experiment 2), because nitrogen fixation rates did not differ significantly among treatments after 2 ($F_{4,58}$=0.63, p=0.64) or 17 days ($F_{4,56}$=0.72, p=0.58). Nitrogen fixation rates in the unvegetated zone were 12 $\pm$ 2.5 μmol C_2H_4 m^{-2} h^{-1}, which were marginally lower than those in the *S. foliosa* zone (t_{70}=-1.60, one-tailed p=0.06). In the unvegetated zone, porewater ammonium levels were significantly higher ($F_{1,77}$=9.00, p<0.01) and 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 in surface and rhizosphere sediments (premanipulation: ANOSIM Global R=0.413, p=0.01; 2 days later Global R= 0.79, p<0.01; 17 days later Global R=0.65, p<0.01) in Experiment 1. However, treatments did not affect diazotroph composition after 2 (Global R= -0.019, p=0.77) or 17 days (Global R=-0.021, P=0.78).

To investigate the possible role of microbial community structure in affecting functional responses to sediment and nitrogen additions, comparisons were made of diazotroph community structures prior to experimental manipulation between *S. foliosa* vegetated (Experiment 1) and unvegetated zones (Experiment 2). Although the diversity (total number of T-RFs) of diazotroph assemblages did not differ between zones (t_{102}=-0.62, p=0.54), the diazotroph composition was distinct (ANOSIM Global R=0.279, p<0.01).

Resistance of plants to sediment and nitrogen additions

No significant effects of treatment or time were observed on plant height (MANOVA treatment: $F_{4,23}$=0.017, p=0.98, time: $F_{2,22}$=1.29, p=0.29) or above- (MANOVA, treatment: $F_{4,14}$=0.49, p=0.74, time: $F_{2,13}$=1.32, p=0.30) or below-ground

biomass (MANOVA treatment: $F_{4,20}$=0.94, p=0.46, time: $F_{2,19}$=1.42, p=0.27) in Experiment 1 (among all dates).

S. foliosa density declined significantly between premanipulation conditions and 17 days later (but was not measured 2 days following the experiment) only in treatment A (paired t_7=4.21, p<0.01). After 17 days, trends of higher *S. foliosa* density were found in plots receiving nitrogen (treatment C) than those to which both sediment and nitrogen (treatment A), or neither (treatment D), were added (C>D: paired t_8=2.02, p=0.08, C>A: t_8=1.99, p=0.08). Nitrogen content of *S. foliosa* shoots increased over the 17-day time period only for treatment C (nitrogen addition) (paired t_2=-21.84, p<0.01).

Effects of sediment and nitrogen inputs on plant-diazotroph interactions

Plant-diazotroph-environment relationships (in Experiment 1) were highly dynamic among treatments and over time. Only significant relationships are detailed below, under pre- and post-manipulation conditions.

Pre-manipulation

Prior to field manipulations (Experiment 1), diversity of diazotrophs in surface sediments was negatively related to above-ground and total biomass of *S. foliosa* plants (Appendix V-1A). Nitrogen fixation rates in marsh sediments showed positive trends with *S. foliosa* shoot nitrogen content (Appendix V-2).

Post-manipulation

Diversity of surface diazotrophs

After 2 days, diazotroph diversity in surface sediments of treatment A was negatively related to *S. foliosa* height, while in treatment B it was negatively related to plant belowground biomass (Appendix V-1B). After 17 days, surface diazotroph diversity of treatments A and D showed positive trends with porewater ammonium (Appendix V-1C, Figure V-4B and C). These and all other significant relationships after 17 days are represented in Figure V-4. In contrast, the diversity of surface diazotrophs within treatment C was positively related to the average height of *S. foliosa* plants (Appendix V-1C, Figure V-4A). Among all Experiment 1 treatments, diazotroph diversity in surface sediments showed significant positive relationships to porewater ammonium concentrations (r^2=0.32, $p<0.01$), the former of which had increased over 17 days.

Diversity of rhizosphere diazotrophs

After 2 days, rhizosphere diazotroph diversity showed positive trends with *S. foliosa* biomass in treatments A and C (Appendix V-1B). After 17 days, rhizosphere diazotroph diversity was lowest at intermediate porewater ammonium concentrations in treatment A and negatively related to porewater ammonium concentrations in treatments C and D (Appendix V-1C, Figure V-4). Positive trends were found between *S. foliosa* height and rhizosphere diazotroph diversity after 17 days in treatment A, while in treatment D significant positive relationships were found between rhizosphere diazotroph diversity and belowground *S. foliosa* biomass (Appendix V-1C, Figure V-4C). Among all

treatments, diversity in rhizosphere sediments was positively related to *S. foliosa* belowground biomass (r^2=0.31, p<0.01) after 17 days.

Nitrogen fixation rates

Nitrogen fixation rates were positively related to *S. foliosa* biomass after 2 days (Appendix V-2) in treatment A. However, in treatment D, nitrogen fixation rates were negatively related to *S. foliosa* height after 2 and 17 days (Appendix V-2, Table V-2, Figure V-4C), possibly because *S. foliosa* height (+, r^2=0.46, p=0.04) and nitrogen fixation rates (-, Table V-2) showed opposite relationships to porewater ammonium in these plots. In treatment C, nitrogen fixation rates were positively related to *S. foliosa* density and aboveground biomass (Table V-2).

Diversity-function relationships

The diversity of diazotrophs in surface sediments of treatment A showed negative trends with nitrogen fixation rates after 17 days (r^2=0.46, p=0.06, Figure V-4B). No other relationships were observed between diazotroph diversity and nitrogen fixation rates during this study.

Laboratory inhibition of nitrogen fixation by ammonium

Nitrogen fixation (acetylene reduction) rates of *S. foliosa* samples in Experiment 3 were reduced by 70 percent in the presence of sodium molybdate (7.6 $\pm$ 2.2 μmol C_2H_4

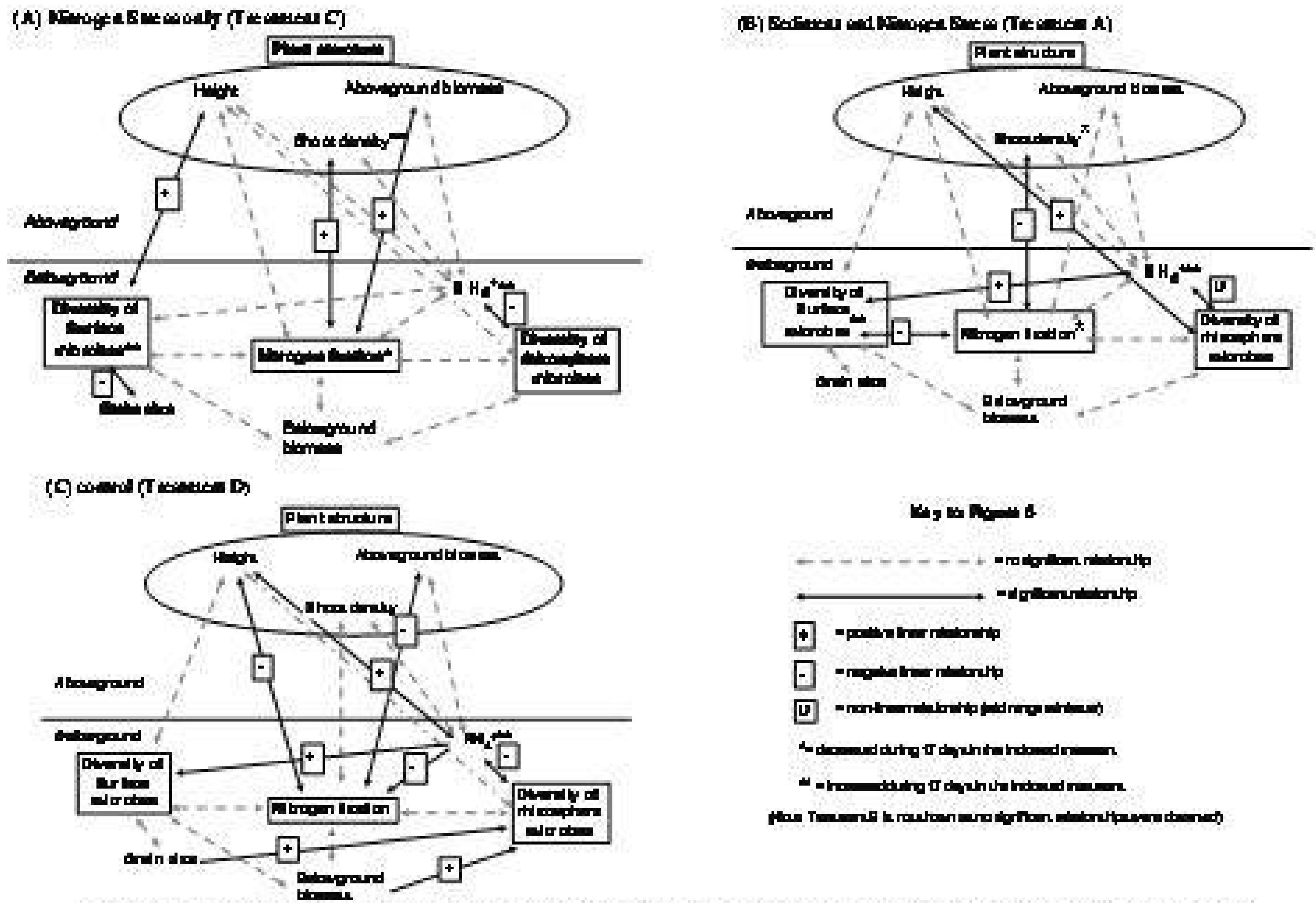

Figure V-4. Diagrams illustrating (linear and nonlinear) regression relationships after 17 days among diversity and plant properties in the S. foliosa zone (Experiment 1) of the [illegible] marsh under conditions of (A) nutrient additions (treatment C), (B) nutrient and sediment additions (treatment A), and (C) control (treatment D). Regression coefficients and p-values are reported in Tables V-2 and V-3.

m^{-2} h^{-1}), a known inhibitor of sulfate reducing bacteria (Welsh 2000), compared to controls with artificial seawater additions (25$\pm$ 5.2 μmol C_2H_4 m^{-2} h^{-1}), and rates were also inhibited by additions of ammonium nitrate (4.1 $\pm$ 2.8 μmol C_2H_4 m^{-2} 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 to 52 above controls) and roots ($\delta^{15}N$: 22 to >3300 above controls) were isotopically enriched within 3 days. Roots showed significantly greater ^{15}N enrichment than shoots (3 days- paired t_2= 4.15, p=0.05; 8 days-paired t_3= 5.85, p=0.01). *S. foliosa* roots exposed to $^{15}N_2$ in the absence of ammonium nitrate additions were about 13 times more enriched in ^{15}N than roots in the presence of exogenous nitrogen after 8 days (Figure V-5, 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 days (t_2=-0.40, p=0.72).

