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Responses of Salt Marsh Plant Rhizosphere Diazotroph Assemblages to Changes in Marsh Elevation, Edaphic Conditions and Plant Host Species

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Abstract An important source of new nitrogen in salt marsh ecosystems is microbial diazotrophy (nitrogen fixation). The diazotroph assemblages associated with the rhizospheres (sediment directly affected by the roots) of salt marsh plants are highly diverse, somewhat stable, and consist mainly of novel organisms. In Crab Haul Creek Basin, North Inlet, SC, the distribution of plant types into discrete zones is dictated by relatively minor differences in marsh elevation and it was hypothesized that the biotic and abiotic properties of the plant zones would also dictate the composition of the rhizosphere diazotroph assemblages. Over a period of 1 year, rhizosphere sediments were collected from monotypic stands of the black needlerush, *Juncus roemerianus*, the common pickleweed, *Salicornia virginica*, the short and tall growth forms of the smooth cordgrass *Spartina alterniflora*, and a mixed zone of co-occurring *S. virginica* and short form, *S. alterniflora*. DNA was extracted, purified and *nifH* sequences PCR amplified for denaturing gradient gel electrophoresis (DGGE) analysis to determine the composition of the diazotroph assemblages. The diazotroph assemblages were strongly influenced by season, abiotic environmental parameters and plant host. Sediment chemistry and nitrogen fixation activity were also significantly influenced by seasonal changes. DGGE bands that significantly affected seasonal and zone specific clustering were identified and most of these sequences were from novel diazotrophs, unaffiliated with any previously described organisms. At least one third of the recovered *nifH* sequences were from a diverse assemblage of *Chlorobia*, and γ-, α-, β- and δ-*Proteobacteria*. Diazotrophs that occurred throughout the growing season and among all zones (frequently detected) were also mostly novel. These significant sequences indicated that diazotrophs driving the structure of the assemblages were diverse, versatile, and some were ubiquitous while others were seasonally responsive. Several ubiquitous sequences were closely related to sequences of actively N_2 fixing diazotrophs previously recovered from this system. These sequences from ubiquitous and versatile organisms likely indicate the diazotrophs in these rhizosphere assemblages that significantly contribute to ecosystem function.

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Introduction

Salt marshes are highly productive [27, 45, 60] nitrogen-limited [42, 62] ecosystems dominated by a small number of plant species [54]. These plants are distributed in discrete zones due to edaphic environmental conditions, interspecific competition and minor differences in elevation in the marsh [2, 11, 49]. Nitrogen limitation of plant productivity

in salt marshes has been demonstrated through nutrient addition experiments [4, 17, 24, 39, 43, 50, 51, 61, 63], as well as ecosystem-level mass balance considerations [24]. Nitrogen availability is supplemented by the introduction of 'new' nitrogen in these ecosystems through plant-associated diazotrophy [18, 43, 66]. This reduction of gaseous dinitrogen to ammonia is apparently carried out by a relatively small number of species in approximately 100 genera of the *Bacteria* and *Archaea* [68]. Diazotrophs associated with salt marsh grasses are present in high numbers (3.4% of the culturable bacteria [41]) and are most active on the roots of these plants [48, 66, 67]. Root exudates, along with decomposition products from dead plant biomass, support the activity of these assemblages [21, 33, 66, 67]. The diazotroph assemblages associated with the rhizospheres of the dominant plant species of estuarine and other highly productive shallow water marine systems are diverse, and consist mainly of presumptive anaerobes and members of the α-, β-, δ-, ε- and γ-*Proteobacteria* [6–8, 36, 38]. Epsilon-*Proteobacteria* diazotrophs have also been detected in the rhizosphere of *Spartina* sp. [40, 65]. Virtually none of the rhizosphere diazotrophs detected to date through molecular biological approaches are closely related to cultivated taxa [16, 36–38].

The diazotroph assemblage associated with the smooth cordgrass, *Spartina alterniflora*, the dominant plant growing at low elevations in salt marshes found along the Atlantic and northern Gulf of Mexico coasts of temperate North America, has received extensive study. This level of attention is due to the contributions these diazotrophs make to the nitrogen supply in these highly productive ecosystems. Previous studies have documented the importance of diazotrophs and have established some parameters affecting their distributions and activities. Though much is known of the variability in the rate of nitrogen fixation in response to abiotic parameters [26, 33, 52, 56, 66] very little is known of how these parameters affect the compositions of the diazotroph assemblages. The assemblage associated with the *S. alterniflora* rhizosphere is quite stable [5, 35, 36, 50, 51]. Molecular profiling of diazotrophs through polymerase chain reaction (PCR) amplification of *nifH* (the structural gene encoding the nitrogenase iron protein), followed by resolution of different *nifH* amplicons through denaturing gradient gel electrophoresis (DGGE), has demonstrated only minor changes in response to long and short-term fertilization and aboveground biomass removal [5, 35, 50, 51].

Host plant species appears to have a greater effect on assemblage composition than variable abiotic parameters. LaRocque et al. [31] and Piceno et al. [52] found similar diazotroph assemblages associated with *Juncus roemerianus* and *S. alterniflora* stands (respectively) that occurred in different locations in the North Inlet (SC, USA) salt marsh. Gamble et al. [20] found that there were also seasonal differences in the structure of the diazotroph assemblage associated with the rhizospheres of short and tall growth form *S. alterniflora*, and that some diazotrophs were responsive to seasonal changes whereas others were not. No significant differences in assemblage composition attributable to the differences in elevation that result in development of the short and tall form *S. alterniflora* zones were detected, confirming the findings of Piceno et al. [52]. In this study, we examined the impacts of plant host, season and marsh elevation on distributions of diazotrophs along the elevation gradient and across several plant zones in a relatively pristine salt marsh.

Materials and Methods

Study Site

This study was conducted in the North Inlet estuary, near Georgetown, SC, USA (33° 20′ N, 79° 12′ W). North Inlet is a high salinity, tidally dominated salt marsh estuary that is relatively pristine [12] and is the benchmark system for the US National Estuarine Research Reserve System. The sampling sites were located along a transect in the Crab Haul Creek basin of the marsh. This transect extended from the terrestrial biome (dominated by loblolly pine, *Pinus taeda*) through a series of readily defined vegetated zones to the banks of Crab Haul Creek.

