Microb Ecol (2010) 59:253–265
DOI 10.1007/s00248-009-9558-0

ENVIRONMENTAL MICROBIOLOGY

Seasonal Variability of Diazotroph Assemblages Associated with the Rhizosphere of the Salt Marsh Cordgrass, *Spartina alterniflora*

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Received: 6 March 2009 / Accepted: 19 June 2009 / Published online: 22 July 2009

Abstract Nitrogen fixation is the primary N source in the highly productive but N-limited North Inlet, SC, USA salt marsh system. The diverse assemblages of nitrogen-fixing (diazotrophic) bacteria associated with the rhizospheres of the short and tall growth forms of *Spartina alterniflora* were analyzed at two sites, Crab Haul Creek and Goat Island, which are in different tidal creek drainage systems in this marsh. The sites differed in proximity to the main channel for tidal intrusion and in several edaphic parameters. We hypothesized that either the differing abiotic environmental regimes of the two sites or the variation due to seasonal effects result in differences in the diazotroph assemblage. Rhizosphere samples were collected seasonally during 1999 and 2000. DNA was purified and *nif*H amplified for denaturing gradient gel electrophoresis (DGGE) analysis of diazotroph assemblage composition. Principal components analysis was used to analyze the binary DGGE band position data. Season strongly influenced assemblage composition and biplots were used to identify bands that significantly affected the seasonal and site-specific clustering. The types of organisms that were most responsive to seasonal or site variability were identified on the basis of DGGE band sequences. Seasonally responsive members of the anaerobic diazotrophs were detected during the winter and postsenescence conditions and may have been responsible for elevated pore water sulfide concentrations. Sequences from a diverse assemblage of Gammaproteobacteria were predominant during growth periods of *S. alterniflora*. Abiotic environmental parameters strongly influenced both the *S. alterniflora* and the diazotrophic bacterial assemblages associated with this keystone salt marsh plant species.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-009-9558-0) contains supplementary material, which is available to authorized users.

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Introduction

Salt marshes of the Atlantic and northern Gulf of Mexico coasts of temperate North America are typically dominated by the keystone angiosperm, *Spartina alterniflora* (smooth cordgrass; *Spartina* hereafter) [48, 49]. *Spartina* marshes are highly productive and may be significant carbon sources for coastal marine waters (e.g., [9, 10, 12, 48]). Highest rates of *Spartina* productivity occur in the summer months [40], during which rates of plant-associated microbial processes are also maximal (e.g., [47]). In

addition to the seasonal cycle of growth and senescence, *Spartina* productivity is also influenced and limited by various edaphic and biotic factors.

A key limiting factor for *Spartina* productivity is nitrogen. This has been demonstrated by ecosystem mass balance estimation of nitrogen inputs and losses, which show that salt marsh systems operate at an apparent nitrogen deficit [7, 9, 16, 17, 53], as well as more directly by fertilization studies, which show strong stimulation of plant productivity in response to added nitrogen [1, 9, 17, 36, 39, 45, 53]. In marshes that do not receive high levels of anthropogenic inputs, nitrogen is supplied primarily by plant-associated nitrogen fixation [11, 39, 55]. Salt marsh diazotrophic (nitrogen fixing) bacteria are found in greatest numbers and maintain highest levels of activity in association with *Spartina* roots [37, 42, 43, 57]. The diazotrophic assemblage and its activity are sustained by carbon exudates from the *Spartina* roots [14, 29, 55, 57], which are expected to vary in quantity, tracking seasonal and trans-site differences in primary production.

Variability in nitrogen fixation rates in response to seasonally variable edaphic and biotic parameters is well established [20, 29, 47, 50, 56]. In contrast, comparatively little is known about variability of the assemblage of diazotrophic organisms responsible for this process. Piceno et al. [47] and Piceno and Lovell [45, 46] profiled the *Spartina* nitrogen-fixing assemblage associated with short growth form *Spartina* using denaturing gradient gel electrophoresis (DGGE) analysis of *nifH* sequences. This profiling method revealed apparent stability of the assemblage in spite of seasonal ecosystem fluctuations [31, 47] or manipulations of nutrients [45] or aboveground plant biomass [46]. Such stability was not anticipated, as rapid and pervasive responses of microorganisms to environmental variability appear to be the norm (e.g., [21, 28, 44]), and salt marsh systems display profound variability in numerous edaphic and biotic parameters. Prior studies of salt marsh diazotroph assemblage stability were relatively short in duration, examined only one sampling site, and were greatly limited by the resolution of the DGGE methods and data analysis techniques employed. Thus, the degree to which the diazotroph assemblage responds to environmental variability is still in question.

Resilience of such an essential bacterial assemblage as diazotrophs would have important implications in ecosystems that, like salt marshes, are subject to strong natural fluctuation in environmental parameters and/or anthropogenic impacts. In this study, we examined variability in the diazotroph assemblage, specifically addressing seasonal, site-specific, and plant growth form (tall- and short-form *Spartina*) associated variability. We hypothesized that diazotrophic assemblages are responsive to the seasonal pattern of *Spartina* production and senescence. In order to assess other potential sources of assemblage variability, two differing *Spartina* sites and both plant growth forms were examined.

Materials and Methods

Sampling Site Description

This study was conducted in the North Inlet Salt Marsh near Georgetown, SC, USA (33° 20′ N, 79° 12′ W). The area is an example of a relatively pristine southeastern North American *Spartina* marsh [4]. The short growth form of this plant occurs on the marsh platform, a relatively broad and flat expanse at the approximate elevation of mean high tide [24, 41], while the banks of the tidal creeks support the tall growth form [52]. Slight changes in elevation in the system, occurring over lateral distances of only a few meters, can result in substantial differences in edaphic parameters. In general, sediments located near the tidal creeks and occurring at elevations below that of the marsh platform experience substantial exchange between sediment pore water and tidal flood waters. Sediments located in the marsh platform or at higher elevations in the system frequently experience pore water stagnation, which, when coupled with evapotranspiration, can produce elevated levels of salinity and soluble sulfide [22], resulting in the stunted short growth form. The North Inlet system is nitrogen-depleted and nitrogen fixation is the primary source of combined nitrogen [39]. Two locations within the estuary were selected on the basis of their similar stands of both tall-growth-form and short-growth-form *Spartina*. The Goat Island site is subject to a larger volume of tidal flood water while the Crab Haul Creek site is further inland from tidal intrusion. Both sites have similar elevations in relation to mean high tide and comparable salinities due to extensive tidal flooding [40].