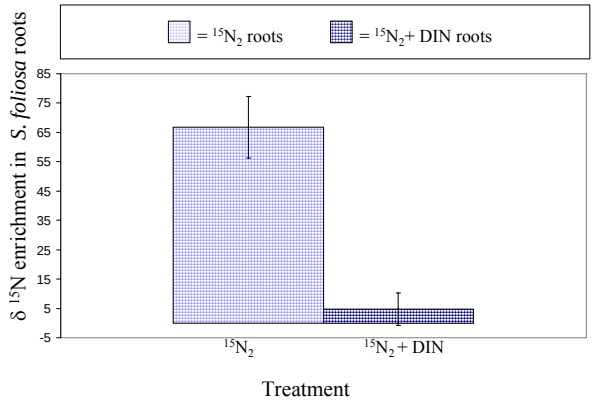

Figure V-5: Isotopic enrichment ($\delta^{15}N$) of *S. foliosa* roots (and standard errors) exposed to $^{15}N_2$ in the presence or absence of (un-enriched) ammonium nitrate (n=2) (Experiment 4). Average background values, 11 $\pm$ 4.0 (roots), have been subtracted.

The uptake of fixed nitrogen by animal consumers was assessed only after 8 days. At that time, significant enrichment was noted among 3 of the 5 taxa that were collected (Table V-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 crustacean, *Corophium* sp., were not enriched (Table V-3).

Discussion

Nitrogen fixation rates were not immediately affected by sediment and nutrient additions in the *S. foliosa* zone (Experiment 1), despite significant increases in porewater ammonium concentrations within 2 days. However, nitrogen fixation rates significantly declined after 17 days among plots receiving nitrogen additions (in the presence and absence of sediment). Increases in porewater nitrogen concentrations were significantly greater and more persistent with sediment and nitrogen added (in treatment A) than with nitrogen added only (treatment C), possibly because porewater nutrients were "sealed" by sediment inputs to the benthos. Although nitrogen fixation rates did not differ between treatments A and C in short term experiments (1 or 2), the potential for longer-term recovery of nitrogen fixation functions in response to sediment and nitrogen loading may be affected by this synergistic interaction between these different types of human impacts.

Greater stability of nitrogen fixation rates in the unvegetated sediments (Experiment 2) suggests that functional responses to sediment and nutrient additions depend on environmental contexts within an ecosystem. Although spatial scales separating *S. foliosa* and unvegetated zones were small (about 30 m), key differences in abiotic properties included higher exogenous nitrogen concentrations in the unvegetated marsh zone. This could result from less frequent tidal flushing and/or less uptake of nutrients from the environment by plants at higher, unvegetated marsh zones. Prior to manipulations, microbial communities in the unvegetated zone (Experiment 2) were fixing nitrogen at lower rates than those in the *S. foliosa* zone (Experiment 1) likely due to higher nitrogen concentrations. Diazotrophs in the higher unvegetated marsh zone may have been adapted to nutrient-rich environments. Composition of diazotroph assemblages did vary among zones; thus, the role of microbial community structure cannot be separated in this study from effects of distinct abiotic environments on functional (nitrogen fixation) responses to sediment and nutrient impacts (Reed and Martiny 2007). Ecosystems with different nutrient regimes may also vary in resistance to multiple human impacts. In particular, wetlands with histories of nutrient loading or reduced tidal flushing may have more stability (albeit lower performance) of nitrogen fixation.

Sediment additions alone did not affect nitrogen fixation rates at the levels employed in this experiment in either the *S. foliosa* (Experiment 1) or unvegetated zone (Experiment 2). Nonetheless, the one-time application of sediment in Experiment 1 and 2 (1 cm) was comparable to the vertical accretion estimated (1.3 cm) to occur annually in

the same marsh in a 5 year period with floods (Wallace et al. 2005). Manipulations were sufficient to produce significant changes in surface sediment grain size and organic content (Table V-1). These results suggest that most nitrogen fixation may have been performed by rhizosphere diazotrophs, which were not expected to be as strongly affected by the direct smothering effects of sediment additions compared to autotrophic cyanobacterial that are active in marsh surface sediments. The results of the laboratory inhibition of nitrogen fixation (Experiment 3) with sodium molybdate (which specifically inhibits sulfate reducing bacteria that would reside in plant rhizospheres, Welsh 1996), further support a dominant contribution of subsurface microbes. This is consistent with observations of nitrogen fixation in plant rhizospheres in previous wetland studies (reviewed in Lovell 2002, Welsh 2000).

The role of microbes in transferring fixed nitrogen to plants via roots has been previously described in similar isotopic enrichment studies of several salt marsh plants (Jones 1974) and seagrasses (O'Donahue et al. 1991, Capone 1988). Nitrogen fixed by both epibenthic microbes (cyanobacteria) and rhizosphere bacteria has been shown to be transferred on short time scales (7 days) to marsh plants, although extents of enrichment varied among plant species (Jones 1974). Stronger contributions of rhizosphere diazotrophs to nitrogen fixation, compared to epibenthic counterparts, have been noted in several wetland ecosystems (reviewed in Welsh 2000, Lovell et al. 2005).

Nitrogen fixing assemblages either responded to ammonium nitrate additions via an increase in diversity (as observed in surface sediments) or by not changing (as found in *S. foliosa* rhizospheres). Despite maintenance (and even increases) of diazotroph diversity, nitrogen fixation rates were not sustained 17 days following the experimental manipulations. These results reveal limits to the functional redundancy of nitrogen fixing microbes and reflect high regulation of this energetically expensive metabolic process. This work demonstrates that the loss of a microbially mediated function can occur independently of significant changes in composition and without declines in diversity of an assemblage. Similar results were found in the context of biological invasions (Moseman et al. *in press*).

The diversification and functional decline (in nitrogen fixation rates) of diazotrophs (in Experiment 1) reflect responses sometimes found among macro-organisms to small scale disturbances, in which removal of dominant members of a functional group or community can produce increased diversity (Sousa 2001). However, the single nutrient and sediment additions in this study may also have benefited some diazotrophs, as some are capable of growth in cultures with ammonium (Fritzche and Niemann 1990), and thus the experimental nutrient additions may not have constituted a disturbance or stress to the microbial assemblages despite declines in nitrogen fixation rates. For microbes, increases in diversity may thus ultimately lead to decreases in the biogeochemical processes that they mediate, through shifts in structures of key functional groups.

Changes in the abundance of particularly active members of the diazotroph assemblage likely occurred (in Experiment 1). Surface diazotroph assemblages exposed to nitrogen loading increased not only in diversity (total average number of T-RFs) but also in evenness (a metric based on relative heights of T-RF peaks among samples) compared to before the experimental manipulations. Increases in evenness among diazotrophs, in combination with decreases in nitrogen fixation rates, suggest that nutrient enrichments may have decreased activities (and possibly growth) of competitive dominants in the diazotroph assemblages, which would have permitted less competitive, ammonium-tolerant diazotrophs to proliferate. Prior studies have shown evidence of functional dominance among diazotrophs, as few of the *nif*H sequences present in coastal ecosystems are expressed at a given time (Short and Zehr 2007). A few dominant players can be responsible for most microbially-mediated functions, including productivity of microphytobenthos in Tijuana Estuary (Janousek et al. 2007). Ecosystem-level consequences of changes in the activity of functionally dominant microbes may depend the abilities of other taxa or anthropogenic nutrient inputs to "substitute" for their lost functions.

Ammonium is considered to be a major factor regulating competitive abilities of diazotrophs (and thus their diversity) (Kolb and Martin 1988). In sorghum rhizospheres, where two nitrogen treatments (12 kg N ha^{-1} and 120 kg N ha^{-1}) were applied, nitrogen additions 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 more than 90% of total diversity) was greatest among soils treated with higher amounts of nitrogen (Coelho et al. 2008), reflecting the results found in the present study of wetland diazotrophs. However, on longer time scales, diazotroph diversity may be expected to 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).

The relative robustness of diazotroph diversity in *S. foliosa* rhizospheres to ammonium nitrate additions is consistent with previous studies. A minor decrease in diversity among diazotrophs in rhizospheres of a related cordgrass, *S. alterniflora*, was only observed after 10 years of repeated nitrogen loading at somewhat lower levels (16.3 g N m^{-3}, Piceno and Lovell 2000). In the present study, rhizosphere diazotroph assemblages were also distinct compositionally (based on T-RF profiles) from those in surface sediments and had higher diversity initially compared to their epibenthic counter parts (prior to increases in surface sediments that resulted from experimental manipulations), potentially reflecting heterogeneity of rhizospheres microenvironments (Bagwell and Lovell 2000, Bowen 1980). Moreover, rhizosphere microbes may have been less strongly impacted in this study than surface dwelling diazotrophs, as the former were not directly smothered by sediments added to marsh surfaces, and nutrient loading impacts were potentially buffered via plant uptake of added nitrogen from the rhizosphere environment.

Plant-diazotroph-environment interactions in this study were dynamic, context dependent, and thus not immune to the influence of human impacts. Plant response to nitrogen inputs was evident only in treatment C (Experiment 1) after 17 days via increases in *S. foliosa* density and shoot nitrogen content. Ammonium additions may have increased *S. foliosa* density in treatment C by triggering new plant growth, which may have stimulated nitrogen fixation in rhizospheres and potentially resulted in the positive spatial relationship between density and nitrogen fixation rates after 17 days (Figure V-4A). Overall nitrogen fixation rates were still low, presumably due to the influence of nitrogen additions. However, porewater ammonium concentrations decreased between 2 and 17 days in treatment C, possibly due to plant uptake, suggesting potential for *S. foliosa* to facilitate recovery of nitrogen fixation functions. Plant-diazotroph interactions differed in conditions of both sediment and nutrient loading, in treatment A, where only negative relationships were observed (Figure V-4C). These differences were most likely not due to minor changes in surface sediment properties, but rather might be attributed to significantly higher porewater ammonium concentrations in treatment A than in other treatments. Despite high ammonium concentrations, plant properties in treatment A did not change during Experiment 1. More substantial plant responses to nitrogen additions may have occurred over longer terms (more than 17 days). Previous nutrient enrichments (at identical levels, 30 g N m^{-2}) of *S. foliosa* marsh in San Diego Bay found structural responses of plants were proportional to the duration of fertilization applications (between 1 and 6 months), with earliest changes 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). Plant growth was likely limited among all treatments due to the winter season in which this experiment was performed. Marsh access at Tijuana Estuary is prohibited during spring which is the breeding season of the endangered Clapper Rail. Nonetheless, experiments (1 and 2) were timed appropriately in this study for mimicking and testing sediment and nitrogen inputs typically associated with heavy winter rains.

Porewater ammonium was a strong mediator of plant-diazotroph relationships. Negative relationships of nitrogen fixation rates and diazotroph diversity to plant structural properties in pre-manipulation stages of the experiment and in control treatments after 17 days (Tables V-2A, V-3) likely reflected influences of porewater ammonium, which was shown in this study and others (i.e.Yoch and Whiting 1986) to inhibit nitrogen fixation while serving as a nitrogen source for plants (i.e. Langis et al. 1991, Valiela 1983). The more than 10-fold decline in ^{15}N enrichment of *S. foliosa* roots (from $^{15}N_2$) in the presence of ammonium nitrate additions (Figure V-5) was likely due not only to a decline in nitrogen fixation, and subsequent translocation from microbes to plants, but also it may have been due 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 mutualistic relationships with diazotrophs, are able to "switch" between nitrogen sources (nitrogen fixation vs exogenous nitrogen) in response to nutrient loading warrants further study as it holds major consequences for ecosystem sustainability, agricultural

practices (Oberson et al. 2007, Rueda-Puente et al. 2003), and restoration in several settings where diazotrophs play key roles (Bashan et al. 1998).