The zones that were sampled included a monotypic stand of black needle rush, *J. roemerianus* (JS), that occurred along the interface of the terrestrial biome with the marsh proper; a monotypic stand of the perennial glasswort, *Salicornia virginica* (SV); a mixed zone of co-occurring *S. virginica* and short growth form *S. alterniflora*. (SS); a monotypic stand of short growth form *S. alterniflora* (S); a patch of *J. roemerianus* that grew within the main stand of short growth form *S. alterniflora* (JP), and a monotypic stand of tall growth form *S. alterniflora* (T). The distribution of these vascular plants is influenced by marsh elevation and governed by biotic and edaphic environmental conditions [1, 10] (Fig. 1). These zones were specifically selected to provide strong contrasts in porewater chemistry and in the types of plants present.

In each plant zone, sampling stations were oriented perpendicular to the transect and each was marked with garden stakes. At each sampling station, six sippers for porewater collection were established at 1 m intervals, defining the centers of six sampling plots.

Sample Collection

Rhizosphere samples were collected by coring within each of these plant zones in June, August and September of 2000

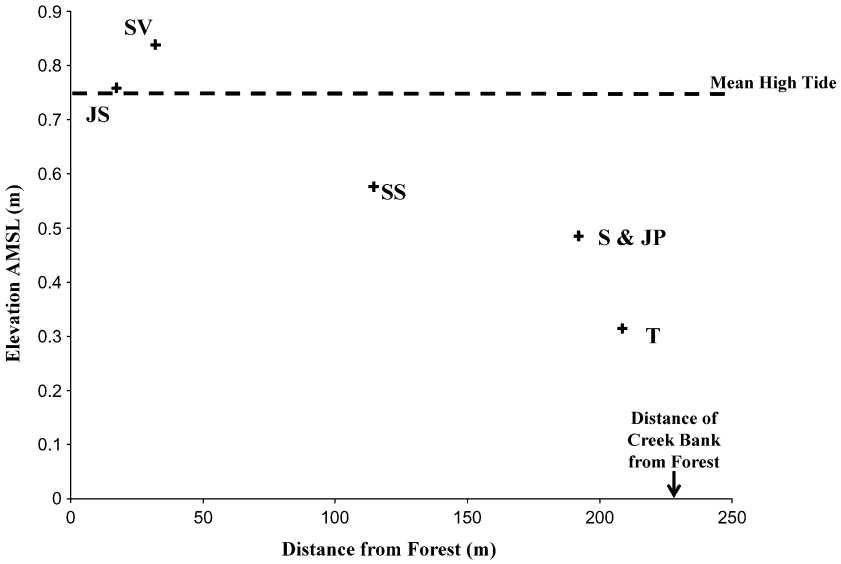


Figure 1 Differences in site elevation above mean sea level (*AMSL*) and distance of each sampling zone from the forest edge. Site designations: *Juncus roemerianus* stand (*JS*); *Salicornia virginica* (*SV*); mixed plant zone of co-occuring *S. virginica* and short form *Spartina alterniflora* (*SS*); short form *S. alterniflora* (*S*); patch of *J. roemerianus* in the S zone (*JP*); and tall form *S. alterniflora* (*T*)

and in February and April of 2001. These five dates were chosen to sample the diazotroph assemblage associated with these plants over a year of growth. June was considered to be a period of high plant productivity. August is within the growing season and September heralds the flowering event and seed production as well as the end of the growing season. During February the plants are senescent and in April marsh plants begin to develop new shoots.

The sediment cores were collected within these plots for DNA extraction (2.4-cm diameter by 6 cm length) and for acetylene reduction assay (1.5-cm diameter by 8 cm length). The cores for DNA extraction were placed in Whirlpack bags (Nasco, Modesto, CA, USA), transported back to Columbia, SC on dry ice and stored at −70°C until processed.

For measurement of acetylene reduction rates, approximately 10 g of fresh sediment was transferred into sterile 40-ml serum vials and 10 ml of sterile artificial seawater at 34‰ salinity were added to the vial [66, 67]. The vials were sealed with sterile butyl rubber septa, acetylene was injected into the headspace (1.5 ml per vial in a headspace of 15 ml), and the vials were incubated at in situ temperature for 24, 48 and 72 h. At each time point the amount of ethylene produced was determined by gas chromatography using a Varian 3700 gas chromatograph (Walnut Creek, CA, USA) equipped with a flame ionization detector and a Carbosphere 80/100 column (Alltech, Deerfield, IL, USA). An ethylene standard curve was used in calculating the rate of acetylene reduction in each sample.

Porewater was also collected within 1 h of low tide on each sampling date except April 2001 [14]. Porewater salinity was measured using a refractometer (Leica Inc, Buffalo, NY, USA) and pH was measured using a portable pH meter (Cole Parmer, Chicago, IL, USA). Samples collected for soluble sulfide ion concentration were fixed in the field in an equal volume of 2 N zinc acetate and concentrations were determined colorimetrically [59]. Samples collected for soluble ammonium ion were fixed in the field with one drop of concentrated hydrochloric acid and concentrations were measured using Technicon, and Bran and Luebbe Autoanalyzers [22]. Statistical analysis of porewater data and acetylene reduction rates was carried out using the repeated measures analysis of variance test with Bonferroni's correction for type 1 errors (α=0.003).

DNA Purification and *nifH* Amplification

DNA extraction and purification from sediment samples followed the direct lysis procedure of Lovell and Piceno [34]. Prior to PCR amplification, extracted DNA was further purified using the Promega Wizard Clean-up Kit (Madison, WI, USA). PCR amplification was carried out using 4 U Taq DNA polymerase in 1× Taq buffer (containing 1.5 mM $MgCl_2$) (Qaigen, Valencia, CA, USA) in a 100 µl reaction mixture containing 25 ng of DNA template, 800 µM of each deoxynucleotide triphosphate (New England Biolabs, Ipswich, MA, USA), 0.5 pmol μL^{-1} of each primer (MWG Biolabs, Huntsville, AL, USA) and 40 µg bovine serum albumin. The GC-clamped *nifH* primers used were designed by Piceno et al. [52], are specific for heterotrophic diazotrophs [38, 52] and contain the artificial nucleotides P [32] and K [15] to reduce primer degeneracy.