Biomass density of *Spartina* is similar but not identical at the two sites and biomass differences between the sites appear to be due to site-specific edaphic phenomena [40]. For example, Crab Haul Creek is a geologically younger site having lower sediment organic matter content [40]. The *Spartina* growing season begins in March, but peak productivity occurs from June through August and is highly dependent on regional tidal flooding and rainfall patterns [40]. September brings a loss of plant mass after the growth period, with final senescence later in the fall. Total productivity varies interannually, based on regional conditions [40, 41]. For this study, samples were collected four times over an annual cycle: October 1999, February 2000, June 2000, and September 2000. October is the beginning of the fall–winter senescence. February is an example of nongrowth winter conditions in the marsh. June is

associated with the growing season and highest productivity. September represents the end of the growing season and the period of plant flowering.

Sediment and Pore Water Analysis

Samples were collected from six 1.0-m^2 plots oriented along horizontal transects in the short- and tall-form *Spartina* zones of Goat Island and Crab Haul Creek. Pore water was collected within 1 h of low tide using sippers that were open at 6-cm depth in the sediment [57]. Pore water samples were collected for determination of soluble sulfide [51] and ammonium [15] concentration. Salinity and pH measurements were made using a refractometer (Leica Inc., Buffalo, NY, USA) and portable pH meter (Cole Parmer, Chicago, IL, USA), respectively.

Acetylene reduction assay (ARA) methods to monitor the rates of nitrogen fixation were modified from Whiting and Morris [56]. Sediment cores of approximately 10-g fresh weight (1.5-cm diameter by 8-cm length) were collected from each plot and aseptically transferred to 40-ml serum vials containing 10 ml of sterile artificial seawater (34-ppt salinity). The serum vials were sealed with butyl rubber stoppers secured with aluminum crimp seals. The vials were injected with acetylene (1.5 ml per vial in a headspace of 15 ml), incubated at in situ temperature, and ARA was determined at 24, 48, and 72 h 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 to calculate acetylene reduction rates. Pore water chemical parameters and ARA rates were statistically analyzed using two-tailed Student's *t* tests.

Recovery and Analysis of *nif*H Sequences

Six sediment cores (2.4-cm diameter by 6-cm length) were collected from each site and plant zone for each sampling date. Cores were frozen on dry ice, transported to Columbia, SC, USA, and maintained at −70°C until extraction. DNA extraction was performed using a previously described direct lysis method [34, 47]. Extracted DNA was further purified using the Promega Wizard DNA Cleanup System (Madison, WI, USA) following the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using a 100-μl reaction mixture containing: 25 ng of template DNA, 6 mM $MgCl_2$, 800 μM each deoxynucleotide triphosphate, 50 pmol of each primer, and 40 μg bovine serum albumin, 0.2U Taq polymerase per microliter (Qiagen, CA, USA) The GC-clamped *nif*H primers for DGGE were designed by Piceno et al. [47] and contained the artificial nucleotides P [27] and K [7]. The amplification program began with a 2-min denaturation step at 94°C followed by two phases of 20 cycles each (phase I 94°C for 45 s, 58°C for 30 s decreasing by 0.5°C with each cycle, 70°C for 30 s followed by phase II 94°C for 45 s, 48°C for 30 s, 68°C for 30 s), then a 2-min elongation step at 72°C. Following PCR, 90 μl of the amplicon solution was concentrated by alcohol precipitation. The remaining 10 μl of the amplicon solution was run on a 1.5% agarose Tris–borate–ethylenediaminetetraacetic acid (EDTA) gel to check for amplification. Amplicon size was approximately 460 bp.

The precipitated amplicons were recovered by centrifugation, washed with 70% ethanol, and dissolved in 10 μl TE (10 mM Tris–HCl, pH 8.0; 1 mM EDTA). DGGE of the *nif*H amplicons was performed as previously described [26]. GC-clamped *nif*H amplicons of *Klebsiella pneumoniae*, *Sinorhizobium meliloti*, and *Azospirillum lipoferum* were used as standards to facilitate gel comparisons. A previously identified artifact band (presumably single-strand DNA and present in every lane; [44]) was also employed. The gel was stained with 15 μl SYBR Gold (Molecular Probes, Eugene, OR, USA) in 250 ml TE for 30 min and an image was acquired using an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA, USA).

Analysis of the DGGE banding patterns was performed using GelCompar II software (BioSystematica, Devon, UK). The gel images were imported and the auto band search function (minimum profiling 4.00% and shoulder sensitivity 1) was used to initially mark bands. A manual check of the identified bands was performed to remove incorrectly marked "bands." Numerical representations of band locations were used to determine the similarities of banding patterns across various gels. After analysis of the total bands for all samples, the bands were given an ultimate band number (UBN) for a total of 50 identified band positions across all gels and samples analyzed. Presence or absence was scored for each of the 50 band positions for every lane and the binary data were compiled for statistical analysis. Gel analysis was not performed on samples that were deemed inadequate due to poor-quality DNA amplification.

Statistical analysis of the DGGE band presence/absence binary data was performed using the Multivariate Statistical Package 3.13 g (MVSP; Kovach Computing Service, Wales, UK) [13]. Eighty (of 96 total) individual lane samples were deemed of adequate quality for analysis and were compiled for the total presence/absence data set. Fifteen samples were used from October with five representing Goat Island Tall (GIT), five representing Goat Island Short (GIS), two representing Crab Haul Creek Tall (CHT), and three representing Crab Haul Creek Short (CHS). All 24 samples were used from February (six replicates from each site and plant growth form). There were 19 samples from June, including five samples each

from GIT, GIS, and CHT and four samples from CHS. For the September samples, 22 lanes were used, six samples each from GIT and CHT and five samples each from GIS and CHS. Lower replicate numbers for some sets reflect poor DNA recovery from some replicates.