Chromic and severe nutrient loading may induce shifts in community composition among primary producers that reflect stress at ecosystem levels, particularly in communities where niche differentiation of nitrogen sources is observed (McKane et al. 2002, Moseman 2004, Kahmen et al. 2006). Shifts among primary producers (i.e. from plants to algae) are frequently observed in coastal ecosystems in response to anthropogenic nutrient loading (Herbert 1999) and changes among consumers have also been noted (Boyer and Zedler 1996, Deegan et al. 2007). This study demonstrates that simultaneous inputs of sediment may determine the long-term extent and duration of nutrient concentrations impacting coastal ecosystems. In this study, ammonium nitrate inputs significantly decreased nitrogen fixation rates, and thus affected the transfer of recently fixed nitrogen from microbes to *S. foliosa* roots (along pathways traced via isotopic enrichment techniques). Longer term nitrogen loading may not only disfavor plant species, such as *Spartina foliosa,* that have developed intimate associations with diazotrophs in nitrogen-limited environments (Moseman 2007), but also they may ultimately affect plant productivity and community structures by favoring strong competitors for exogenous nitrogen, including *Salicornia* (or *Sarcocornia*) species and algae.

Broader consequences of declines in nitrogen fixation in response to sediment and nutrient loading include not only shifts in plant-microbe interactions, and cascading

consequences for plant community composition, but also effects on ecosystem food webs (Brietburg et al. 1999). Isotopic enrichment studies revealed uptake of fixed nitrogen, traced via $^{15}N_2$, among most of the macrofaunal species collected (Table V-3). Animals may obtain fixed nitrogen by consumption of cyanobacteria, as previously reported in this marsh (Moseman et al. 2004). Cyanobacteria are components of microalgal communities known to be major food sources, particularly in invaded (Levin et al. 2006) and developing (Currin et al. 1995) wetland ecosystems. Rhizosphere diazotrophs such as sulfate reducers could also be consumed by subsurface feeding animals, such as *Capitella* spp. (Table V-3). Faunal grazing on ^{15}N-labelled microbial biomass was observed via isotopic enrichment of dissolved inorganic nitrogen, urea, and amino acids in an intertidal mud bank (Veuger et al. 2007). Plants may mediate other possible trophic pathways for fixed nitrogen to reach animal consumers including transfer via organic exudates from roots or via plant detritus (Levin et al. 2006, Whitcraft et al. 2006), the latter of which may increase in importance on longer time scales than those investigated in the present study.

A growing volume of studies highlight 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 (Klironomous et al. 2000, Reynolds et al. 2003). As sedimentation increasingly plagues coastal regions (Thrush et al. 1994), and anthropogenic inputs of nitrogen increase on global scales (Galloway 1995, Howarth et

al. 2006), significant shifts in the function and structure of key microbial groups can occur and may have cascading consequences for dynamics of many ecosystems.

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Tables

Table V-1: Environmental properties (average ± standard error) in the Friendship marsh before and after sediment and nitrogen additions (A) in the *S. foliosa* zone (Experiment 1) and (B) in the unvegetated zone (Experiment 2).

(A) Experiment 1: *S. foliosa* zone

	Premanipulation Bulk sediment	2 days later Bulk sediment	Top 2 cm sediment*
Sediment % clay (% grain size < 63 μm)	70 ± 0.04	A: 45± 13 B: 69 ±11 C: 40± 15 D: 51 18	A: 85 ±3.7 B: 82 ± 3.2 C: 94 ± 1.1 D: 92 ± 1.6
Sediment % organic matter	8.4 ± 0.5	A: 7.7± 1.6 B: 9.9 ± 3.1 C: 17 ± 5.0 D: 15 ± 6.8	A: 5.4 ± 1.0 B: 4.4 ± 0.8 C: 8.8 ± 0.3 D: 8.7 ± 0.9
	Premanipulation	2 days later	17 days later
Porewater ammonium (μM)	52 ± 8	A: 420 ± 120 B: 25 ± 6 C: 260 ± 99 D: 13 ± 2	A: 359 ± 90 B: 45± 16 C: 54 ± 16 D: 53± 13

(B) Experiment 2: Unvegetated zone

Unvegetated Bulk sediment	Premanipulation	2 days later
Sediment % clay	65 ± 0.09	A: 35 ±0.14 B: 31 ± 0.42 C: 46 ± 0.13 D: 48 ± 0.17
Sediment % organic matter	5.2 ± 0.6	A: 12 ± 4 B: 8.3 ± 0.8 C: 8.8 ± 0.8 D: 8.3 ± 0.3
Porewater ammonium (μM)	195 ± 40	A: 1353 ± 124 B: 352 ± 58 C: 861 ± 109 D: 396 ± 102

* sediment properties after 17 days were analyzed for top 2 cm only, while on other dates bulk sediments (0-6cm) were measured

Table V-2: Regression coefficients, p-values, and types of relationships (in "?" column) for linear or quadratic relationships of nitrogen fixation rates (dependent variable) to plant and abiotic factors (independent variable) (best fit shown) after 17 days (Experiment 1); U= non linear relationship with minimum nitrogen fixation rates at intermediate levels of the given environmental factor; + = positive, - = negative, o= none; Significant relationships are in bold.

	A			B			C			D		
	r	p	?	r	p	?	r	p	?	r	p	?
Height	0.18	0.24	o	0.17	0.63	o	0.18	0.23		0.78	0.01	_
Density	0.44	0.04	-	0.28	0.14	o	0.59	0.02	+	0.09	0.45	
shading	0.07	0.50	o	<0.01	0.99	o	0.11	0.37	o	0.50	0.03	+
Below-ground biomass	<0.01	0.82	o	0.19	0.28	o	0.01	0.81	o	0.41	0.35	o
Above-ground biomass	0.34	0.35	o	0.28	0.14	o	0.44	0.01	+	0.37	0.06	_
Total biomass	0.02	0.73	o	0.02	0.74	o	0.08	0.53	o	0.35	0.10	_
Shoot N content	0.57	0.25	o	0.42	0.41	o	0.04	0.68	o	0.58	0.13	o
NH4+	<0.01	0.91	o	0.04	0.28	o	0.12	0.34	o	0.45	0.07	_
% clay	0.11	0.38	o	<0.01	0.82	o	<0.01	0.95	o	0.04	0.70	o
% org. content	0.30	0.13	o	0.42	0.15	o	0.01	0.89	o	0.10	0.40	o

Table V-3: $\delta^{15}N$ values observed for macrofaunal taxa in enclosures exposed to subsurface $^{15}N_2$ for 8 days (Experiment 4)

Taxa		**$\delta^{15}N$**	**control**
Polychaete	capitellid	127	12
		1864	
		13	
		14	
	spionid	1642	10
		18	
Insect	chironomids and dolichopods	599*	19
Gastropod	*Cerithidea californica*	14	7
		8	
		9	
		7	
		28	
		18	
Crustacean	*Corophium* sp.	12	11

*= both insect taxa combined in one sample (for sufficient mass for analyses) and control values are available from dolichopod only

**= control values were not available in this experiment and are thus presented from prior work in the Friendship marsh during 2002 (L.Levin, unpublished)

Appendix V-1: Regression coefficients, p-values and types of relationship (in "?" column) between diversity of surface (0-1 cm) and rhizosphere (4-5 cm) diazotrophs and plant or abiotic factors in the *S. foliosa* zone (Experiment 1) (V-1A) prior to and (V-1B) 2 days and (V-1C) 17 days following manipulations: (+)= positive linear, (-)= negative linear, (u)= nonlinear, o= no relationship

Appendix V- 1A

	A			B			C			D		
	r	p	?	r	p	?	r	p	?	r	p	?
Height	0.18	0.26	o	0.17	0.63	o	0.18	0.25		0.78	0.01	_
Density	0.44	0.04	-	0.28	0.14	o	0.59	0.02	+	0.09	0.45	
shading	0.07	0.50	o	<0.01	0.99	o	0.11	0.37	o	0.50	0.03	+
Below-ground biomass	<0.01	0.82	o	0.19	0.28	o	0.01	0.81	o	0.41	0.35	o
Above-ground biomass	0.34	0.35	o	0.28	0.14	o	0.66	0.01	+	0.37	0.06	_
Total biomass	0.02	0.73	o	0.02	0.74	o	0.08	0.53	o	0.35	0.10	_
Shoot N content	0.57	0.25	o	0.62	0.61	o	0.04	0.68	o	0.58	0.13	o
NH4+	<0.01	0.91	o	0.04	0.28	o	0.12	0.36	o	0.45	0.07	_
% clay	0.11	0.38	o	<0.01	0.82	o	<0.01	0.95	o	0.04	0.70	o
% org. content	0.30	0.13	o	0.62	0.15	o	0.01	0.89	o	0.10	0.60	o

Appendix V-1B

	Treatment A (2 days)			Treatment B (2 days)			Treatment C (2 days)			Treatment D (2 days)		
0-1 cm	r	p	?	r	p	?	r	p	?	r	p	?
Individual height	**0.85**	**<0.01**	-	<0.01	0.93	O	0.12	0.83	O	0.03	0.71	O
Below-ground biomass	0.01	0.79	O	**0.58**	**0.05**	-	0.30	0.34	O	0.29	0.27	O
Above-ground biomass	**0.49**	**0.05**	-	0.47	0.38	O	0.13	0.87	O	0.77	0.48	O
Total biomass	0.04	0.12	O	0.25	0.39	O	0.30	0.45	O	0.83	0.27	O
Pore-water ammonium	0.25	0.42	O	0.07	0.52	O	0.10	0.50	O	0.09	0.79	O
% clay (< 63 μm) sediment	0.38	0.10	+	<0.01	0.94	O	<0.01	0.96	O	0.29	0.59	O
% organic content of sediment	0.03	0.66	O	<0.01	0.92	O	0.10	0.49	O	<0.01	0.92	O

	Treatment A (2 days)			Treatment B (2 days)			Treatment C (2 days)			Treatment D (2 days)		
4-5 cm	r	p	?	r	p	?	r	p	?	r	p	?
Individual height	<0.01	0.02	O	<0.01	0.92	O	0.02	0.78	O	0.24	0.26	O
Below-ground biomass	0.34	0.17	?	0.06	0.63	O	0.59	0.07	+	0.01	0.85	O
Above-ground biomass	**0.58**	**0.05**	+	0.30	0.70	O	0.34	0.42	O	0.30	0.45	O
Total biomass	**0.64**	**0.03**	+	0.04	0.79	O	0.24	051	O	0.61	0.43	O
Pore-water ammonium	0.25	0.20	O	0.02	0.95	O	0.02	0.75	O	<0.01	0.98	O
% clay (< 63 μm) sediment	0.29	0.50	O	0.26	0.30	O	0.25	0.31	O	<0.01	0.87	O
% organic content of sediment	0.08	0.53	O	0.16	0.44	O	0.15	0.39	O	<0.01	0.97	O