PCR amplification was initiated with a 2 min denaturation step at 94°C followed by a touchdown program of 20 cycles of – 94°C for 45 s, 58°C for 30 s, decreasing 0.5°C per cycle, and 70°C for 30 s. The touchdown program was

followed by an amplification program that consisted of 20 cycles of – 94°C for 45 s, 48°C for 30 s and 68°C for 30 s. The PCR program was ended with a final extension step of 2 min at 72°C. Ninety microlitres of the reaction mixture were concentrated by isopropanol precipitation. Amplicons were recovered by centrifugation for 30 min at 10,000×*g*, washing in 70% ethanol and recovery in 10 μl of TE (pH 8.0; 10 mM Tris–HCl, 1 mM disodium ethylenediamine tetraacetate).

Denaturing Gradient Gel Electrophoresis

The GC-clamped *nifH* amplicons were electrophoresed on DGGE as described by LaRocque et al. [31]. Standards used for gel comparisons included GC-clamped *nifH* amplicons of *Klebsiella pneumophila, Sinorhizobium meliloti* and *Azospirillum lipoferum*. The gels contained an artifact band in each lane that was likely single stranded DNA [52] and was used as an additional reference. Gels were stained with 15 μl of SYBR Gold (Molecular Probes, Eugene, OR, USA) in 200 ml 1× TE for 30 min and images were acquired using an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA, USA).

Analysis of Band Patterns

Gel analysis was accomplished using GelCompar II software (BioSystematica, Devonshire, UK). Gel images were uploaded and the auto band search function (minimum profiling 4.00% and shoulder sensitivity 1—modifications were made based on DNA quality) was used to initially mark bands. Band scoring was carried out as previously described by Gamble et al. [20]. A total of 53 identified band positions were scored and presence or absence of each of the band positions for every lane (each replicate) was determined and the binary data compiled for statistical analysis. Gel analysis was not performed on samples that were deemed inadequate due to poor quality DNA amplification.

The Multi-Variate Statistical Package 3.13 g (MVSP; Kovach Computing Service, Wales, UK) [19] was used to statistically analyze the presence/absence binary data. One hundred and twenty-five individual lane samples were compiled for the total presence/absence data set. Twenty-three samples were used from June 2000 with six replicates from each of the following plant zones—JS, S and T, and five replicates from the SS zone. Thirty samples were used from August 2000 with six replicates from each plant zone. Twenty-four samples were used from September 2000, including six replicates from each of the following plant zones—SV, SS, S, and T. For the February 2001 samples there were 23 lane samples used, six replicates each from JP, S, and T plant zones and, five replicates from JS plant zone. The April 2001 sample set included 25 lane samples, six replicates each from SS and S plant zones, five replicates each from JS and SV plant zones and three replicates from T plant zone. Lower replicate numbers for some sets reflect samples not included due to poor DNA recovery or quality.

Principal components analysis (PCA) of the presence/absence data using a covariance matrix and Kaiser's Rule was performed, and results were plotted both with and without Euclidean bi-plot. The Euclidean bi-plot provided eigenvectors indicating DGGE bands that controlled clustering. Data by plant zone and data by date were compared and figures were produced for all comparisons. MVSP produces a sign condition (+/−) causing some groups to cluster to the left of the main axis whereas a similar data group clusters to the right of the main axis. It has been previously determined that this is an artifact of the program and not a result of the data [20], therefore the data were corrected for the axis shift.

A distance measurement between clusters was used to establish the significance of clustering [20]. A basic formula $\left(\sqrt{\left[(x_1-x_2)^2+(y_1-y_2)^2\right]}\right)$ was used to calculate the average point for each plant zone and date. The distance was calculated for each PCA data point from all the PCA data points within that group cluster as well as between each condition's average PCA data point. A two-tailed student's *t* test (α=0.001) was used to determine the significance of the distances between the various date and plant type groups. Eigenvectors produced from the Euclidean bi-plot were used to identify individual bands that contributed to formation of plant type and seasonal PCA clusters. The sum of the absolute values of the *x* and *y* coordinates for each eigenvector were used to classify the bands as strong or weak determinants. A threshold value of 0.4 was used to determine a strongly affecting band, meaning the longer the vector the stronger the effect.

Sequence Analysis of 'Significant Bands' from DGGE Gel Analysis

DGGE bands deemed 'significant' due to their recurring presence in DGGE banding patterns or considered 'strongly affecting' bands based on Euclidean bi-plot analysis (bands producing long eigenvectors) were selected for sequencing. DGGE gel bands were sampled and the bands in question were sequenced using previously described methods [20, 38]. Sixty-two sequences were deposited with the National Center for Biotechnology Information (NCBI) GenBank (accession numbers HM182106—HM182167).

Nucleotide sequences were imported into ClustalX version 2.0.12 (The Pennsylvania State University, University Park, PA, USA) [30, 57], aligned and edited to remove primer sequences. The sequences were manually converted

into a format recognized by Molecular Evolutionary Genetics Analysis software version 4.0 (MEGA4) [55]. A neighbor-joining phylogenetic tree with 20 *nifH* reference sequences was constructed in MEGA4 using the Jukes–Cantor correction for nucleotide sequences, complete deletion of missing data and gaps, and 1000 bootstrap replications. The outgroup taxon used was the *Methanothermobacter thermoautotrophicum nifH* sequence (GenBank accession number X87971). A BLAST study was performed to identify sequences appropriate for comparison. Based on the results of this study, a neighbor-joining phylogram was also created using sequences recovered in this study as well sequences presented in Brown et al. [16] and Gamble et al. [20].

Results

Porewater Chemistry

Salinity was lowest in the high marsh zone, (*J. roemerianus*, JS) and highest in the mid-marsh zones, (*S. virginica*, SV, and the mixed *S. virginica*/short *S. alterniflora* zone, SS) (Table 1). The low marsh zones (Short form, S, and Tall form *S. alterniflora*, T) consistently had higher salinities than the high marsh zones. Salinities were highest during the summer months and lowest in the winter, ranging from 20.2‰ to 57.3‰. The influence of plant zone and sampling date on salinity was statistically significant ($p<0.003$). pH measurements (Table 1) were consistently higher in the mid to low marsh zones compared with the high marsh and there was a statistically significant influence of sampling date ($p<0.003$). pH ranged from 6.42 to 7.53 during the growing season but was significantly lower during February 2001.