Principal components analysis (PCA; MVSP) of the presence/absence data using Kaiser's rule was performed and results were plotted both with and without Euclidean biplot. The Euclidean biplot provided eigenvectors that indicated DGGE bands that controlled clustering. Figures were produced for the following comparisons: all data, data by date, data by location, and data by plant growth form.

A distance measurement between clusters was used to establish the significance of clustering. Using the basic distance formula (formula 1), the average point for each date and site condition was determined and the distance was calculated for each PCA data point from all the PCA data points within that group cluster as well between each condition's average PCA data point. A two-tailed Student's *t* test was used to determine the significance of the distances between the various date, site, and plant-type groups.

$$\sqrt{(x_1 - x_2)^2 + (y_1 - y_2)^2}$$

Formula 1

This is the basic distance formula where x_1 and y_1 are the (x, y) coordinates for the average point within a cluster and x_2 and y_2 are (x, y) coordinate values for a point being analyzed.

Eigenvectors produced from the Euclidean biplot were used to identify individual bands that contributed to formation of seasonal and site-specific PCA clusters. The sums of the absolute values of the x and y coordinate for each eigenvector were used to classify the bands as strong or weak determinants. A threshold value of 0.3 was used to identify a band that strongly influenced formation of PCA clusters (the longer the eigenvector the stronger the effect).

DGGE Band Sequence Analysis

The most intense and frequently observed DGGE bands were sampled. Highly resolved DGGE gel lanes having intense bands often represented high quality, rather than quantity, of sample DNA. Consequently, the strongest bands are likely more due to effective PCR amplification than to actual quantities of each target sequence in a sample. Band stabs were taken using wide bore 200-µl pipette tips and frozen in 100 µl TE. DNA from band stabs was reamplified as above. Amplicons were then used for cloning and sequencing and a replicate DGGE gel to correlate band position with the previously determined 50 band positions. While there were few extraneous bands in positions surrounding the original band position, all bands yielded intense signals in their original DGGE gel position.

DGGE band sequence amplification and cloning were performed as described by Lovell et al. [32, 33]. Recombinant plasmids were purified using the Qiagen Plasmid Mini Kit (Santa Clarita, CA, USA). PCR using primers specific for the T7 and Sp6 RNA polymerase binding sites of pGEM-T and sequencing were performed as described previously [32]. A total of 219 *nif*H sequences were deposited with National Center for Biotechnology Information (NCBI) GenBank (accession numbers FJ294926–FJ395144).

Reference NCBI GenBank *nif*H sequences from a variety of diazotrophs were included in phylogenetic analyses. Nucleotide sequences were aligned using Clustal X (version 1.81, The Pennsylvania State University, University Park, PA, USA). Neighbor-joining phylogenies using the Jukes–Cantor model, complete deletion of gaps and missing data, and 1,000 bootstrapping replications were performed using MEGA version 3.1 [25].

Results

Edaphic Parameters

Salinity ranged from 22.7‰ to 38.8‰ during the period studied (Table 1). Salinities were significantly different for all dates ($p<0.001$) and increased from January through June followed by a slight decrease in September. The short-form *Spartina* zones had higher summer salinity for both sites, presumably due to the higher elevation and subsequent pore water stagnation. The Crab Haul Creek salinities in the short form were higher than that of the tall form for all dates sampled. Goat Island salinities were lower in the short form than tall form for all dates with the exception of June. Other than the increased values for the short form in Crab Haul Creek, salinity was not a significant indicator of site effects.

Soluble sulfide concentrations were significantly different for all seasonal comparisons ($p<0.001$) except between October and June and between October and September (Table 1). Sulfide concentration differences between sites were significant for October ($p<0.001$). Sulfide concentrations were also significantly different for all sites ($p<0.01$). Overall, Goat Island had higher levels of soluble sulfide at all dates and for both plant growth forms.

Ammonium concentrations were higher in the short-form zones on all sampling dates except for September in Crab Haul Creek (Table 1). Ammonium concentrations were significantly different between October and June and between February and June ($p<0.01$) but were not significant for between-site comparisons on any date.

Table 1 Pore water chemical parameters for the Goat Island (GI) and Crab Haul Creek (CH) tall (T) and short (S) *S. alterniflora* zones

Site	Salinity (‰)	pH	H_2S (mM)	NH_4^+ (mM)
10-99 GIT	24.7 (1.4)	7.5 (0.2)	3.36 (0.34)	187.13 (56.82)
10-99 GIS	22.7 (5.2)	7.4 (0.1)	2.95 (1.50)	621.81 (387.98)
2-00 GIT	27.8 (0.8)	7.4 (0.4)	0.45 (0.45)	231.73 (154.70)
2-00 GIS	25.7 (2.4)	7.5 (0.3)	0.52 (0.73)	826.35 (476.43)
6-00 GIT	36.2 (1.6)	7.4 (0.2)	2.17 (0.91)	ND
6-00 GIS	38.5 (2.7)	7.5 (0.2)	2.17 (0.98)	4,707.20 (4,971.53)
9-00 GIT	32.0 (0.6)	7.1 (0.1)	4.17 (0.33)	N/S
9-00 GIS	31.7 (2.2)	7.0 (0.1)	3.92 (0.77)	N/S
10-99 CHT	23.1 (1.3)	7.3 (0.1)	0.38 (0.31)	124.60 (19.51)
10-99 CHS	26.5 (1.2)	7.6 (0.4)	1.77 (1.00)	204.68 (41.40)
2-00 CHT	25.8 (2.6)	7.2 (0.3)	0.00 (0.00)	41.25 (99.77)
2-00 CHS	28.7 (0.8)	7.3 (0.2)	0.23 (0.30)	186.65 (200.40)
6-00 CHT	36.7 (1.6)	7.4 (0.1)	0.97 (0.42)	917.30 (1311.72)
6-00 CHS	38.8 (1.2)	7.4 (0.1)	2.82 (0.94)	3,111.30 (2,047.53)
9-00 CHT	32.0 (0.6)	8.0 (1.4)	1.87 (1.35)	201.77 (149.44)
9-00 CHS	34.0 (1.1)	7.0 (0.1)	3.88 (0.96)	174.60 (108.19)

The numerical site codes indicate the date of sampling, (i.e., 10-99 indicates October 1999). Average values are based on six replicates per site and date. Standard deviations are shown in parentheses

N/S no sample, *ND* below detection limits

The pH was generally similar for all dates, locations, and plant types (Table 1). There was no significant seasonal, site, or plant growth form effect related to pH ($p>0.05$).