Appendix V-1C

	Treatment A (17 days)			Treatment B (17 days)			Treatment C (17 days)			Treatment D (17 days)		
0-1 cm	r	p	?	r	p	?	r	p	?	r	p	?
Ind. height	0.04	0.60	O	0.01	0.77	O	0.12	0.82	O	0.02	0.95	O
Below-ground biomass	0.15	0.31	O	0.24	0.21	O	0.04	0.69	O	0.36	0.21	O
Above-ground biomass	0.13	0.34	O	0.02	0.74	O	0.12	0.40	O	0.05	0.64	O
Total biomass	0.16	0.27	O	0.01	0.77	O	0.01	0.85	O	0.15	0.44	O
NH_4^+	0.40	0.09	+	0.21	0.49	O	0.40	0.63	O	**0.61**	**0.04**	+
% clay	0.02	0.72	O	0.24	0.22	O	**0.56**	**0.04**	-	0.10	0.61	O
% organic content	0.09	0.43	O	<0.01	0.98	O	0.05	0.60	O	0.07	0.72	O

4-5 cm	r	p	?	r	p	?	r	p	?	r	p	?
Ind. height	0.13	0.42	O	<0.01	0.93	O	0.31	0.39	O	0.24	0.26	O
Below-ground biomass	0.18	0.34	O	0.14	0.73	O	0.51	0.11	O	**0.81**	**0.01**	+
Above-ground biomass	0.28	0.22	O	0.03	0.68	O	0.03	0.70	O	0.26	0.24	O
Total biomass	0.28	0.22	O	0.01	0.98	O	0.51	0.11	O	0.10	0.54	O
NH_4^+	**0.88**	**0.01**	**U**	0.32	0.14	O	**0.50**	**0.05**	-	0.50	0.08	-
% clay	0.49	0.09	-	0.06	0.88	O	0.45	0.22	O	**0.83**	**0.03**	+
% organic content	<0.01	0.98	O	0.43	0.16	O	<0.01	0.96	O	0.28	0.47	O

Appendix V-2: Regression coefficients, p-values and types of relationship (in "?" column) between nitrogen fixation rates and plant or abiotic environmental factors prior to and 2 days after initiation of Experiment 1: (+)= positive linear relationship, (-)= negative linear relationship (u)= nonlinear relationship, o= no relationship

	Pre			2A			2B			2C			2D		
	r	p	?	r	p	?	r	p	?	r	p	?	r	p	?
Height	0.10	0.09	O	<0.01	0.85	O	0.62	0.09	U	0.09	0.82	O	0.82	<0.01	-
Density	<0.01	0.66	O	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Below-ground biomass	<0.01	0.63	O	0.75	0.01	+	0.36	0.15	O	0.18	0.40	O	0.28	0.22	O
Above-ground biomass	<0.01	0.88	O	038	0.31	O	001	0.82	O	0.60	039	O	<0.01	0.96	O
Total biomass	<0.01	0.92	O	0.21	0.25	O	0.85	0.15	O	0.51	0.28	O	0.28	0.46	O
Sheet N content	0.23	0.06	+	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
NH4+	<0.01	0.98	O	0.16	0.32	O	0.62	0.14	O	0.36	0.21	O	0.40	0.35	O
% clay	0.14	0.02	-	0.11	0.43	O	0.29	0.60	O	0.80	0.01	+	0.25	0.31	O
% org. content	<0.01	0.74	O	0.38	0.10	-	0.16	0.30	O	0.14	0.41	O	039	0.19	O

CHAPTER VI

FUNCTIONAL DISPARITY IN NITROGEN FIXATION AMONG RESTORED AND NATURAL SALT MARSHES OF THE VENICE LAGOON, ITALY

Abstract

To test the effects of site and successional stage on nitrogen fixation rates in salt marshes of the Venice Lagoon, acetylene reduction assays were performed with *Salicornia veneta*- and *Spartina townsendii*- vegetated sediments from 3 restored (6-14 years) and 2 natural marshes of the Venice lagoon. Average nitrogen fixation (acetylene reduction) rates ranged from 31 to 343 μmol C_2H_4 m^{-2} h^{-1} among all marshes, roughly 3 times higher than those reported from southern California marshes of similar Mediterranean climate. Nitrogen fixation rates did not consistently vary between natural and restored marshes within a site (Fossei Est, Tezze Fonde, Cenessa) but were negatively related to plant biomass among all marshes. Highest nitrogen fixation rates were found at Tezze Fonde, where plant biomass was lowest, suggesting early stages of marsh succession in both natural and restored marshes at this location.

Introduction

Nitrogen transformations are a key part of wetland ecosystem functions. In these ecosystems which are frequently nitrogen-limited, microbes (diazotrophs) sustain nutrient pools by converting N_2 in the atmosphere to reduced forms that can be used by plants and algae in the process of nitrogen fixation (Herbert 1999). Most studies of nitrogen fixation

have focused on *S. alterniflora*-dominated marshes of the Atlantic coast of the U.S. (reviewed in Lovell et al. 2005). Patterns of nitrogen dynamics may vary in marshes such as those of the Mediterranean and Pacific coasts, where quite different plant species dominate landscapes (Hopkinson and Giblin *in prep*).

Wetlands of Mediteranean climate (in Europe and southern California) experience much hotter and drier conditions that could induce higher salinity stress. As nitrogen is a requirement for osmoregulation as well as growth (Cavalieri and Huan 1981), nitrogen fixation may more important to wetland plants under these climatic conditions than those in moister climatic regions (i.e. U.S. Atlantic coasts). Contrasts of nitrogen fixation rates across climatic regions might also be useful for predicting potential responses of wetland nitrogen dynamics to global warming.

Wetlands of the Venice Lagoon (Italy) may particularly reveal functional responses of these ecosystems to climatic stress. In addition to warm and arid Mediterranean climates, marshes (and cities) of the Venice Lagoon are experiencing exacerbated subsidence rates (Ferla et al. 2007) that may be used to predict potential influences of anticipated sea level rise on functional dynamics of wetland ecosystems.

No prior studies of nitrogen fixation are known from the Venice Lagoon. However, studies of restored and natural salt marshes in southern California which experience a similar Mediterranean climate have shown low but variable rates of nitrogen

fixation (Langis et al. 2001, Moseman 2007, Moseman et al. submitted). In San Diego Bay (CA), rates of nitrogen fixation were higher in a natural *Spartina foliosa* marsh than a 7- year old restored one and were attributed to lower organic matter content in the early succession ecosystem. In contrast, the opposite pattern was observed in Tijuana Estuary, with lower nitrogen fixation rates in a natural *S. foliosa* marsh being attributed to higher pools of porewater ammonium than in the 5-6 year old restored marsh (Moseman et al. *submitted*), as organic content of the marsh sediments were comparable. In order for restoration efforts to appropriately identify targets for functional equivalency among restored and natural marshes, such site-specific variability among nitrogen fixation rates and its controls must be better constrained. Further, current dogma regarding controls on nitrogen fixation rates, based on *S. alterniflora* marshes of the Atlantic U.S. coasts, may not apply to wetlands in other regions of the world.

The following questions were addressed regarding nitrogen fixation in salt marshes of the Venice Lagoon:

1) Do nitrogen fixation rates vary as a function of site (Tezze Fonde, Fossei Est, and Cenessa) in different parts of the Venice Lagoon?
2) At each site in the Venice Lagoon (Tezze Fonde and Fossei Est), do nitrogen fixation rates differ between restored and natural marshes?
3) What role might nitrogen fixation play in salt marsh succession in the Venice Lagoon?
    a) Are rates related to plant biomass or shoot tissue nitrogen content?

4) How do Venice salt marsh nitrogen fixation rates compare to those reported from salt marshes in other regions?

Methods

Nitrogen fixation activity was measured via acetylene reduction in two pairs of natural and created salt marshes, at Fossei Est and Tezze Fonde, in the Venice Lagoon, Italy during June 2006 (Figure VI-1).

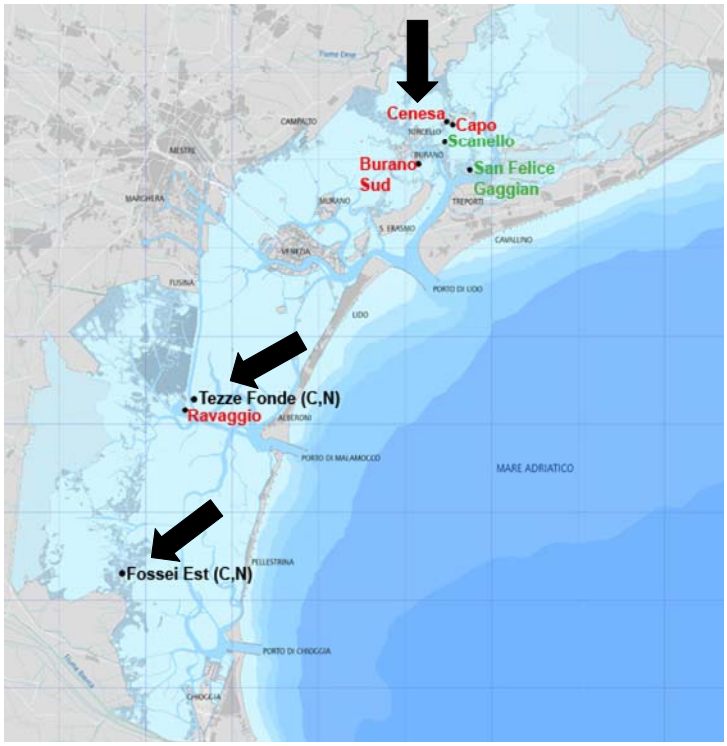

Figure VI-1: Map of marshes within the Venice Lagoon, Italy with field sites marked by arrows

Restored marshes at Fossei Est and Tezze Fonde were 12-14 years old. One single restored 6 year-old marsh, at Cenesa, in the northern part of the lagoon was also studied. The dominant plants in these salt marshes were *Puccinellia palustris, Sarcocornia*

fruticosa, and *Salicornia veneta* at high, middle, and low elevations, respectively. This study focused on nitrogen fixation activity within *Salicornia* patches, typically found fringing unvegetated ponds in both the natural and created marshes (Figure VI-2).

Figure VI-2: Image of *Salicornia veneta* plant in pond habitat characteristic of those sampled in this study (which frequently featured visible microalgal and bacterial mats)

Cores (2.1 cm, approximately 5 cm deep) were centered around single *Salicornia veneta* plants (except as described below) and extracted to include sediment as well as root material. The numbers of samples varied among marshes, and some *Spartina townsendii* samples were collected when the invasive species was found at Fossei Est. Samples collected for acetylene reduction assays are described in Table VI-1.

Table VI-1: Summary of the location and type of vegetated sediment cores collected for acetylene reduction assays in this study (between June 19-June 21, 2006).

Site	Marsh type	*Salicornia veneta* cores (count)	*Spartina townsendii* cores (count)
Fossei Est	Natural	7	5
Fossei Est	restored	10	2
Tezze Fonde	Natural	7	0
Tezze Fonde	Restored	7	0
Cenessa	Restored	10	0

Sampling sites matched those used for macrofaunal sampling by the Levin research group. At Cenessa, with 4 samples containing *Salicornia* within ponds were taken and the rest taken from slightly higher elevations. One sample in each marsh was used as a blank to check for natural ethylene production rates. Bottoms of samples were sealed with plastic-wrapped aluminum foil and cores were stored on ice for transport to the laboratory. They were processed within 5 hours of field sampling.

All plants were dried at 60 ^{o}C and weighed for determination of total plant biomass. A section of *Sarcocornia veneta* shoot tissue was extracted from a subset of these plant samples (Fossei Est n=6, Tezze Fonde n=3-5, Cenessa n=3), acidified, dried and analyzed for percentage of nitrogen content at the SIO analytical facility. Bulk sediment properties (grain size and organic content) were determined from surface sediment scoops collected by the Levin research group (n=7) and were processed as previously described (Moseman et al. 2004).