Sulfide concentrations (Table 1) were highest in the low marsh zones during the growing season and, overall, concentration levels were lowest in February 2001. The influence of plant zone and sampling date on soluble sulfide was statistically significant ($p<0.003$). Ammonium concentrations were also determined and they were lowest at the beginning of the growing season, increased as the season progressed, and were highest during the winter months. Sampling date significantly influenced ammonium concentration ($p<0.003$).

Acetylene Reduction Rates

Acetylene reduction rates were determined in all zones for all dates except April 2001 (Table 1). Overall, they were highest during the months of August and September 2000 and lowest during February 2001. JS and SV zones had the highest rates throughout the sampling dates—June 2000

Table 1 Porewater chemistry and acetylene reduction assay results

	Sampling Date	JS	SV	SS	JP	S	T
ARA ($nmol^{-1}h^{-1}ml^{-1}$)	June 2000	0.7 (0.7)	1.3 (0.2)	0.9 (0.4)	0.1 (0.1)	0.5 (0.1)	0.1 (0.1)
	August 2000	26.6 (12.8)	4.0 (1.1)	23.7 (10.8)	9.3 (9.3)	20.2 (12.5)	6.7 (6.7)
	September 2000	38.3 (38.3)	7.8 (2.4)	19.7 (12.9)	0 (0)	0 (0)	0 (0)
	February 2001	0 (0)	0.2 (0.1)	0 (0)	0.1 (0.1)	0 (0)	0 (0)
Salinity (‰)	June 2000	24.2 (3.4)	ND	57.3 (1.1)	49.0 (0.6)	38.8 (0.5)	36.7 (0.7)
	August 2000	28.4 (1.0)	54.3 (1.2)	49.5 (1.6)	34.5 (0.4)	36.2 (0.4)	33.2 (0.4)
	September 2000	26.7 (1.5)	47.3 (1.1)	43.5 (1.0)	31.0 (0.6)	34.0 (0.4)	32.0 (0.3)
	February 2001	20.2 (1.7)	35.7 (1.8)	37.8 (1.3)	33.0 (0.5)	33.0 (0.3)	31.8 (0.2)
pH	June 2000	6.9 (0.3)	ND	7.2 (0.2)	7.1 (0.1)	7.4 (0.1)	7.4 (0.1)
	August 2000	6.6 (0.3)	6.4 (0.2)	7.5 (0.3)	7.4 (0.1)	7.5 (0.1)	7.5 (0.1)
	September 2000	7.0 (0.3)	6.7 (0.1)	7.4 (0.1)	7.4 (0.1)	7 (0.1)	7.0 (0.1)
	February 2001	5.9 (0.1)	6.8 (0.1)	7.0 (0.3)	6.1 (0.6)	6.9 (0.1)	5.9 (1.4)
H_2S (mM)	June 2000	0 (0)	ND	0 (0)	0.5 (0.1)	2.8 (0.4)	0.9 (0.2)
	August 2000	0.2 (0.1)	0.2 (0.1)	0.4 (0.1)	0.3 (0)	2.9 (0.3)	1.2 (0.3)
	September 2000	0.3 (0)	0.3 (0)	0.4 (0)	0.5 (0.2)	3.9 (0.4)	1.9 (0.6)
	February 2001	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
NH_4^+ (mM)	June 2000	167.2 (43.1)	ND	35.2 (6.9)	11.9 (10.9)	77.8 (20.9)	22.9 (13.4)
	August 2000	288.8 (67.7)	98 (39.9)	200 (65.4)	224.7 (51.9)	130.7 (17.2)	53.3 (12.3)
	September 2000	221.2 (480)	215.2 (42.9)	174.4 (35.6)	292.3 (49.6)	174.6 (44.2)	201.7 (61.0)
	February 2001	144.3 (100.3)	163.9 (83.1)	120.2 (115.9)	482.5 (190.3)	88.2 (55.8)	485.4 (249.7)

Values are averages based on six replicates for each zone per date; values in parentheses are standard error

ND no data

and February 2001 for SV, August and September 2000 for JS. T and JP zones had consistently low rates of acetylene reduction during all dates of sample collection. Sampling date significantly influenced acetylene reduction rates ($p<0.003$) whereas plant zone did not.

Gel Analysis

PCA analysis was carried out on DGGE bands obtained on the same date for all plant zones to quantitatively assess the contributions of individual bands to observed patterns (Fig. 2 and Electronic Supplementary Fig. 1a, b). Some samples were eliminated due to low numbers of bands (<4 bands) in the banding pattern. Samples from the same plant zone either strongly clustered together ($p<0.001$) or they clustered with samples from the same region of the marsh—low, mid or high. S and T samples frequently clustered together as both zones are in the low marsh and experience comparatively similar abiotic conditions. SS and SV samples clustered together; the SS zone contains both *S. virginica* and short form *S. alterniflora*. JP and JS samples clustered together as both zones contained the same dominant macrophyte, however in August 2000 when there were no analyzable JS samples, the JP samples clustered with the SS samples. The distinct clustering within and among zones having the same or similar plant type(s) was evident for all dates and seasons.

To determine if there was a seasonal effect on clusters, samples from all plant zones for each date were also analyzed (Fig. 3 and Electronic Supplementary Fig. 2a, b). For all plant zones there was significant clustering by date ($p<0.001$) with samples from each date clustering with themselves or with samples from another date in the same season. Samples from June and August 2000 frequently clustered together and samples from April 2001 clustered with June 2000. April 2001 had 18 days with maximum air temperatures greater than 20°C; June and August 2000 samples had maximum air temperatures greater than 20°C for each day in both months [47]. One S lane from February 2001 clustered with the August 2000 samples; this lane had the lowest number of bands (7) for that sample set (all other lanes had between 14 and 20 bands). The trends in clustering were the same whether the analysis was run using all samples obtained on each sampling date or all samples obtained for each plant type.