Acetylene reduction rates were only measurable during the June and September sampling dates, with the exception of the October GIS growth form samples (0.37 nmol ethylene per square meter per day). Acetylene reduction activity was measured in all sites and plant growth forms for June (GIT 0.33, GIS 0.45, CHT 0.12, and CHS 0.52 nmol ethylene per square meter per day), but only measured in Goat Island samples during September (GIT 0.99 and GIS 1.17 nmol ethylene per square meter per day). Nitrogen fixation activity was not significantly different between June and September, but both dates differed from February ($p<0.01$). Acetylene reduction activity was also significantly different between sites in September ($p<0.01$).

Diazotroph Assemblage Characterization by PCA

PCA was used to develop a quantitative assessment of individual DGGE band contributions to the observed patterns of seasonal variability. The combined data (Fig. 1) exhibited primary clustering by season with stronger clusters for the June and September samples. Only June and September were significantly different based on cluster distances ($p<0.001$). The October and February samples formed a diffuse cluster. Within the combined data PCA plot, the June cluster showed separation of the Goat Island and Crab Haul Creek samples. Plant growth form was also differentiated within these Goat Island samples. September samples lacked site separation. Two September samples fell near the large December and February cluster. The October and February samples were mixed for both site and growth form. The combined data analysis indicated a strong seasonal effect on the banding pattern.

To further examine the possible effects of site or plant growth form, the data were also examined by date (Fig. 2). GI and CH clusters were significantly different ($p<0.001$) within each date PCA profile. The October samples were separated very well by site, but plant growth form separation was not observed. This site group is the smallest with only five usable lanes and two of these had identical band profiles. Goat Island samples were significantly separated by plant growth form ($p<0.01$). February samples were separated by site with some plant growth form separation within the Crab Haul Creek samples ($p<0.01$). June samples were not significantly separated by either site or plant growth form. September samples had site-dependent separation and strong separation of plant growth form associated with the Crab Haul Creek samples ($p<0.01$). Data were also examined by site as well as growth form using PCA (data not shown). However, seasonality was clearly the strongest influence.

Euclidean distances with Euclidean biplots were also used to examine the data and this method presented the same profiles.

Seasonally Responsive Diazotrophs

To further elucidate the diazotroph taxa most responsible for the seasonal shifts in the composition of the diazotrophic assemblage, *nifH* sequences from DGGE band stabs were compared. The dates for the band stab analysis were chosen to evaluate bacterial community changes among the nongrowth winter (February; $n=17$), summer (June; $n=6$), the postsummer flowering (September; $n=16$), and the postflowering early senescence (October; $n=15$) periods.

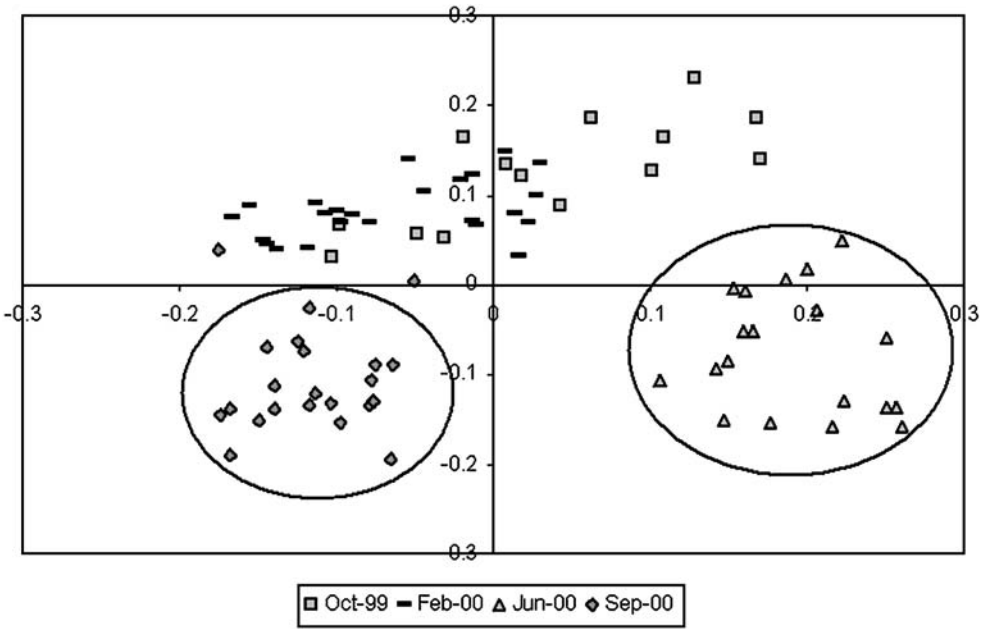

Figure 1 Principal component analysis of all DGGE band data. *Circles* indicate significance ($p < 0.001$) for clustering. *Gray squares* indicate October 1999 samples; *black dashes* indicate February 2000 samples; *gray triangles* indicate June 2000 samples and gray diamonds indicate September 2000 samples. *Axis 1* represents 17.67% of the variation and *Axis 2* represents 12.25% of the variation

Variability in the numbers of band stabs taken was due to differences in DNA quality, PCR amplification, and band resolution in the gels.