Acetylene reduction assays

Samples were extruded into 250-ml flasks which were sealed at top with rubber stoppers and gas-tight tape as previously described (Moseman 2007). The assays were conducted under aerobic conditions at Thetis laboratories (Venice, Italy). Flasks containing the samples were incubated outdoors in an open plastic tub placed on a grass lawn in indirect sunlight. Ambient temperatures and those in the flask were measured and remained below 27 ^{o}C. Incubations were initiated within 3 hours of sample collection and processed immediately upon return to the laboratory. Incubation times were 7 pm for the Fossei Est samples, although assays were completed prior to sunset. Samples from Cenesa and Tezze Fonde were assayed at 3 and 4 pm respectively. Subsamples (2.5 ml) of the headspace gases were collected immediately and 2-2.5 hours after initiation of the assay. They were stored in gas-tight Vacutainers and analyzed at SIO on an FID-equipped gas chromatograph in peak height mode (Capone and Montoya 2001).

Statistical analyses

Nitrogen fixation rates were compared between all marshes via a one-way ANOVA. Natural and restored marsh pairs were compared with t-tests, or in cases where variances were unequal, non-parametric Wilcoxon tests were applied. Relationships between nitrogen fixation rates and plant biomass or tissue nitrogen content were tested via linear regressions. All statistics were performed with JMP 4.0 software.

Results

Nitrogen fixation rates were significantly higher in Tezze Fonde than in Fossei Est and Cenessa (Figure VI-3, $F_{4,41}$=3.33, p=0.02). At Fossei Est significantly higher nitrogen fixation rates were found in the natural marsh than its restored counterpart (Wilcoxon z=-3.28, p< 0.01), while rates did not differ between restored and natural marshes of Tezze Fonde (t_6=0.88, p=0.41).

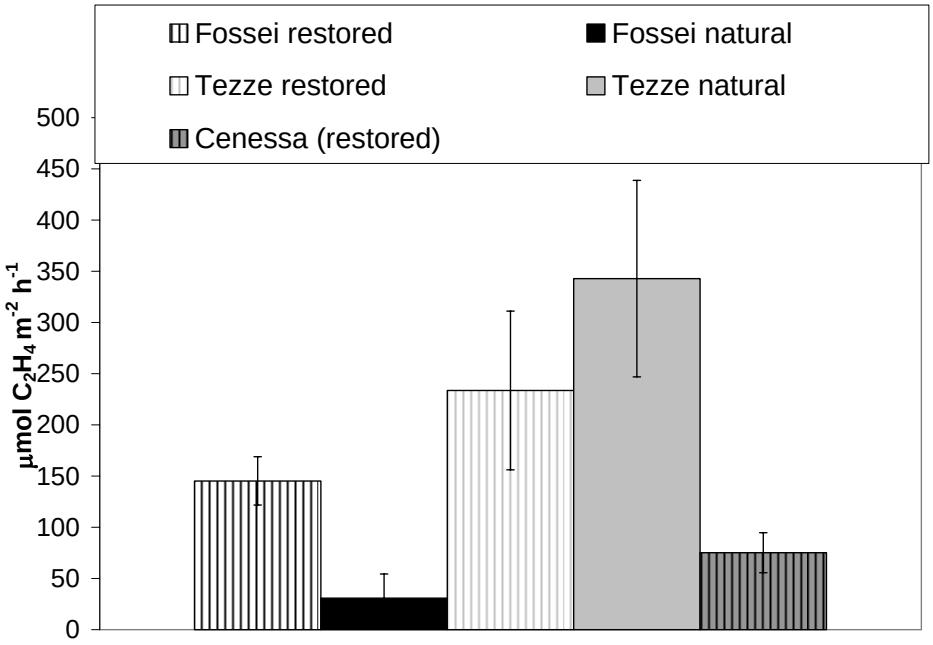

Figure VI-3: Average nitrogen fixation (acetylene reduction) rates and standard error bars in salt marshes of Venice Lagoon, Italy

Nitrogen fixation rates were negatively related to *S. veneta* biomass (r^2=0.11, p=0.06, n=32) across all of the Italian marshes in this study but were unrelated to tissue nitrogen content in *S. veneta* shoots (r^2=0.14, p=0.13, n=17). The natural marsh of Fossei Est, which had lowest nitrogen fixation rates, also had significantly greater belowground biomass ($F_{4,45}$=4.67, p<0.01) than all other marshes.

Sediment organic content was significantly higher in the natural marsh at Fossei Est than in both marshes at Tezze Fonde ($F_{3,19}$= 4.21, p=0.02). Sediment grain size was greater, with less clay content (< 63 μm), in the created marshes at Tezze Fonde and Fossei Est than the natural marshes at those sites ($F_{3,19}$=3.10, p=0.05, Table VI-2). Sediment bulk properties were not determined for Cenessa.

When invasive cordgrass (*Spartina townsendii*) samples from Fossei Est were excluded from analyses, *S. veneta* tissue nitrogen content was significantly greater in the natural marshes of Fossei Est and both marshes of Tezze Fonde than in the created marshes of Fossei Est and Cenessa ($F_{4,18}$=3.45, p=0.04). Nitrogen fixation rates in sediments of Fossei Est containing the invasive cordgrass did not differ from those of uninvaded *S. veneta*- vegetated sediments (Wilcoxon z=-1.21, p=0.22).

Table VI-2: Summary of average sediment and plant parameters (and standard errors) in salt marshes of the Venice Lagoon; nd= not determined

Bulk Sediment properties	Tezze Fonde restored	Tezze Fonde natural	Fossei Est restored	Fossei Est natural	Cenessa (restored)
Clay content (%)	71 ± 6	86 ± 3	83 ± 3	87 ± 2	nd
Organic content (%)	21 ± 1	25 ± 2	22 ± 1	34 ± 0.5	nd
Plant biomass* (g)	0.6 ± 0.3	0.3 ± 0.06	0.5 ± 0.2	1.3 ± 0.2	0.4 ± 0.09
Shoot N content (%)	3.2 ± 0.4	3.1 ± 0.3	2.3 ± 0.9	3.8 ± 0.3	2.8 ± 0.1

* Total biomass of the plant collected within acetylene reduction sample

Discussion

Significant differences in nitrogen fixation were found between regions of the Venice Lagoon, with substantially higher rates in the central marshes at Tezze Fonde than in those of the southern region of the lagoon at Fossei Est. These differences in nitrogen fixation rates among sites of the Venice Lagoon may reflect lower porewater nutrient concentrations of sediments at Tezze Fonde than Fossei Est, as suggested by prior work in southern California (Moseman et al. *submitted*). Among all marshes, sediment organic content was highest in the natural marsh at Fossei Est, which could indicate sufficient successional development in this marsh for porewater nutrient reserves to accumulate (and thus limit nitrogen fixation rates). Sediments used in the construction of restored marshes Fossei Est were from distinct sources and had higher peat and humic content than those in central and northern sites (A. Rismondo, personal communication). Sediments at Fossei Est may have offered more nutrients than sediments of central and northern marshes via regeneration of organic matter.

Sediment chemistry may also be a factor affecting nitrogen fixation rates and plant growth. The high organic matter content at Fossei Est may not only indicate possible accumulation of ammonium but also of sulfide as both are products of organic matter regeneration. Among Italian classification schemes of sediment toxicity, the marsh of Tezze Fonde contained type A and B sediments, while Fossei Est only contained those of type A (Apitz et al. 2007). It is also possible that properties of type B sediments stimulated nitrogen-fixing microbes more than those of type A sediments, not just that type A properties inhibited them. Factors known to promote nitrogen fixation rates

include labile organic carbon for heterotrophic diazotrophs in plant rhizospheres and light availability for autotrophic diazotrophs like cyanobacteria. An abundance of the latter was visibly evident among all marshes in this study (Figure VI-2).

Nitrogen fixation is often a significant component of wetland ecosystem function. The observation that the two marshes with the highest nitrogen fixation rates (at Tezze Fonde) also had among the highest nitrogen content of plant shoots (Table VI-1) suggests that nitrogen fixed by microbes may support primary producers, particularly in early successional ecosystems. As higher nitrogen fixation rates have been noted in developing marshes with little organic matter, low nutrient reserves, and poor development of plant canopies (Tyler et al. 2003, Moseman et al. submitted), the higher activities in Tezze Fonde (Figure VI-3) may indicate both marshes at this site are in earlier successional stages than other those in other sites of the lagoon. Specifically, the low plant biomass in sediment samples collected Tezze Fonde, compared to Fossei Est (Table VI-1), is consistent with less developed plant cover, possibly resulting from disturbance or stress. Negative relationships between nitrogen fixation rates and plant biomass among all of the Italian marshes further support notions that as plant cover develops, in advanced stages of marsh succession, nitrogen fixation rates decrease. Plants may be competing with nitrogen-fixing microbes on surfaces of marsh sediments by blocking light or usurping space for microbial mat development. Trends of higher rates were observed in *Salicornia virginica* marshes of southern California among clipped plots compared to unclipped ones (t_5=1.88, p=0.06, S. Moseman unpublished data). In addition to offering a source of

nitrogen to primary producers, nitrogen fixing microbes can play key trophic roles (MAGIS, ACQUE, SIOSED 2008).

The broader significance of nitrogen fixation to nutrient cycling will depend on relative magnitudes of this process compared to denitrification. In the northern region of Venice lagoon (near Cenessa), rates of denitrification (< 1 mg N m^{-2} h^{-1}) during summer months (Eriksson et al. 2003) were comparable to rates of nitrogen fixation reported here (0.7 mg N m^{-2} h^{-1}). Temporal and spatial dynamics of both of these nitrogen transformations require better quantification to fully understand functions of wetlands as sources or sinks of nitrogen in the Venice Lagoon.

Functional equivalence of restored and natural marshes will depend on regional contexts including histories of stress and disturbance. After 13 years, nitrogen fixation rates were still higher in the restored than the natural marsh at Fossei Est, while they did not differ at Tezze Fonde where activities were high among both marshes. The greater proximity of Tezze Fonde marshes to the city of Venice (Figure VI-1) compared to those at Fossei Est offers the possibility that urbanization differentially affected marshes at that site (Bettiol et al. 2005, Apitz et al. 2007). However, mechanisms of anthropogenic influence were not addressed in this study. Nonetheless, the high nitrogen fixation rates in the natural marsh at Tezze Fonde compared to Fossei Est likely reflect a more general effect of anthropogenic impacts in shifting “reference” marks against which successional progress of restored marshes can be evaluated. The restored marsh at Tezze Fonde could

be incorrectly considered equivalent in function to a natural marsh by comparison only to that at Tezze Fonde which itself seems to be in early developmental stages, as reflected by high nitrogen fixation activities. Thus, studies which address functional equivalence of wetlands in the context of restoration or disturbance must explicitly address human roles on natural systems, which are increasingly affecting marine and coastal ecosystems on global scales (Halpern et al. 2008).

