Biol Invasions
DOI 10.1007/s10530-008-9227-0

ORIGINAL PAPER

Diversity and functional responses of nitrogen-fixing microbes to three wetland invasions

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Received: 21 October 2007 / Accepted: 12 February 2008

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

Keywords Asian mussel · Diazotroph · Mangrove · Salt cedar · Functional redundancy

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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 1999). 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).

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°47′35′N, 117°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 5,000–10,000 individuals m^{-2} (Morton 1974; Crooks and Khim 1999), is a dominant species in the inter- and subtidal benthos of the bay (Crooks 1996, 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°34′N, 117°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 three hybrids of Tamarisk have become established in salt marsh habitats along brackish streams (Whitcraft et al. 2007). Native to Eurasia and Africa, at least seven 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).

Methods

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 six 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 m 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 mussels, via the presence of byssus cocoons, might affect 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 below. 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 (seven sediment and three cocoon samples) taken from the same transect in KF-NWP in December 2004. The three cocoon samples were paired with underlying sediment samples. All samples were kept on ice, returned to the laboratory and frozen at −80°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, pp. 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 (1.3 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° until analyzed for diversity of nitrogen fixers 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, pp. 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 five blocks established according to the distribution of the five 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 m 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, three 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).

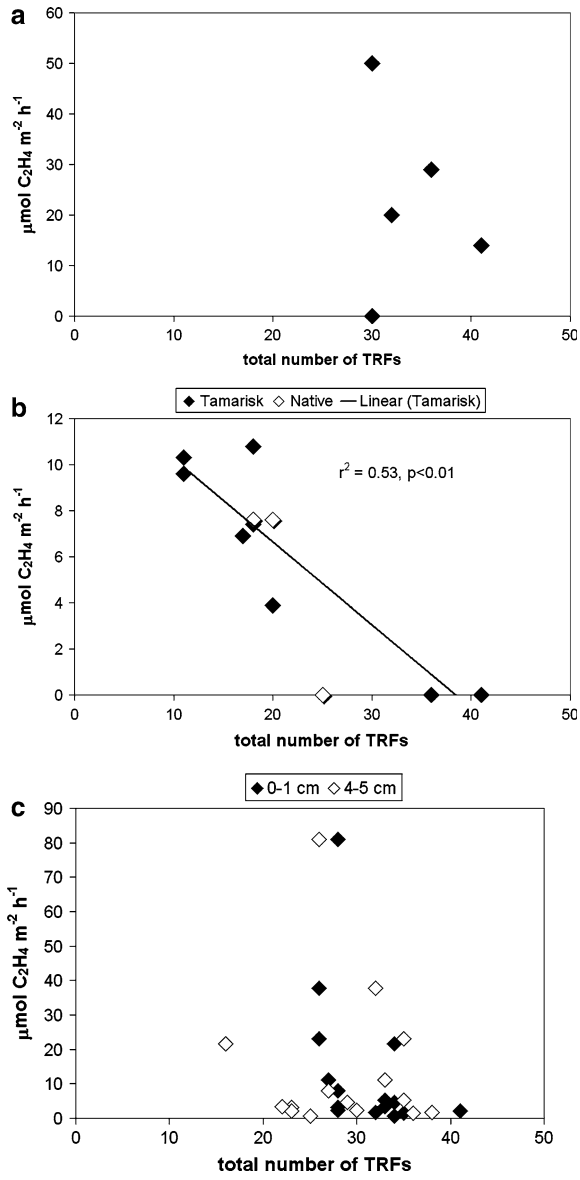

Fig. 1 The relationship between activity (micromole C_2H_4 m^{-2} h^{-1}) and diversity (total number of TRFs) of nitrogen fixing microbes in ecosystems invaded by (**a**) *M. senhousia*, (**b**) Tamarix spp. and (**c**) *A. marina*

Acetylene reduction assays

In experiments with *M. senhousia* (Fig. 1a, b), 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°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 h (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°C. In this experiment, headspace samples were collected after only 2 h, 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 h 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°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 (Solorzano 1969). 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 (*nif*H1) following the protocol of Zani et al. (2000). The PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit (TaKaRa) and digested at 37°C for 6 h 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 Multi-dimensional 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 two-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 ± 2.8μmol 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 nitrogen 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 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%, while that among mussel cocoon material was 60%. The dissimilarity between these sample types however was only 33%.

No significant linear relationship was found between nitrogen fixer activity and diversity in *Z. marina*-vegetated sediments invaded by *M. senhousia*, but

Table 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

highest activity rates were found in samples with lowest nitrogen fixer diversity (Fig. 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 ($r^2 = 0.54$, $P = 0.02$) (Fig. 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 $\rho = 0.419$), followed by the combination of nitrogen fixation (acetylene reduction) rates and mussel density ($\rho = 0.371$).