Sequencing showed that these bands consisted of multiple *nif*H sequences, as seen previously [30, 34]. Each band stab contained an average of five different *nif*H sequences. Cloning and *nif*H sequencing of the bands provided 217 total sequences with 96 from February, seven from June, 89 from September, and 25 from October. Phylogenetic analysis (Supplementary Fig. 1) was employed to determine which *nifH* sequences were seasonally responsive or nonresponsive, based upon their cluster-

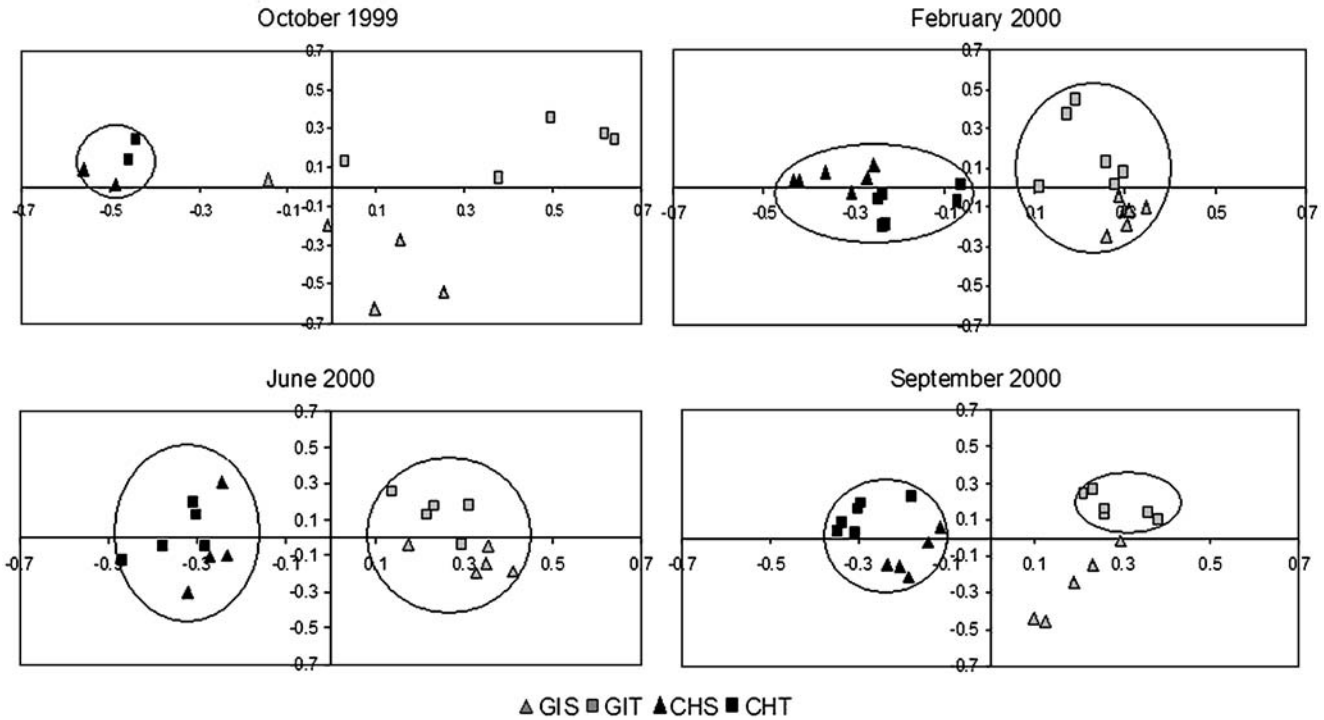

Figure 2 Principal component analysis of October 1999, February 2000, June 2000, and September 2000 samples by date. *Circles* indicate significance ($p < 0.01$) for clustering. *Gray triangles* indicate Goat Island short *Spartina* samples; *gray squares* indicate Goat Island tall *Spartina* samples. *Black triangles* indicate Crab Haul Creek short *Spartina* samples and *black squares* indicate Crab Haul Creek tall *Spartina* samples

ing. A cluster of sequences was designated seasonally responsive if it contained sequences from only one sampling date. Clusters that contained sequences from multiple sampling dates were considered to represent frequently recovered or nonresponsive taxa. There were a few exceptions where clusters contained sequences from one date and also included a single sequence from another date; in this case, all sequences within the cluster were designated as seasonally responsive. This evaluation was performed at the individual sequence level. After separating the sequences into responsive and nonresponsive groups, additional phylogenetic analyses were performed and included reference sequences employed by Lovell et al. [32] and *nif*H messenger RNA (mRNA) sequences presented in Brown et al. [6]. The nonresponsive phylogram (Fig. 3) contains 129 band stab sequences and the seasonally responsive phylogram (Fig. 4) contains 88 band stab sequences. Phylogenetic analyses were also performed using the sequences from the current study and those from clone libraries determined by Lovell et al. [30], in order to further assess the types of organisms represented in the seasonally responsive and nonresponsive groupings.

Phylogenetic analyses were also performed using translated peptides, pairwise deletion of gaps and missing data, and maximum parsimony and minimum evolution models; however, all analyses exhibited the same trends. For ease of discussion, only the nucleotide sequence, neighbor joining, Jukes–Cantor-corrected, 1,000 bootstrap replicates, and complete deletion of gaps and missing data version are presented.

The nonresponsive group included numerous sequences affiliated with pseudomonads as well as a clearly defined *Vibrionaceae* clade. There were also sequences associated with *K. pneumoniae*, *Sulfitobacter* sp., and *Azoarcus*. Within the anaerobe grouping, there were clusters of sequences associated with *Desulfosporosinus* and *Desulfovibrio* sequences. A large percentage of the sequence collection was not strongly allied with known diazotroph reference sequences.

The seasonally responsive group included sequences affiliated with pseudomonads and other Gammaproteobacteria. There was also a small cluster associated with *Herbaspirillum seropedica.* Within the anaerobe grouping, sequences clustered with various presumptive *Desulfovibrio* sequences. Once again, many sequences did not cluster strongly with known diazotroph sequences.

After correlating the Euclidean biplot data and the *nif*H DGGE band sequence analysis of the 50 band positions, 11 were noted as relatively invariant (nonresponsive) and unique seasonal bands were also identified for each date. Two bands were attributed to clustering for October, four for February, five for June, and six for September; these seasonally variable bands are noted in bold in Table 2. In total, there were 35 band positions from October samples, 28 from February samples, 38 from June samples, and 34 from September.

Discussion

The diazotroph assemblage associated with *Spartina* consisted of both a seasonally nonresponsive group detected at all times of year and a seasonally responsive group of organisms that were undetected at some times of the year. Seasonality is the strongest predictor of diazotroph assemblage shifts, with site effects following and plant growth form being the least significant. The seasonally responsive assemblage appears to be determined, directly or indirectly, by edaphic variables, such as sulfide concentration or salinity. Edaphic conditions correspond to seasonal differences in diazotroph communities. Thus, seasonally responsive diazotrophs grouping with known anaerobes were represented during senescence while the seasonally variable Gammaproteobacteria were more readily detected during the *Spartina* growing season. The increase in detectable diazotroph diversity during the *Spartina* growing season is reflected by measurable ARA rates during the summer months.