Analysis of Significant Sequences

Analysis of banding patterns and calculations of Euclidean distances, Euclidean biplots (data not shown), and eigenvectors were performed for each of the DGGE gels generated for the samples collected in June, August and September 2000, and February and April 2001. These analyses identified the bands that were responsible for the longest eigenvectors and which were present throughout the sample set either in all plant zones on a particular sampling date or in one plant zone on every sampling date (frequently detected). Bands were sequenced to determine the types of organisms that most influenced the structures of these assemblages. Not all sequences or bands could be recovered due to poor DNA quality, inadequate PCR amplification or poor resolution in the DGGE gels for some samples. Twenty-three of the frequently detected bands and 39 of the bands responsible for long eigenvectors were recovered and sequenced.

The sequences recovered did not group together; frequently detected (FD) sequences were interspersed with

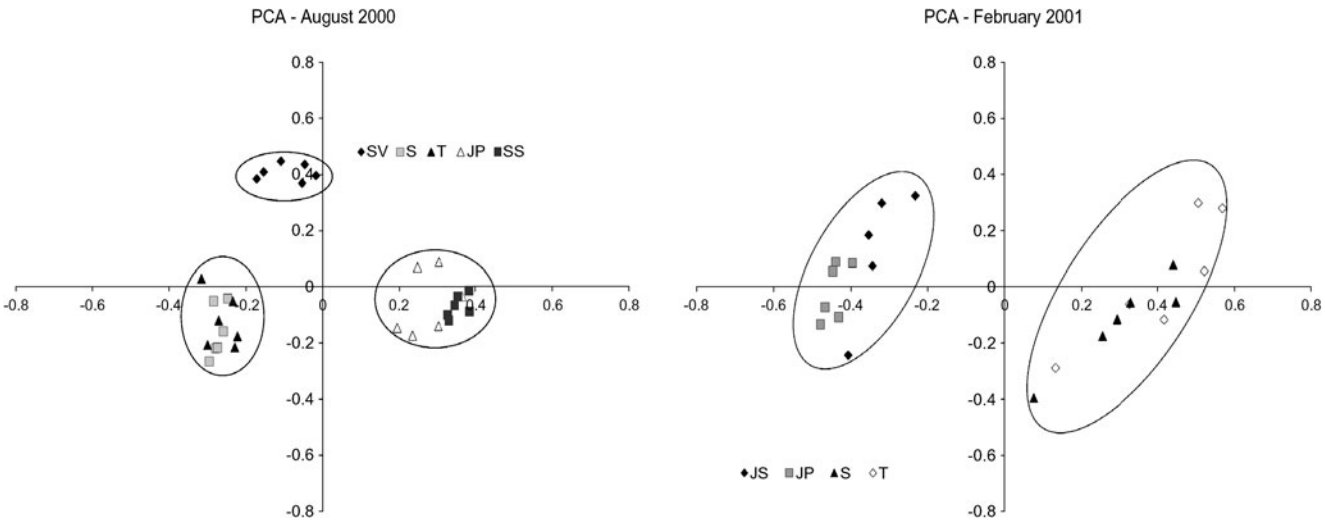
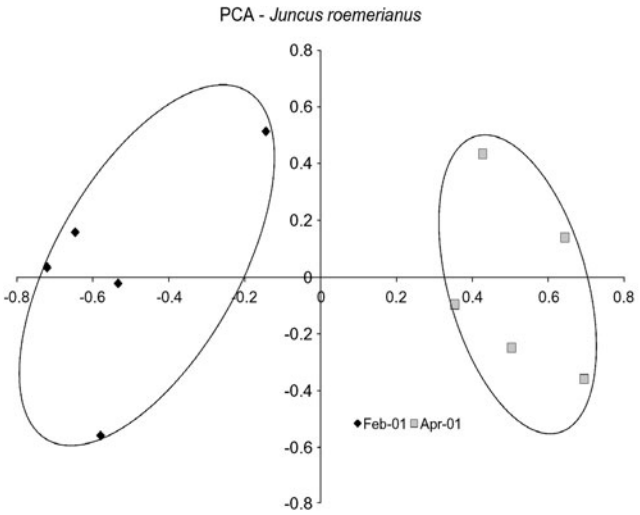


Figure 2 PCA results for dates August 2000 (*SV*, *SS*, *JP*, *S* and *T*) and February 2001 (JS, JP, S and T). Circles denote significance ($p<0.001$) for clustering. For August 2000 Axis 1 represents 31.2% of the variance and Axis 2 represents 20.8% of the variance. For February 2001 axis 1 represents 47.4% of the variance and axis 2 represents 10.9% of the variance



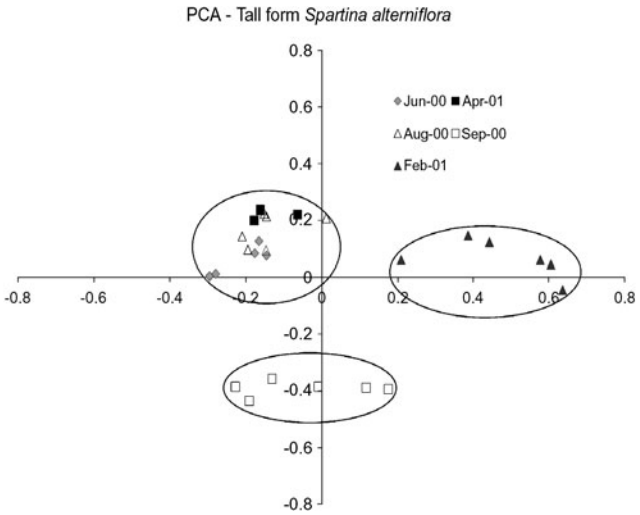


Figure 3 PCA results for *Juncus roemerianus* stand and tall form *Spartina alterniflora* on all dates. Circles denote significance ($p < 0.001$) for clustering. For *J. roemerianus* stand Axis 1 represents 31.2% of the variance and axis 2 represents 20.8% of the variance. For tall form *S. alterniflora* axis 1 represents 25.8% of the variance and axis 2 represents 15.5% of the variance