Nitrogen fixation rates in this study were 3-6 times greater than those found among southern California marshes of Mediterranean climate (Langis et al. 1991, Moseman 2007, Moseman et al. *submitted*). As similar methods were applied across these systems (described in Moseman 2007), these measurements reflect true differences in microbial activity. Seasonality and higher temperatures (about 3^{o}C) likely contributed to differences in nitrogen fixation rates, as the measurements reported in this study were taken during the summer, while those in southern California were measured in the winter or fall months (Moseman 2007, Moseman et al. submitted), and prior work has shown significant seasonal variability in nitrogen fixation rates (Langis et al. 1991, Moseman et al. submitted). However, nitrogen fixation rates may also be higher in Venice Lagoon than in southern California due to distinctions in microbial abundance (that were visibly higher in Venice Lagoon, Figure VI-2) as well as substantial differences in plant cover (lower in Italy than in California), both of which likely reflect combined effects of marsh subsidence and anthropogenic stress. Subsidence may limit plant growth via stressful effects of inundation and it may also reduce concentrations of porewater nutrients via

tidal dilution or flushing, which could stimulate nitrogen fixation. Nitrogen fixation rates among *Salicornia veneta* marshes in Italy (7 to 77 mg N m^{-2} d^{-1}) were among the highest reported for coastal marshes (Hopkinson and Giblin *in prep*), perhaps because of their warmer climates and lower elevations (as in Moseman 2007).

In the context of global warming, most coasts may experience sea level rise in combination with warmer climate, likely under much moister conditions than those in Mediterranean marshes today. Effects of these complex changes on nitrogen fixation rates may be constrained by increased inputs of anthropogenic nitrogen (Howarth et al. 2006). Production and respiration in marsh sediments have been found to decline in response to experimental sea level rises (Miller et al. 2001). However, anthropogenic effects on wetland plant cover and community composition, via urbanization and land conversion (Whitcraft and Levin 2006, Thrush et al. 2004, Chapter V) and biological invasions (Moseman et al. *in press*), will likely play key roles in moderating microbial responses to global climate change (Rogers et al. 1994, Reynolds et al. 2003).

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CHAPTER VII

CONCLUSIONS

Nitrogen is a key nutrient for plant growth in most major agricultural and natural ecosystems (Vitousek and Howarth 1991). In order to obtain nitrogen, some plants benefit from interactions with microbial communities capable of converting abundant atmospheric nitrogen (N_2) into biologically available forms (NH_4^+) through the process of nitrogen fixation (Lovell et al. 2005, Rosch et al. 2005). In coastal wetlands, primary production is often nitrogen limited (Covin and Zedler 1988, Boyer and Zedler 1998, Boyer et al. 2001, Traut 2005) but is essential to functions such as water purification, coastal stabilization, and habitat provision for animals (including commercially valuable fishes and federally endangered birds) (Figure VII-1, Zedler 1991, Levin et al. 2001). Efforts to conserve and restore functional aspects of wetlands have been constrained, however, by a need to better understand controls on microbially mediated processes (Deevey 1970) and dynamics of interactions between plants and microorganisms. As human populations grow along coastlines of the world, they exert increasing stresses on coastal wetlands through introduction of invasive species (Crooks and Ruiz 2001) and modifications of watersheds (associated with urban development and deforestation) that exacerbate sediment and nutrient loading to these ecosystems (Thrush et al. 1994). The independent and combined effects of these impacts on ecosystem structure and function are likely to be mediated in part by nitrogen fixing microbes.

This dissertation research characterized interactions between nitrogen-fixing microbes (diazotrophs) and vascular plants in coastal wetlands in an effort to understand environmental controls on nitrogen fixation and its consequences for ecosystem structure and function. Nitrogen-fixing microbes in wetlands include photosynthetic cyanobacteria (on marsh surface sediments) and plant root (rhizosphere)-associated sulfate reducing bacteria (Figure VII-1). Combinations of biochemical, molecular, and experimental field techniques were applied to explore how several types of human impacts (biological invasion, restoration, nutrient loading, and sedimentation) affect the diversity and activity of diazotrophs as well as their interactions with wetland plants.

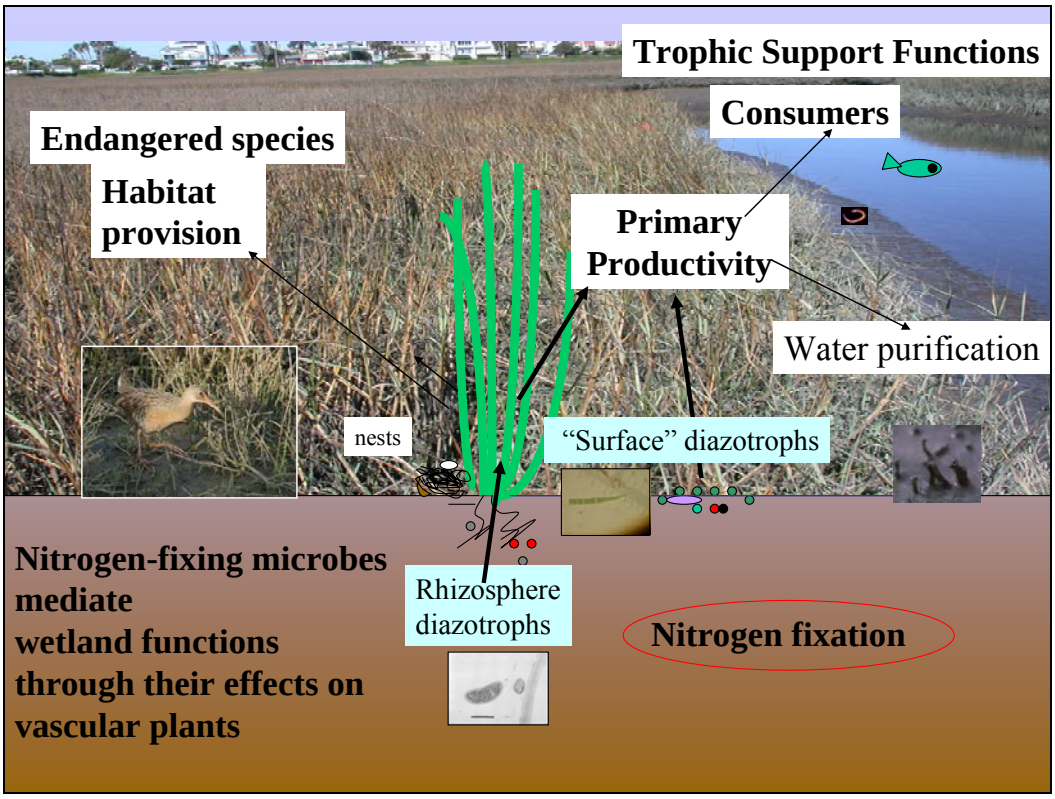

Figure VII-1: Illustration of interactions by which nitrogen fixing microbes indirectly support a range of plant-dependent functions in wetland ecosystems

A summary of results from this dissertation is presented below, followed by a review of relationships found between diazotroph diversity and function, and a discussion of implications from this work for wetland management.

Summary

Nitrogen fixation activity displays opposite temporal patterns among the two dominant wetland plants in Pacific coast marshes, the cordgrass, *Spartina foliosa*, and the pickleweed, *Salicornia virginica* (also known as *Sarcocornia pacifica*, Chapter II). Daytime rates of nitrogen fixation are significantly higher in *S. foliosa*-vegetated sediments and also more tightly coupled with photosynthetic activities than in those of *S. virginica*. The findings offer a new mechanism to explain the relatively poor ability of *S. foliosa* to compete against *S. virginica* for exogenous nutrients in the environment (Boyer and Zedler 1999), as ammonium inhibits nitrogen fixation. These results also highlight a new way that nitrogen sources may be partitioned between these key species, suggesting that *S. foliosa* has a greater reliance on nitrogen fixation. These results yield predictions that cordgrasses may be susceptible to competition with other higher-marsh plants such as pickleweed under conditions of excessive nutrient loading (i.e. eutrophication) due in part to stronger interactions with nitrogen-fixing microbial communities.

Invasive species (accidentally or intentionally introduced to wetlands by humans) also vary in their interactions with nitrogen fixing microbes. Key invaders in southern California wetlands are salt cedar (*Tamarix* spp.), a mangrove (*Avicennia marina*), and an

Asian mussel (*Musculista senhousia*, Moseman et al. 2007). Mensurative experiments reveal that effects of these invasive plant and animal species on nitrogen fixation rates in wetland sediments vary substantially and depend on the environmental context of the sites they invade (Chapter III). Nonetheless, common trends of lower diazotroph diversity among *Tamarix*- invaded marsh sediments and cocoons of the invasive *M. senhousia*, compared to native marsh sediments, demonstrate the potential for invaders to alter wetland communities at the most fundamental (microbial) levels. Although redundancy of microbes within key groups is proposed to sustain their functions against disturbance (Bagwell and Lovell 2000), this research reveals limits in the extent to which diazotroph diversity confers functional stability. Further, shifts in interactions of plants with nitrogen-fixing microbes may constitute new mechanisms by which invasions modify wetland ecosystems (Hawkes et al. 2005).

At Tijuana Estuary (CA), higher nitrogen fixation rates are observed during Fall seasons in an early successional, restored marsh than its natural counterpart (Chapter IV). Differences in nitrogen fixation rates between marshes reflect disparities in the composition of diazotroph communities and in plant structure. These patterns are consistent with diazotroph facilitation of plant growth in early stages of marsh development. Within the early successional marsh at Tijuana Estuary, novel positive relationships between the diversity of nitrogen fixers, nitrogen fixation rates, and height of the cordgrass, *S. foliosa* (Chapter IV), link microbial assemblages to wetland productivity, and habitat-provision functions of plants. These results tie diazotrophs to

nesting success of the endangered Light-footed Clapper Rail in southern California, which needs tall plants for protection of nests that float with incoming tides (Zedler 2001). In Atlantic-coast marshes, similar links may tie microbes to higher trophic levels including the mummichog, *Fundulus heteroclitus*, which reproduces by attaching eggs to *Spartina alterniflora* plants at particular heights (5-10 cm) above the marsh surface (Taylor et al. 1977). Changes in microbial functions may thus have cascading consequences that indirectly affect highest consumers in these ecosystems, and management approaches may benefit from facilitating positive interactions between nitrogen fixers and plants among restored marshes (i.e. by minimizing nitrogen inputs via pollution).

Nitrogen fixation activity is not always a simple function of marsh age, as illustrated by differences among 2 natural and 3 restored *Salicornia veneta* marshes of Venice Lagoon, Italy (Chapter VI). A natural and a restored marsh at Tezze Fonde both display significantly high nitrogen fixation rates, suggesting an influence of anthropogenic activities in maintaining these marshes in early successional stages. An observed negative relationship between plant biomass and nitrogen fixation rates among marshes of both Italy and southern California (Figure VII-2) may relate to subsidence in Italy, which can restrict *Salicornia* growthdue to stress of inundation, but may stimulate nitrogen fixation through flushing and dilution of porewater ammonium concentrations with overlying tidal waters. Porewater ammonium concentrations are not known in the marshes of the Venice Lagoon, but they decline with elevation between marsh zones of

southern California (Chapter V) and thus may vary between marshes of different elevations as well. A combination of low plant cover, high light availability, and low nutrient (ammonium) pools may stimulate microbial abundance and activity, to produce the high nitrogen fixation rates in Italy (Chapter VI). Temperatures are also higher in Italy (by about 3 ^{o}C) than in southern California and likely enhanced rates as well. In the process of marsh succession, nitrogen fixation rates may display negative relationships to plant biomass (similar to that in Figure VII-2, Appendix I), as nutrient pools accumulate in marsh sediments, and plant growth shades the benthos, decreasing light availability for photosynthetic diazotrophs such as cyanobacteria.