Among the seasonally variable parameters that may impact the diazotroph assemblage are sulfide concentrations, which significantly differed for all dates compared and are related to increased primary production and organic matter availability [18, 23]. Highest concentrations were observed in September and lowest in February, correlating well with the seasonal changes in organic matter availability due to the growth and senescence phases of *Spartina*. Highest organic matter availability will likely occur during senescence when the plant moves energy resources to storage roots in the rhizosphere from which they are released through root death and turnover. The increased organic matter availability and abundant sulfate from tidal flood water provides significant resources for the sulfate-reducing bacterial assemblage, which includes many diazotrophic taxa [18, 23, 32, 33].

Salinity and temperature correlated with overall seasonality, i.e., increasing summer temperatures promote increased evapotranspiration and thus increased summer salinity. Seasonal salinity fluctuations are typical of salt marshes and have been shown to affect *Spartina* productivity [5, 38]. Primary productivity by *Spartina* is maximal at 20 ppt, but summer pore water salinities in this study exceeded 35 ppt and can reach values of 50 ppt or greater [39]. Lower than average temperatures occurred during summer 2000 and significant rainfall events occurred on May 25, 2000 and June 29, 2000, with the largest rainfall event of the year on September 18, 2000 [3]. Sampling dates fell prior to these rainfall periods, but pore water salinity could have been affected by rainfall, tidal effects, or lower evaporation due to lower than average temperatures. Salinity may influence plant exudate supply [19, 54] and, indirectly, the diazotroph assemblage that depends upon exudates for carbon and energy [14, 29, 56]. This effect

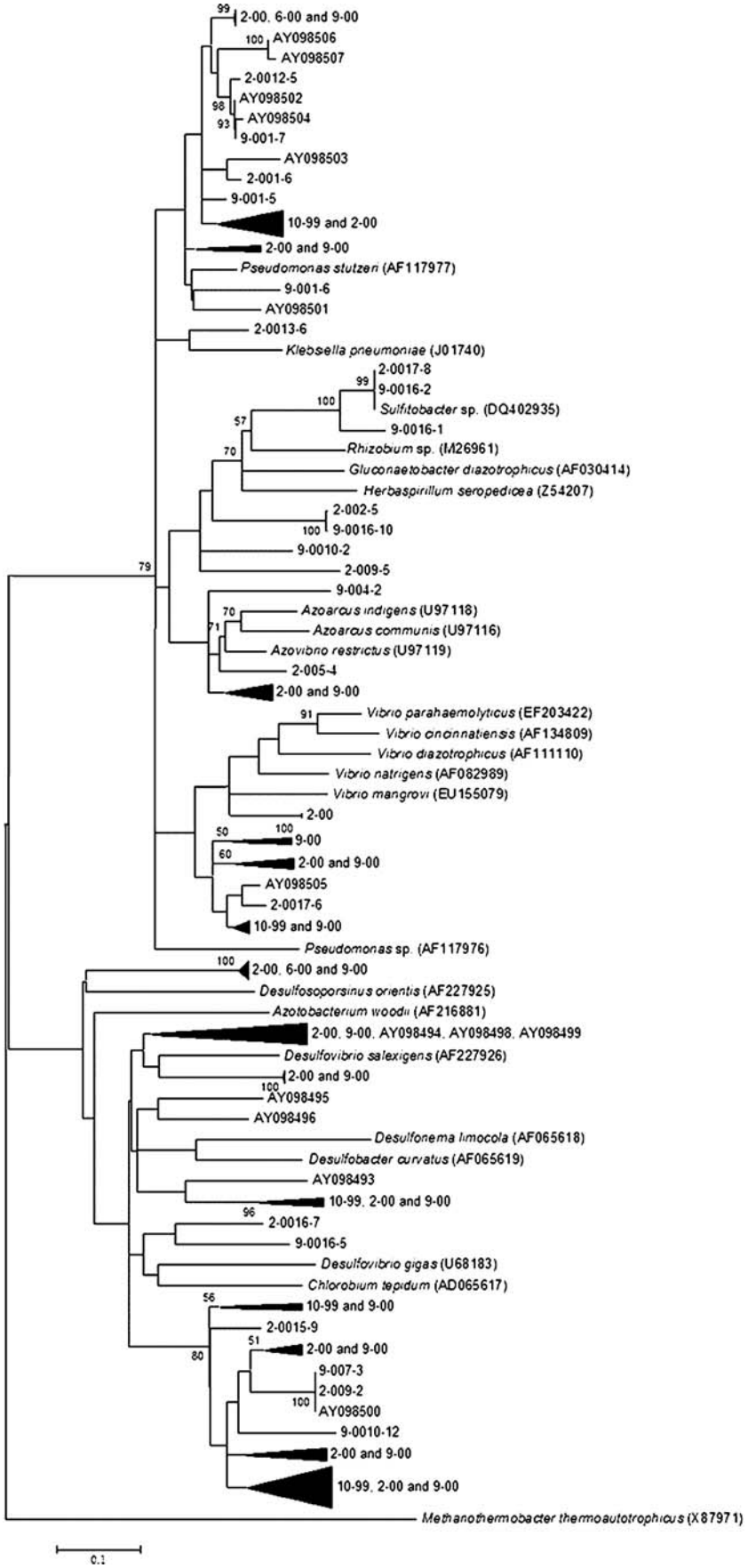

Figure 3 Phylogenetic analysis of seasonally nonresponsive bands from band stab sequence analysis (nucleotide sequences, neighbor joining, 1,000 bootstrap replicates, Jukes–Cantor correction, and complete deletion of gaps and missing data)