the sequences driving the longest vectors (LV) in the neighbor-joining phylogram (Fig. 4). Approximately two thirds of these sequences did not group with *nifH* sequences from reference organisms (formally described diazotrophs) and apparently represent novel diazotrophs. Thirteen of the FD and LV sequences grouped strongly with *Chlorobia* or α-, β-, δ-, and γ-*Proteobacteria* reference sequences and likely belong to these groupings (all marked with asterisks in Fig. 4) but none were identical to the reference sequences. Two FD sequences (SV-17-3-FD and SV-17-12-FD) grouped strongly with *Azotobacter vinelandii* (γ-*Proteobacteria*) and another (6-00-37-8-FD) grouped strongly with *Chlorobium tepidum* (*Chlorobia*). Of the LV sequences, two sequences grouped with the α-*Proteobacteria* reference sequences, one sequence grouped with *Azoarcus communis* (β-*Proteobacteria*), two sequences grouped with *Halorhodospira halophila* (γ-*Proteobacteria)*, one sequence grouped with two δ-*Proteobacteria* reference sequences (*Desulfovibrio vulgaris* and *Desulfomicrobium baculatum*) and one sequence grouped with *Pelobacter carbinolicus* (δ-*Proteobacteria*). Two FD sequences and one LV sequence grouped with the *Vibrionaceae* reference sequences (γ-*Proteobacteria*). Several of the sequence clusters with greater than 95% bootstrap values consisted of sequences having less than 6% difference and were thus scored as identical (sensu Venter, [37, 64]) (data not shown). Only three of these groups contained both LV and FD sequences (Fig. 4, bracketed {}) whereas all other clusters of identical sequences contained one type of sequence, either FD or LV. There were also numerous FD sequences which grouped with but were not identical to LV sequences, indicating they were of the same taxonomic lineage.

A neighbor-joining phylogram was also created using the seasonally responsive and seasonally non-responsive sequences of uncultured diazotrophs presented in Gamble et al. [20] and the *nifH* messenger RNA (mRNA) sequences presented in Brown et al. [16] (Electronic Supplementary Fig. 3a, b). These sequences were also obtained from *S. alterniflora* rhizosphere samples taken in the North Inlet salt marsh system and were included to further elucidate any correlation between sequences of potentially active diazotrophs and those sequences that significantly influenced the diazotroph assemblages, frequently detected, and seasonally responsive and/or non-responsive members of the assemblage. A number of well-defined clusters containing sequences with less than 6% differences (identical, sensu Venter [37, 64]) appeared throughout the tree. Most of these clusters contained sequences from the same study; however, there were many mixed clusters (Electronic Supplementary Fig. 3a, b; clusters 1, 7, 12, 13, 14, 15, 19, 22, 27, 31, 38, 40 and 45) with sequences from two or more studies. Some seasonally non-responsive and seasonally responsive sequences were identical to FD and LV sequences and some mRNA sequences were identical to seasonally non-responsive sequences. Other mRNA *nifH* sequences grouped with, but were not identical to seasonally non-responsive and responsive sequences as well as FD and LV sequences.

Discussion

DGGE analysis clearly indicates that there are plant host and seasonal effects on the distributions of diazotrophs throughout the marsh. It was expected that the differences