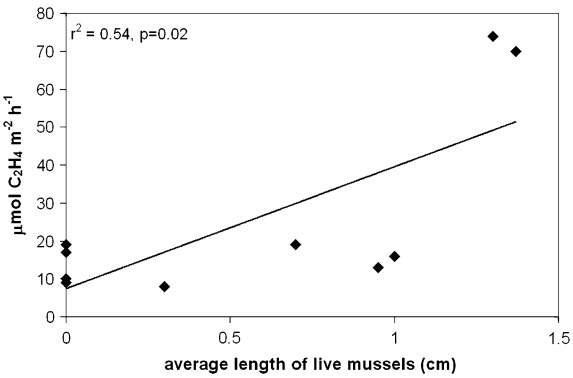

Fig. 2 The relationship between nitrogen fixation (acetylene reduction) rates and length of live *M. senshousia* mussel in the assayed sample (containing *Z. marina* vegetated sediment and mussels with byssus cocoons)

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 ± 1.5 µmol C_2H_4 m^{-2} h^{-1} while those of native, *S. pacifica*-vegetated sediments were 3.1 ± 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 ± 3.9 T-RFs) than in sediments vegetated by native plants (37 ± 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 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 *nif*H TRFs), in combined native and invaded sediments (Fig. 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 (Fig. 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 (Fig. 4a, b, respectively). However, diversity was only related to temperatures

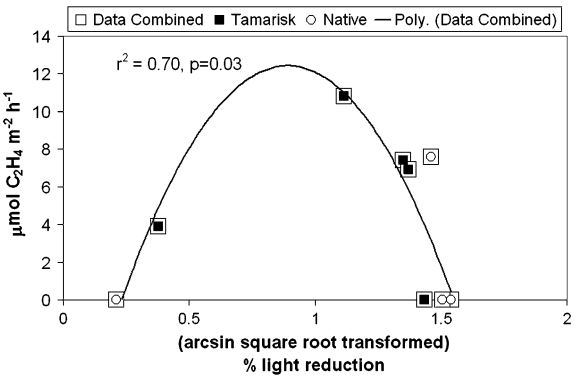

Fig. 3 The relationship between light reduction by plants (during Fall) and nitrogen fixation (acetylene reduction) rates (in Winter 2003) Tijuana Estuary

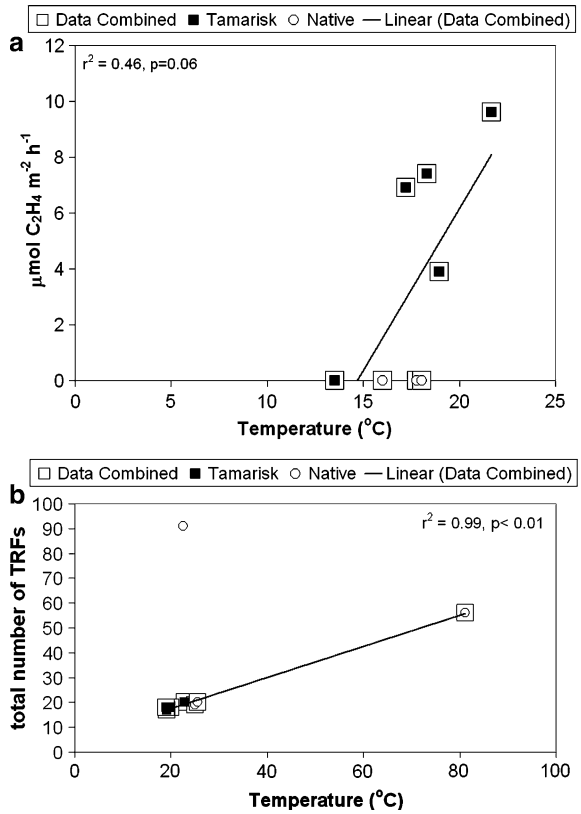

Fig. 4 (**a**) The relationship between activity of nitrogen fixers (acetylene reduction rates) and temperature of sediment of Tijuana Estuary salt marsh (Winter 2004). (**b**) Relationship between diversity (total number of TRFs) of nitrogen fixers in Winter 2003 and sediment temperature (determined in Fall 2003) in the Tijuana Estuary salt marsh (outlier is shown but excluded from regression)

measured in Fall 2003 (excluding one outlier in native sediments,) when sediments in Tamarisk invaded plots were cooler 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) (Fig. 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 $\rho = 0.636$). The combination of

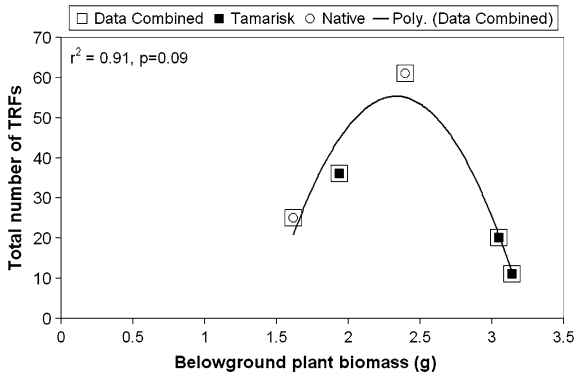

Fig. 5 Relationships of belowground plant biomass with diversity (total number of TRFs) of nitrogen fixers in Tamarisk-invaded salt marsh of Tijuana Estuary

these factors and sediment redox was the next strongest correlate with microbial community profiles (BIOENV $\rho = 0.616$).