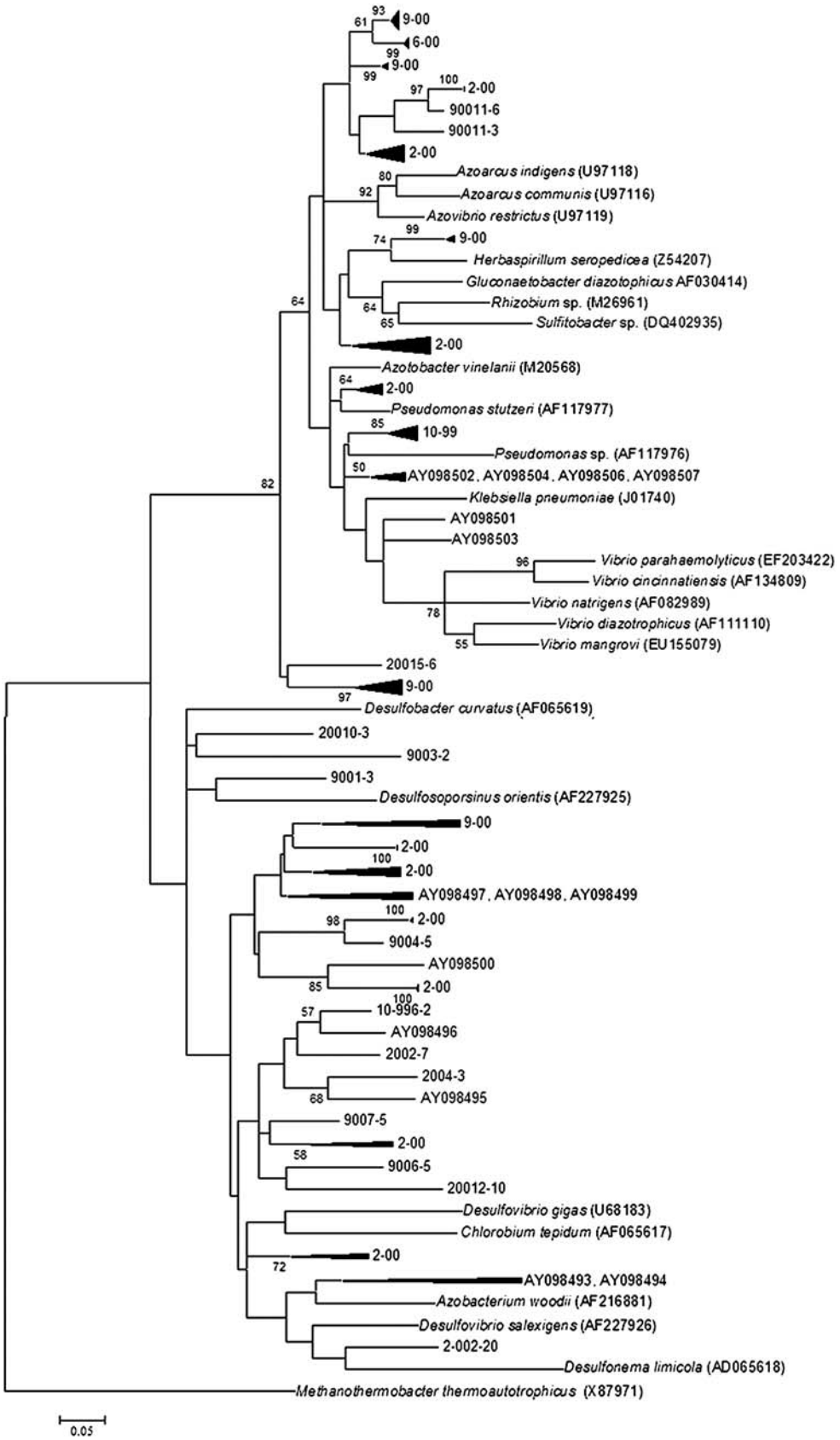

Figure 4 Phylogenetic analysis of seasonally responsive bands (nucleotide sequences, neighbor joining, 1,000 bootstrap replicates, Jukes–Cantor correction, and complete deletion of gaps and missing data)

Table 2 *nifH* denaturing gradient gel electrophoresis bands

UBN	10-99	2-00	6-00	9-00
1	1			1
2	2	*1*	1	2
3	3	*2*	*2*	3
4	4	3		4
5	5	4	3	5
6	*6*	5		6
7	7	6	4	7
8	8	7	5	8
9	9	8	6	9
10	10	9	7	10
11	11	10	8	11
12	12			12
13	13			13
14	14			14
15	15	11	9	15
16			10	16
17	16	12	11	
18	17		12	
19	18	13	13	17
20		14	14	
21			15	
22	19	15	16	18
23	20	16	17	19
24		17	18	*20*
25			19	*21*
26			20	*22*
27			21	*23*
28			22	*24*
29			23	25
30			*24*	26
31			25	
32			*26*	
33	21		27	
34	22	18	*28*	27
35	23	19	29	28
36	24		30	29
37	25		31	
38	*26*		32	*30*
39	27	20	33	31
40	28			
41	29	21	34	32
42	30		35	
43	31			
44	32	22	36	33
45	33	*23*	*37*	34
46	34	24	38	
47	35	*25*		
48		26		
49		27		
50		28		

Ultimate Band Number (UBN) values in italics represent the 11 seasonally nonresponsive bands. Bands marked in italics for each date represent the seasonally responsive bands

would be predicted to be stronger at the Crab Haul Creek site, due to its higher elevation and thus less frequent and shorter-duration tidal flooding.

Pore water ammonium concentrations also varied with season and were comparable for both sampling sites on each date sampled. June ammonium concentrations were significantly higher than during the *Spartina* nongrowth periods. North Inlet is a known nitrogen-limited system and ammonia is the product of diazotrophy, as well as a product of decomposition [33]. This condition may be a contributing factor to the seasonal differences seen in the bacterial community during the June sampling, when rates of diazotrophy were highest. Ammonium concentrations increased seasonally but some extremely high values (6-00 GIS) could have resulted from elevated decomposition rates during the warm season prior to *Spartina* senescence.

In this study, diazotrophy was only quantifiable during the active growing season of *Spartina*. Increased organic carbon availability during *Spartina* growth is likely necessary for the activity of the rhizosphere diazotroph assemblage which ultimately supports the nitrogen demand of the entire estuarine system [39]. The pronounced seasonality of nitrogen fixation activity might be correlated with the seasonal changes seen in the diazotrophic assemblage between the February/October and June/September DGGE profiles. The combined DGGE band data exhibited primary clustering by season with stronger clusters for the June and September samples, most likely due to stimulation of the rhizosphere diazotroph assemblage by *Spartina* productivity during those dates. The organisms depicted by the February/October DGGE profiles represent the persistent base assemblage diazotrophs, while the June/September profiles represent the (presumably) active and dynamic diazotroph assemblage. More DGGE bands were visible during the active growth or senescence period as compared to the nongrowth winter condition, as expected from the higher rates of nitrogen fixation during this time.