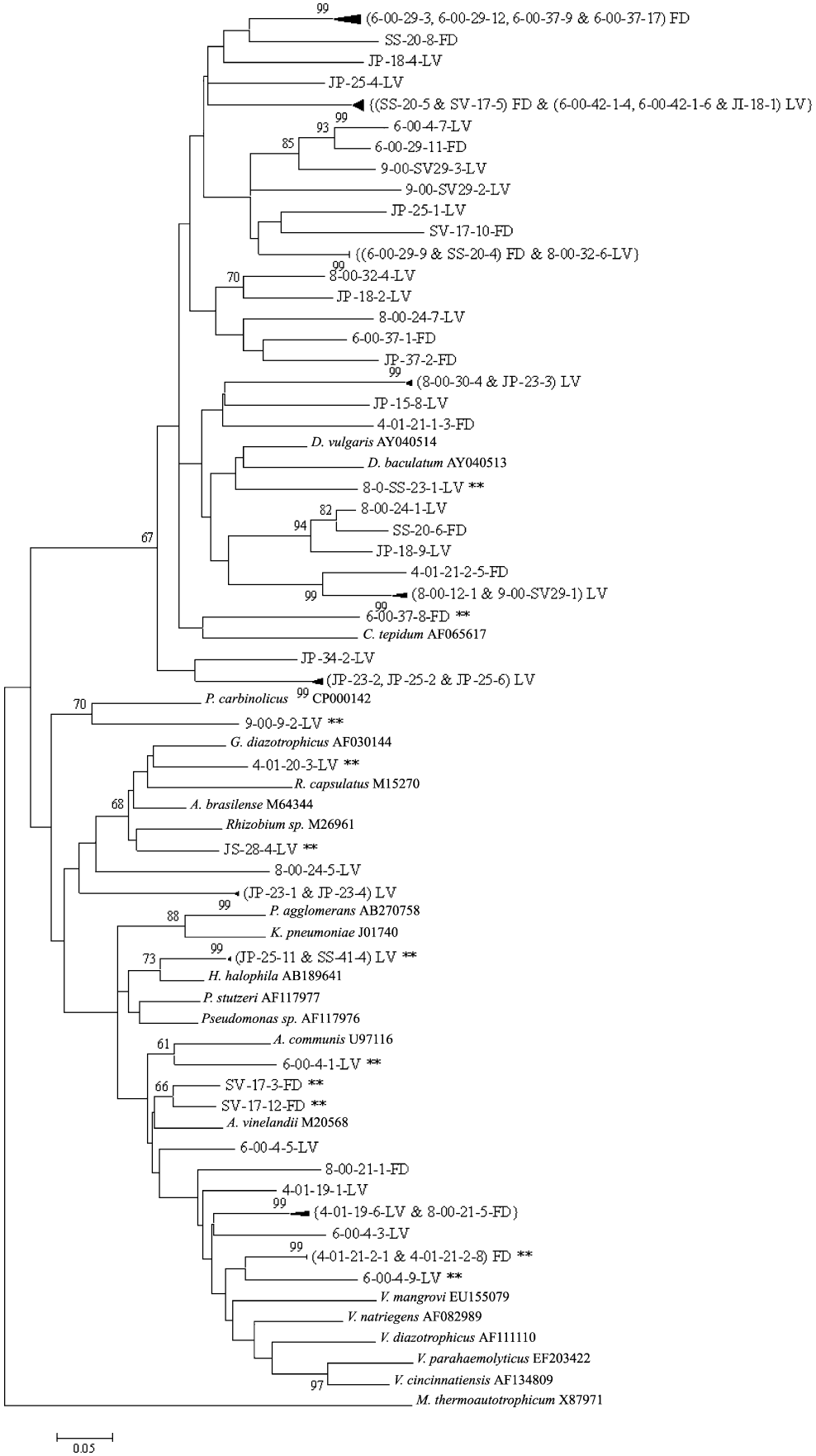


Figure 4 Phylogram of sequences from rhizosphere samples (nucleotide sequences, neighbor-joining, Jukes–Cantor correction, 1,000 bootstrap replicates, complete deletion of gaps and missing data). Frequently detected (*FD*) and longest eigenvector (*LV*) sequences are designated. Sequence designations depict whether they were from a seasonal (date prefix, e.g. 8-00 for month and year) or plant effect analysis (plant zone abbreviation, e.g. JS for *Juncus roemerianus* stand). *Numbers following the prefix* in the sequence designation are ultimate band number and clone designation. FD sequences with a seasonal prefix are sequences from bands that are present in all zones on that sampling date and those with a plant zone prefix are sequences from bands that are present in all dates in that plant zone. *Bracketed clusters* (*{}*) indicate clusters with identical FD and LV sequences

in the seasons, porewater chemistry and air temperature especially, would produce distinct differences in the diazotroph assemblages. With the environmental gradient and physical differences between the zones it was expected that there would be some effect on the distribution of the diazotrophs throughout the site. Of particular interest were the diazotroph assemblages associated with the plant species occurring in the two sections of the marsh having substantial differences—low- and high-elevation marsh. Porewater chemistry parameters such as salinity and soluble sulfide concentration are the most notable differences in these areas of the marsh with both higher in the low marsh. Duration of tidal flooding is also a distinct physical difference; the low marsh is more frequently submerged and for longer durations than the high marsh. These differences were expected to impact the composition of the assemblages and the data support this expectation. The seasonal effect data support previous results from *S. alterniflora* [20] but the observation of the influence of the host plant on diazotroph diversity across 6 different plant zones is the first of its kind.

Analysis of porewater chemistry data indicated the types of fluctuations in abiotic parameters to which the diazotroph assemblages associated with the marsh macrophytes are likely exposed. All measured constituents varied seasonally, however soluble sulfide and salinity varied with plant zone. High concentrations of soluble sulfide were recorded at the end of the growing season (August and September 2000). This indicates that the presumptive sulfate reducers were active at this time. It can also be inferred based on these data that sulfate reduction rates were relatively low during the winter and consistently lower in the higher marsh zones. These trends have been reported in previous studies [53]. Sulfide concentration and sulfate reduction are correlated to season; an increase in these solutes at the beginning of the growing season indicates an increase in metabolic activity in response to dissolved organic compounds in the sediment due to the onset of plant growth [23, 28, 29, 53]. Though sulfate reduction occurs in all plant zones it occurs at markedly higher rates in the short growth form *S. alterniflora* [28], a trend consistent with observations in this salt marsh ecosystem.

Salinity was at its highest value (for this data set) at the beginning of the growing season (June 2000). Salinity measurements were consistently higher in the mid-marsh and lowest in the high marsh. The low marsh is exposed to a tidal influx twice daily, whereas the high marsh experiences less tidal flooding. The mid-marsh areas can be much more stagnant and evapotranspiration can produce higher salinities there. High salinities (>50‰) are known to decrease the productivity of *S. alterniflora*, though this grass is capable of surviving salinities of up to 60‰ [43]. The measurements in this data set were mostly below the 50‰ threshold and the areas of the marsh in which higher salinities were measured were exposed only briefly as high salinities did not last throughout the season. Salinities at this site are known to reach values in excess of 50‰ in summer months but in 2000 the temperatures were lower than normal and heavy rainfall events could also have contributed to lower salinities [20].

The end of the growing season (August and September) heralded high rates of acetylene reduction (a proxy for nitrogen fixation), and increasing concentrations of ammonium. Though there is a correlation between carbon metabolism and nitrogen fixation rates [13] this was not examined in this study. Acetylene reduction rates did peak in the later portion of the growing season for some plant zones but at the end of the season where a significant increase in nitrogen fixation rates is expected [22], this process was undetectable in the low marsh. This could be explained by interannual variability in plant productivity that is well documented for this site [45]. Morris et al. [44, 46] found that above ground *Spartina* productivity was correlated with mean sea level and salinity. During the sampling year (2000–2001) the site experienced a low mean sea level for the summer months and therefore plant productivity was low. Ammonium concentrations were high throughout the sampling period as is typically observed in this system. The increase in porewater ammonium during the fall and winter sampling seasons may be due to the production of ammonia from decomposition processes [58].

The distinct clustering of samples from the plant zones in the different areas of the marsh (high or low elevation) indicates that the assemblages present within these zones are similar. The assemblages in the S and T zones are very similar, consistent with findings of Gamble et al. [20] in sites of short and tall form *S. alterniflora* in other locations in the North Inlet system. The assemblage in the JS zone is specific to *J. roemerianus* dominated zones. On dates where samples from both the JS and JP zones were available the samples clustered strongly; otherwise the samples from the JS zone separated into a distinct and homogenous cluster. The assemblage in the JP zone is also similar to the SS zone indicating it is influenced by both host and environmental parameters. This similarity is likely due to the similar trends in porewater chemistry that were observed (Table 1). The porewater parameters in the JP zone did not nearly attain the maximal levels that were observed in the S zone. Samples that were collected during the warmer months (June, August and September 2000) frequently clustered together, or samples from a particular date segregated from all others. Samples from April 2001 sometimes clustered with June or September 2000 samples as that month was particularly warm with a mean maximum temperature of 23°C [47]. The samples from the winter date

(February 2001) did not cluster with samples from any of the warmer months, including April 2001.