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}) (Fig. 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

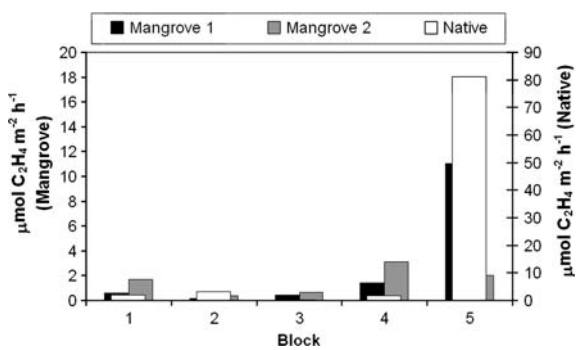

Fig. 6 Nitrogen fixation (acetylene reduction) rates of sediments in five blocks of paired mangrove (*A. marina*) plots and adjacent (single) plots with native *S. pacifica* vegetation in Mission Bay

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 0–1 cm or 4–5 cm depths (Table 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 $\alpha = 0.025$). Highest nitrogen fixation rates were found in sediments with intermediate nitrogen-fixer diversity (Fig. 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 (Fig. 7). Redox was also the environmental factor most strongly correlated to nitrogen-fixing community structure (T-RF profiles) among mangrove-invaded plots ($\rho = 0.685$). 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 ($\rho = 0.673$).

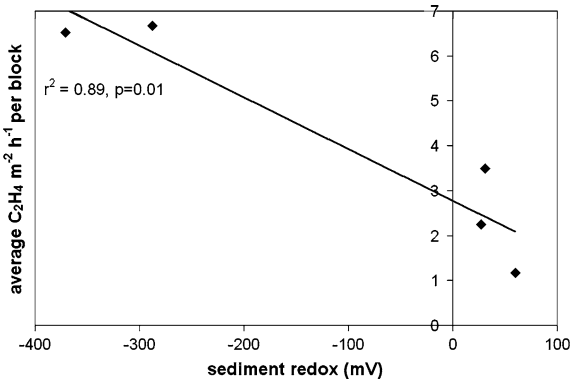

Fig. 7 The relationship between nitrogen fixation (acetylene reduction) rates and sediment redox in mangrove-invaded sediments of Mission Bay

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 (Fig. 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 (Fig. 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 et al. 1992) and inhibit nitrogen fixation rates (Zhaoyong et al. 2006). The positive relationship of native belowground plant biomass with nitrogen fixation (Fig. 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 (Fig. 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, pp. 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, pp. 80–89).

Nitrogen-fixing bacteria were relatively resistant to changes in community composition based on comparisons in invaded and native sediments in all three 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 (Fig. 4b); temperature has been found to be reduced in Tamarisk invaded portions of high salt marsh habitats (Whitcraft 2007, pp. 80–89). Trends of lower diversity among *M. senhousia* cocoons than in sediments were consistent across diversity metrics (Table 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 the single date examined 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 (Fig. 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 *nif*H gene expression via reverse transcriptase PCR of *nif*H (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, pp. 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.

Acknowledgements The authors thank Christine R. Whitcraft, Jeffrey A. Crooks, and Amanda J. Demopolous for providing environmental data. Generous support from Pei-Yuan Qian of the Hong Kong University of Science and Technology enabled T-RFLP applications and collaborations. Funding for this research was provided by the National Science Foundation, the National Estuarine Research Reserve System (Graduate Research Fellowships for Serena Moseman), and the University of California Natural Reserve System (Mildred Mathias Student Research Grant). The Michael M. Mullin and Mia Tegner Memorial Funds and the Graduate Department of Scripps Institution of Oceanography also provided financial support. Helpful comments and guidance were provided by Carolyn Currin, and James Leichter. Facilities and gas chromatography equipment were provided by Lihini Aluwihare. Equipment for DNA extractions were provided by the Center for Marine Biodiversity and Conservation at Scripps Institution of Oceanography. Guillermo Mendoza provided guidance with Primer software operation and applications. Pat McMillan gave valuable formatting assistance. Tracy Washington and Maria del Carmen Rivero assisted with laboratory analyses.

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