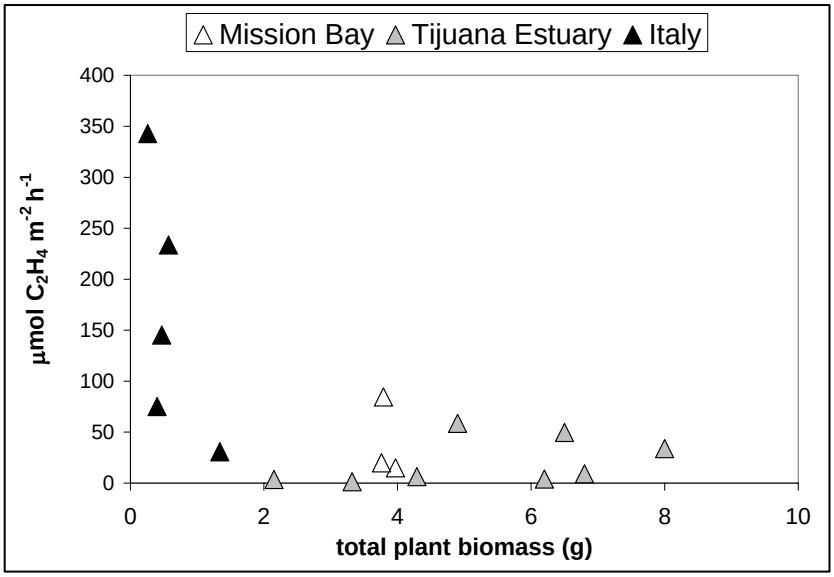

Figure VII-2: Relationship between nitrogen fixation (acetylene reduction) activities and plant biomass across all early and late succession marshes studied

Given the key roles of diazotrophs in wetland ecosystems, the resistance of their diversity and function to increasing sediment and nutrient loads are particularly relevant

for predicting functional responses of wetlands in Tijuana Estuary to construction of the Triple Border Fence. In field additions of sediment and nitrogen at Tijuana Estuary, nitrogen additions rapidly and strongly decrease nitrogen fixation rates (Chapter V), while sediment inputs exacerbate the duration and magnitude of changes in porewater nitrogen concentrations. In exploration of the broader significance of nitrogen fixation to wetland ecosystems, isotopic enrichment techniques reveal the fates of newly fixed nitrogen, which is rapidly acquired by *S. foliosa* and animal consumers. Uptake of newly fixed nitrogen (as $^{15}N_2$) decreases in the presence of ammonium nitrate loading (Chapter V), consistent with declines in nitrogen fixation rates that suggest potential for anthropogenic nutrient loading to affect plant-microbe interactions. Longer term changes in nutrient sources, which may occur via simultaneous sediment loading, may profoundly affect the structure and function of wetlands through shifts in such key ecological interactions.

Diversity and function among wetland diazotrophs

Patterns of diazotroph diversity and function are described across a range of spatial scales from inter-marsh zones of native and invasive species (plant and animal) to intermarsh comparisons of early and late succession ecosystems. Within the wetlands studied, few relationships are observed between nitrogen fixation rates and diazotroph diversity. One exception is a positive trend between diazotroph diversity in *S. foliosa* rhizospheres and nitrogen fixation rates in the early succession wetland of Tijuana Estuary during a Fall season corresponding to plant growth (and thus high nitrogen

demand) (Chapter IV). In this same wetland, a negative trend is observed between diazotroph diversity in surface sediments and nitrogen fixation rates after 17 days of nitrogen loading (Chapter V). These shifts in diversity-function relationships are driven by differential effects of experimental nitrogen additions on the diversity and activity of these microbes.

To represent diversity-function relationships on a larger scale, average diversity and nitrogen fixation rates are shown as single points in Figure VII-3 for all dates, treatments, zones, or marshes in which both data existed. Peaks in the function of nitrogen fixation are found among sediments with low to intermediate levels of diazotroph diversity in both surface and rhizosphere sediments.

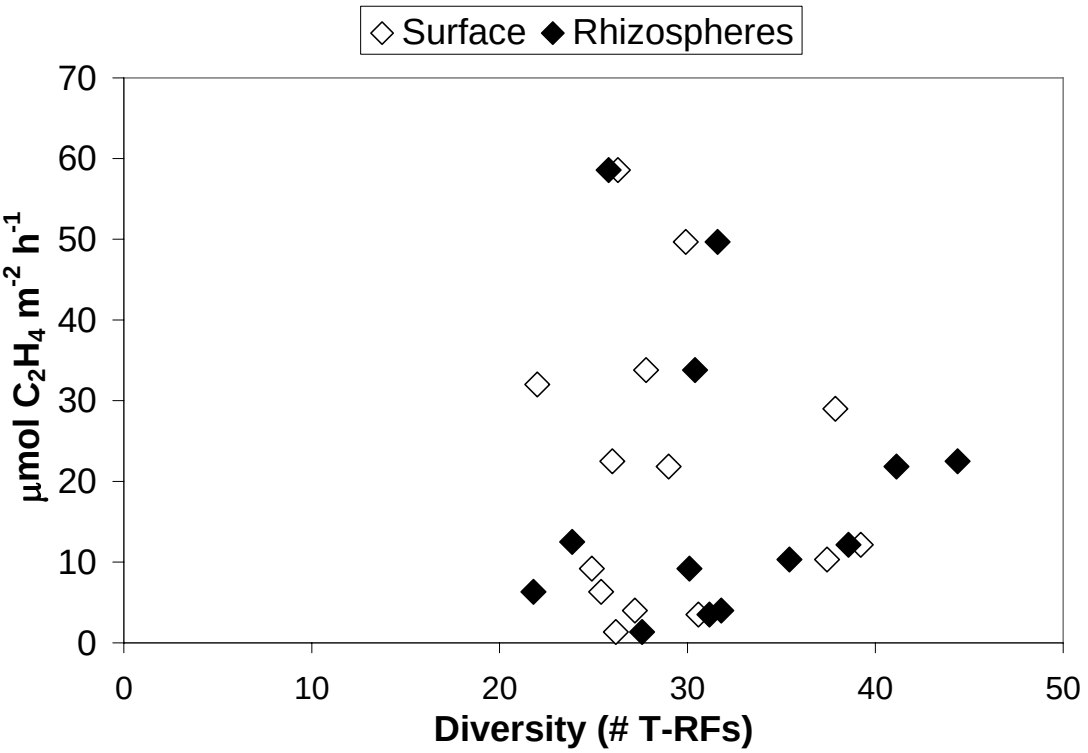

Figure VII-3: Relationships between the function (nitrogen fixation rates) and diversity (# T-RFs) of diazotrophs across all studies (each point represents averages of treatments, most n=10)

Ammonium availability exerts a strong influence on the diversity and function of nitrogen fixers. Diazotroph diversity in both surface and subsurface sediments is lowest at intermediate porewater ammonium concentrations (200 μM) among all experiments, which corresponds to levels observed in the unvegetated marsh zone (among *S. virginica*) (Figure VII-4A). Higher diversity at maximal ammonium concentrations may be due to displacement of competitive-dominant diazotrophs (most active in nitrogen-poor environments) as well as to increased representation of ammonium-tolerant diazotrophs in nitrogen-rich conditions. Diversification of surface-dwelling diazotrophs, in response to small-scale experimental nitrogen inputs (Chapter V), may also reflect the ability of some diazotrophs to assimilate dissolved inorganic nitrogen (Fritzsche and Niemann 1990).

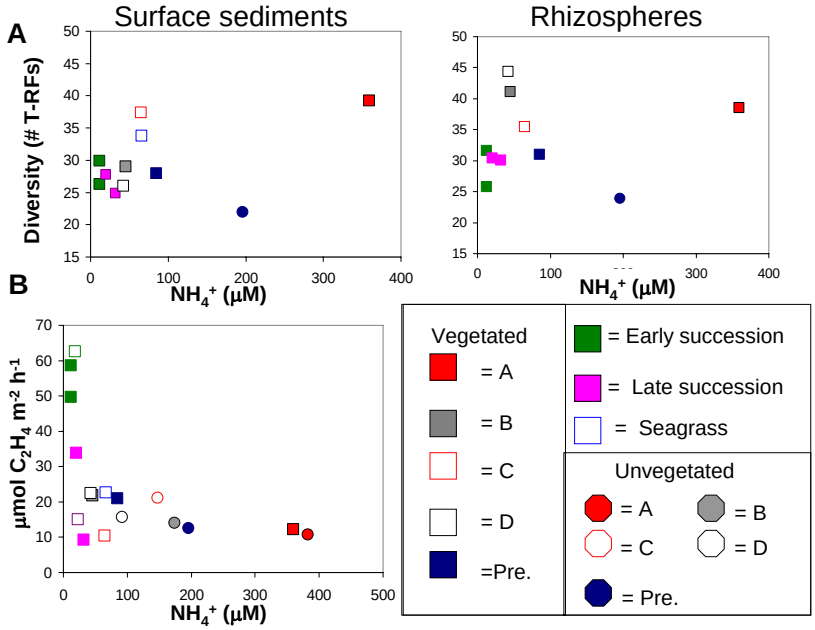

Figure VII-4: Relationships between porewater ammonium and (A) diversity or (B) function of nitrogen fixers among all treatments applied in this thesis research (each point represents an average all replicates within the treatment indicated in the legend (most n=10)

Relationships of nitrogen fixation rates to porewater ammonium concentrations across all experiments are also summarized (Figure VII-4B). Higher porewater ammonium concentrations correspond to lower nitrogen fixation rates (Figure VII-4B). Different responses of diazotroph diversity (Figure VII-4A) and function (Figure VII-4B) to changes in porewater ammonium concentrations highlight the ability of nutrient availability to affect relationships between diversity and function among diazotrophs, perhaps inducing the left skews in diversity and function plots (Figure VII-3). Plants may also potentially influence diversity-function relationships among nitrogen- fixing microbes by ameliorating the influence of nutrients, as indicated by plant uptake of experimental ammonium nitrate additions (in Chapter V).

Other microbial functions may be more tightly linked to diversity of functional groups or total microbial communities (i.e. productivity, Smith et al. 2007). However, given the complexity of microbial roles in wetlands, the significance of their diversity is likely to be understood only through consideration of interactions among a suite of ecosystem functions and cross-guild interactions.

Messages from microbes for wetland management

Microbes have long been overlooked from management and conservation perspectives, but they can substantially facilitate efforts to protect and restore wetland ecosystems. The roles of microbial communities within most ecosystems have frequently

been reduced to a mysterious "black box" (Tiedje et al. 1999) due to their invisibility, intractability, and complexity. Through a combination of molecular, biochemical, and experimental approaches, this dissertation research reveals basic patterns and controls of nitrogen-fixing microbes (diazotrophs) in wetlands across a range of spatial scales and highlighted their importance to wetland ecosystem functions.