Despite seasonal differences, distinct clustering of samples based on host plant (JS and JP, S and T, SV and SS) occurred, indicating that there are host specific diazotrophs present in the assemblages associated with the rhizospheres of the host plants. Host specific diazotrophs have been examined in this system previously but those studies employed culture-based protocols and the findings did not represent the entire diazotroph assemblage [4, 7, 9, 31]. The distinct clustering based on host plant type occurred regardless of season and regardless of the number of samples available for analysis. Host specificity of the diazotroph assemblages is credible as microorganisms tend to be specific for root exudates produced from photosynthetic activity and nitrogen fixation is tightly coupled to plant photosynthesis [66]. Previously in this system, Bagwell et al. [4] found distinct physiological differences between pure cultures of rhizoplane diazotrophs from plants in the high-elevation marsh and those from the low elevation marsh. Gamble et al. [20] also found no significant difference between diazotroph assemblages associated with the rhizosphere of *S. alterniflora* at two different sites within the North Inlet system. LaRocque et al. [31] reported similar findings of host specificity based on physiological data from rhizoplane diazotrophs and *nifH* DGGE profiles from root and rhizosphere samples from *J. roemerianus* in two sites with very different abiotic environmental parameters. The data presented in this paper strongly support previous findings of host specificity of rhizosphere diazotrophs and provide clear evidence for this relationship across host plant types growing along an environmental gradient.

Differences in the root structure of each macrophyte leads to different levels of ventilation of the rhizosphere. *S. alterniflora* has well-anchored rhizoidal roots that oxygenate its rhizosphere via an aerenchyma system that draws oxygen from above ground sources to its below ground roots and rhizomes [25]. *J. roemerianus* has a network of tillers that support radial introduction of oxygen into its rhizosphere and infrequent flooding leads to longer exposure of surficial sediments overlying its rhizosphere. *S. virginica* does not have a true root system but has root hairs extending off older stems containing xylem cells and is unable to efficiently ventilate its rhizosphere [3]. Based on these differences in host plant root structure, abiotic environmental parameters, and physical differences in the marsh structure; the diazotroph assemblage exhibits clear differences among plant zones.

The sequences that contributed to the differences among the assemblages (LV) were elucidated and the organisms they represent were mostly novel, but some belonged to the classes α-, β-, δ- and γ-*Proteobacteria*. These sequences that produced the longest eigenvectors indicate the taxa that contribute significantly to the structure of the diazotroph assemblages. Though most of the sequences were novel, it is clear that the families *Acetobacteraceae*, *Ectothiorhodospiraceae*, *Pelobacteraceae*, *Pseudomonadaceae*, *Rhizobiaceae* and *Vibrionaceae* contain diazotrophs that strongly influence the structure of these assemblages. Organisms that were present throughout all seasons or in all zones in each season (FD) were also mostly novel, though some belonged to the classes *Chlorobia*, and the γ-*Proteobacteria*, specifically the families *Chlorobiaceae*, *Pseudomonadaceae* and *Vibrionaceae*. The three clusters containing both FD and LV sequences that were greater than 94% similar to each other indicate that there are some diazotrophs that are capable of maintaining detectable populations in all seasons, are present in all zones in the marsh and contribute to the structure of the assemblages. There is a large breadth of diversity in these significant sequences indicating that there is no one particular group of diazotrophs that drive the structure of the assemblages or is present both throughout the seasons and in all plant zones.

The recovery of virtually identical (>94% similar) sequences from three different studies ([16, 20], this study) that were sampled on different dates and in different locations in the marsh indicates that sequence recovery is highly reproducible and that these sequences represent taxa that are typical of this salt marsh ecosystem. Seasonally non-responsive sequences from Gamble et al. [20] (FJ) appeared in clusters with FD sequences and LV sequences (Electronic Supplementary Fig. 3a, b) confirming that there are diazotrophs present in the assemblages that are non-responsive to seasonal changes, capable of tolerating all the edaphic conditions across the marsh landscape and that contribute strongly to the structure of the assemblage. These diazotrophs would be predicted to be versatile in their use of carbon sources to survive in the different macrophyte dominated zones of the salt marsh. The presence of *nifH* mRNA sequences (GenBank accession numbers beginning with AY) in groupings with FD sequences (bracketed in the Electronic Supplementary Fig. 3a, b) indicates that some diazotrophs that are potentially active are included in the group of versatile, seasonally non-responsive sequences. It is therefore reasonable to assume that within the assemblage there are ubiquitous diazotrophs having the potential to actively fix nitrogen under all abiotic conditions occurring along the environmental gradient of this marsh and during all seasons. These presumptive ubiquitous and versatile diazotrophs are potentially of great importance to ecosystem function. Such organisms would not be dependent on a specific plant host or set of edaphic conditions and could be present and capable of activity in the marsh at all times. Nitrogen fixation is tightly coupled to plant photosynthetic

activity and necessary for plant productivity in a nitrogen-limited marsh such as this one. The ability of a diazotroph to be present and active at all times or in association with any plant host species indicates an organism that is likely to contribute significantly to ecosystem function.

Seasonally responsive sequences (FJ) also appeared in clusters with FD and LV sequences confirming that also present in the assemblage are frequently detected diazotrophs that were responsive to seasonal changes and contributed to the structure of the assemblage. These seasonally responsive sequences could represent the key organisms responsible for seasonal nitrogen fixation dynamics, promoting plant growth and ecosystem vitality at different times of year. These data highlight the complexity of the cosmopolitan diazotroph assemblage [4, 5, 8, 16, 38] and give the first insight into the distributions of important organisms in the diazotroph assemblages associated with the rhizospheres of salt marsh plants.

Seasonal changes and differences in marsh elevation affected the structure of the rhizosphere diazotroph assemblages. Marsh elevation clearly contributed to differences in diazotroph assemblages that corresponded to plant zonation patterns. The rate of microbial nitrogen fixation activity is correlated to plant photosynthetic activity [66] and the variety of carbon sources associated with each plant species can clearly influence the structure of the diazotroph assemblage. The diazotroph assemblages are diverse indicating that diazotrophy is functionally redundant and is not limited to a small number of microorganisms. Host plant-specific diazotrophs are present in the assemblages and are at least somewhat tolerant of differences in abiotic parameters. Versatile diazotrophs are also present that can utilize a variety of plant exudates, have broad tolerances for edaphic variability, and are thus present throughout the marsh at all times. These versatile diazotrophs could be very important to ecosystem function and contribute to the resiliency of salt marsh ecosystems. Some diazotroph taxa maintain detectable populations in the rhizosphere of salt marsh plants as indicated by the frequent recovery of their *nifH* sequences in several studies. Future studies will address the roles of specific diazotrophs in the assemblages and how these roles may vary across the marsh landscape.

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