Fundamental differences among plant species in their interactions with microbes must be considered when predicting impacts of disturbance, stress, or restoration efforts on wetland ecosystems. Although plant-diazotroph relationships are dynamic, nitrogen fixation offers more promise for meeting plant nitrogen demands than costly fertilization strategies in agricultural contexts (Rueda-Puente et al. 2003). Innoculations of the wetland plant, *Salicornia bigelovii* (a crop in arid soils) (Rueda-Puente et al. 2003), and of mangrove seedlings (Bashan et al. 1998) are currently being applied with some success for agricultural and restoration purposes, respectively. Nitrogen fixers may also play roles in mediating effects of "fertilization" via heavy anthropogenic inputs of nitrogen that have degraded several wetland ecosystems (Deevey et al. 2007). For example, run-off bearing nutrient-rich fertilizers may initially promote growth of most wetland plant species (McCray 2001). However, chronic exposure to nutrient loading can substantially disfavor the cordgrass, *S. foliosa,* relative to the succulent, *S. virginica* (Boyer and Zedler 1999), possibly because the former is more tightly coupled to nitrogen-fixing microbes (Chapter II). Declines in nitrogen fixation and diversification of surface diazotrophs in response to a small-scale nutrient enrichment (Chapter V) suggest that nitrogen-fixing

microbes may switch to roles as nitrogen assimilators, which could ameliorate ecosystem-level effects of nitrogen loading.

Nitrogen fixation plays key roles in supporting the structural development of plants in nitrogen-limited marshes and may thus indirectly sustain multiple plant-dependent functions of wetland ecosystems (Chapter IV). The relative importance of diazotrophs and nitrogen fixation may eventually decline in response to natural accumulation (Chapter IV) or anthropogenic enhancement of wetland nitrogen pools (Chapter V). Thus, nitrogen fixation rate measurements may aid marsh restoration efforts targeting functional equivalence (rather than just structural similarity) between natural and restored marshes, by indicating the biogeochemical status of wetlands.

Changes in nitrogen fixation rates may also reveal functional impacts of human-altered nitrogen dynamics in natural wetlands. Shifts in interactions of diazotrophs with native wetland plants should also be considered as a potential consequence or mechanism of biological invasion (Chapter III). Invasive species, such as *Caulerpa taxifolia,* that significantly stimulate nitrogen fixation (Chisholm and Moulin 2003) may need to be prioritized in eradication efforts, because of their greater potential for widespread growth and habitat modification in nitrogen-limited wetlands. Introduced species that decrease nitrogen fixation rates may also be detrimental, particularly in developing marshes where this nutrient source is vital. Further, high rates of nitrogen fixation among marshes of Venice Lagoon may be indicative of wetland subsidence beneath sea level (Chapter VI).

Such changes will affect wetlands worldwide, as global warming lowers effective wetland elevations beneath rising sea levels.

The number of simultaneous human impacts affecting coastal ecosystems should be minimized. Interactions between sediment and nutrient loading in Tijuana Estuary exacerbated the magnitude and duration of increases in porewater ammonium concentrations that were key controls on nitrogen fixation and diazotroph diversity (Chapter V). Currently, sewage contamination plagues the Tijuana Estuary region despite decades of bi-national efforts to control the problem (King 2003). In the case of the Triple Border Fence, managers attempting to reduce impacts of sedimentation may need to build and expand domestic and international infrastructure to prevent nitrogen-laden sewage from accompanying sediment loads.

Declines in nitrogen fixation resulting from increasing loads of anthropogenic nitrogen (i.e. sewage, runoff) may disfavor productive cordgrasses compared to more competitive marsh plants such as pickleweeds. In southern California, reduced flushing due to construction of highways and raidroads across the mouths of most estuaries and lagoons has further added to cordgrass vulnerability via promotion of hypersalinity and hypoxia (Mudie 1976). In southern California, loss of the cordgrass, *S. foliosa,* may drive species such as the endangered Clapper rail to extinction, as it has historically relied on this plant for nesting success (Zedler 1991, Zedler 2001, Figure VII-6), and may induce shifts in food webs that could fail to sustain a number of higher consumers (Moseman et

al. 2004). Loss of *Spartina* detritus within food webs, resulting from cordgrass declines, may reflect the inverse of effects observed with *Spartina* hybrid invasions (Levin et al. 2006). Overall productivity of wetlands may also decline, as succulents are not as productive as tall and fast growing grasses (Figure VII-5, arrow pointing left), and coastal stabilization may suffer since cordgrasses have more extensive rhizomes than their higher- elevation counterparts.

Future work must address the extent to which exogenous nutrients, particularly those associated with anthropogenic nitrogen loading and eutrophication can effectively "substitute" for declines that they induce in nitrogen-fixation and associated wetland functions. In other words, "Are all forms of nitrogen ecologically equal?" The species-specificity of plant-microbe interactions that sustain nitrogen fixation, its temporal coupling to plant production on diel (Chapter II) and seasonal cycles (Chapter III), and the intimate proximity of diazotrophs to plant rhizospheres, are unlikely to be matched by anthropogenic nutrient loads. In contrast to human-driven nutrient inputs, nitrogen fixation may promote plant diversity by offering a means of resource-based niche partitioning. For example, the two major vascular plant species in southern California marshes, *S. foliosa* and *S. virginica* dominated sediments with significantly different nitrogen fixation rates (Chapter II) and harbored distinct diazotroph communities (Chapter V). Further, the functions of nitrogen fixers in marsh ecosystems extend beyond simple provision of nutrients to include trophic support for macrofauna (Chapter V, Moseman et al. 2004), sediment stabilization (through cyanobacterial mat formation),

and nutrient cycling through sulfate reduction (a key process in organic matter regeneration). Thus, declines among diazotrophs that could occur as a consequence of chronic nutrient loading would have multiple ramifications for wetland ecosystems. Given the diversity of these microbes, and the lack of ability to culture most representatives, it is also likely that many other functions of diazotrophs in wetland ecosystems remain to be identified.

In the context of global climate change, multiple stresses are likely to impact wetland ecosystems (Figure VII-6). First, succulents (C-3 plants) are anticipated to be favored under the high CO_2 concentrations that are driving global warming (Choi and Wang 2001), while seagrasses and cordgrasses with C-4 metabolisms may decline. Second, sea level rise is anticipated to drown low elevations of wetlands where these grasses are found, which may enhance nitrogen fixation rates and promote their ability to colonize higher elevations as they subside (Chapter VI). However, the ability of grasses to recruit to higher elevations may be constrained by competition with higher marsh plants (Bertness 1994) or algae if anthropogenic nutrient loading continues to grow. They may also be affected by increasingly prevalent invasive species, which may induce changes in nitrogen fixation that affect nutrient regimes for native species (Chapter III), possibly in combination with effects of anthropogenic nutrient loading (Rickey and Anderson 2004).

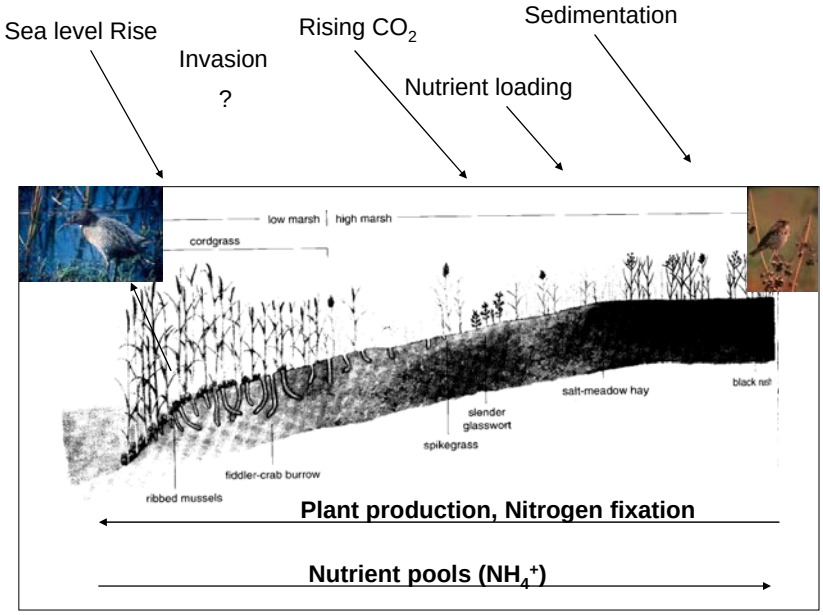

Figure VII-5: Spatial gradients in nitrogen fixation, plant production, and nutrient pool concentrations in a salt marsh supporting distinct plants and animals (endangered Clapper Rail and Beldings Savannah Sparrow are shown at the left and right, respectively) along an elevational gradient. Arrows at the top point to higher marsh elevations to indicate hypothesized effects of several anthropogenic impacts in favoring higher elevation plant species. (Effects of invasion are uncertain).

Under scenarios of future declines in nutrient loading (improved water quality) and fewer interacting stresses on wetlands, microbes may mediate alternative responses of these ecosystems to impending global changes. For instance, nitrogen fixation represents a sustainable nutrient source for wetland plants that, in contrast to anthropogenic nitrogen loading, may prevent eutrophication through tighter links of nutrients to plant production. Increases in nitrogen fixation may also promote greater sequestration of CO_2 into recalcitrant biomass. Practices that might similarly promote CO_2 returns to soils are being explored and may possibly increase terrestrial carbon sinks

by 100 billion tonnes by 2050 (Reay et al 2007). Future research may address roles of interactions between plants and diazotrophs in maintaining wetland structures and functions by mitigating rising CO_2 levels that drive global sea level rise, a major long term threat to coastal wetlands (Choi and Wang 2001, Friborg et al. 2003).

Both humans and the plant and animal inhabitants of wetlands benefit from maintaining functions of these coastal ecosystems against numerous growing threats. This thesis marks a beginning step towards breaking down the "black box" which has too long shrouded roles of microbes in negotiating mutual fates with wetland plants and animals upon which valuable ecosystem functions depend. The fates of macrofauna, fishes, and even endangered bird species are tied to those of wetland plants and ultimately to the microbes. My studies offer new tools and perspectives by which to predict future responses of these productive and valuable ecosystems to regional and global disturbances. The function or failure of most ecosystems (terrestrial, coastal, and marine) in response to impending environmental changes may largely depend on the dynamics of microbe-"macrobe" interactions.

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APPENDIX I: Nitrogen fixation among clipped and unclipped *Salicornia virginica* plants

Nitrogen fixation rates were measured among *Salicornia virginica* (also known as *Sarcocornia pacifica*) plots that had been experimentally clipped in 1 m^2 plots on a weekly basis between May 2005 and May 2006 and compared to those in adjacent unclipped plots (Whitcraft 2007?) in the Kendall Frost Reserve, Mission Bay. These plots were distributed in 8 blocks according to places where *S. virginica* covered more than 90% of the marsh surface (Whitcraft et al. 2007). Measurements of nitrogen fixation rates were made in November 2006 but little plant re-growth was observed, with striking visible differences in plant cover between clipped and unclipped plots. From each block, two sediment samples containing *S. virginica* plants were collected from each clipped and unclipped plot and were processed as described for aerobic, daytime assays in Chapter I. The nitrogen fixation rates were averaged by treatment in each block, and then compared among all blocks with a paired t-test in JMP 4.0.

Trends of higher nitrogen fixation rates among the clipped plots than the unclipped ones were observed (t_5=1.88, p=0.12, one-tailed p=0.06). These results are consistent with declines in nitrogen fixation rates in the process of succession among marshes (Chapter IV, Chapter VI), and would likely have been more significant if measured during periods of plot maintenance.