As has been seen in previous studies [32, 33, 35], there were two major clusters within the seasonally responsive tree, one including sequences similar to those from known diazotrophs in the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria classes and another cluster of sequences similar to those from known anaerobic diazotrophs. Sequences within the anaerobe cluster were recovered from the February, September, and October samples, with a strong predominance of February sequences, indicating a core group of diazotrophic sulfate reducers that are maintained over the winter period. All sequences reported here corresponded to those previously analyzed by Lovell et al. [32] in a comprehensive study of *nifH* sequences recovered from *Spartina* rhizosphere. Rarefaction analysis indicated that the recoverable *Spartina*

rhizosphere *nifH* pool was fully sampled by Lovell et al. [32], implying that the current sequence set reflects this recoverable sequence pool.

Within the seasonally responsive phylogram, sequences from the same band stabs often grouped together, indicating similarity of sequences contained in each band. This similarity of sequences within a band stab is not as clearly defined for the seasonally nonresponsive sequences, indicating that seasonal changes in a small group of taxa in the diazotroph assemblage are responsible for the changes in the DGGE profile. The smaller clusters typically include sequences from the same or nearby band stabs, indicating a similarity of band migration based on melting profile [35] and perhaps a similarity of unknown bacterial species contributing to changes in the seasonal community composition.

The seasonally nonresponsive tree also contained a large cluster of sequences from anaerobes that were represented in all sampling dates. These sequences reflect an assemblage of anaerobic diazotrophs that are maintained over the entire annual cycle. It should also be considered that sequences such as the February anaerobe group identified as "seasonally responsive" are able to maintain viable populations during the active growth period of *Spartina* and to compete successfully for resources during *Spartina* senescence. These anaerobes are therefore detectable during periods of *Spartina* senescence, i.e., February. Thus, the nonresponsive anaerobes represent a base assemblage of anaerobes that are detected during all seasons, likely indicating robust populations, but seasonally responsive sequences also reflect organisms that maintain populations that may fall below detection limits of the methods employed here, but most likely persist. Using these molecular methods, a seasonally significant assemblage was depicted that can be classified as "opportunists" or the members of the assemblage that maintain populations and potential activity through periods of decreased substrate availability.

Also represented in the nonresponsive tree is a large Gammaproteobacteria cluster containing sequences obtained from all sampling dates and that cluster with various authentic Gammaproteobacteria *nif*H sequences. As expected, *nif*H sequences from Gammaproteobacteria are readily recovered throughout the annual cycle, and the organisms they reflect constitute a diverse segment of the rhizosphere diazotroph assemblage. The diversity of Gammaproteobacteria in the rhizosphere is consistent with findings from previous studies [2, 6, 32, 35]. Interestingly, a *Vibrionaceae* clade has been identified from both a large *nifH* clone library analysis [32] as well as the current DGGE band sequence analysis. All sequences within this clade were greater than 79.4% similar to one or more of the *Vibrio* reference sequences. The lowest similarity between *nifH* from the known diazotrophic reference *Vibrio* species was 79.4% between *Vibrio natriegens* and *Vibrio cincinnatiensis*, strongly supporting the placement of the DGGE sequences in the *Vibrio* clade. This finding is significant in that a great variety of diazotrophic *Vibrionaceae* have been isolated from this estuary through culture-based techniques [1, 2] and these organisms are versatile, fast growing (under appropriate conditions), and ubiquitous in estuarine systems. This is the first phylogenetic analysis to show that diazotrophic *Vibrionaceae* are stable and consistent members of the salt marsh diazotrophic assemblage. Further, two previously reported *nif*H mRNA sequences clustered within the *Vibrio* clade, indicating that these organisms can express *nifH* and presumably support diazotrophic activity in the *Spartina* rhizosphere [6].

These *Vibrio* sequences may have been present in earlier phylogenetic studies but unrecognized. Recent reference sequences added to the database have improved resolution of *nif*H sequences from this family of bacteria. Future studies will examine seasonality of *Vibrio* species within this environment. Since *Vibrionaceae* are known to be highly responsive to temperature (e.g., [8]), it is likely that temperature-driven seasonal trends may be controlling their population sizes and activity. Additionally, this family of bacteria may also use the rhizosphere as a refugium from extremely cold temperatures during the winter months and would therefore be persistent members of the winter assemblage, as they are identified in the seasonally nonresponsive tree.

A small alphaproteobacterial cluster is also seen in the nonresponsive tree. Only four sequences are represented in this cluster and none in the seasonally responsive tree. This is in agreement with previous studies that also reported very few *nifH* sequences from diazotrophic Alphaproteobacteria [30, 35] and no recovery of expressed mRNA from Alphaproteobacteria [6]. Alphaproteobacteria do not appear to be important members of the *Spartina* rhizosphere diazotroph assemblage.

While a majority of the seasonally responsive sequences are clearly not from formally described diazotrophs and do not appear to be closely related to known diazotrophs, there are significant seasonal assemblages of Gammaproteobacteria represented by specific DGGE bands. Also, a distinct winter anaerobe assemblage was identified from these analyses. Sequence analysis confirms that DGGE banding patterns depict both the nonresponsive fraction of the assemblage and seasonally responsive members of the assemblage. Further studies will be required to identify the key taxa in these seasonal shifts and these studies will target those taxa expressing *nifH* mRNA.

Piceno and Lovell [45, 46] showed that nutrient additions [47] and clipping of aboveground biomass [46] over a short time period (2–8 weeks) did not significantly change the composition of the diazotrophic community.

Piceno et al. [45] also reported a stable diazotroph assemblage that did not change through the annual cycle of *Spartina* production and senescence. Using the methods employed at that time, band pattern changes were not detected. Similarly, analysis of phospholipid fatty acid profiles from the same experiments lent support to the argument that the *Spartina* rhizosphere diazotroph assemblage was remarkably stable [31]. Primarily due to improved DGGE techniques and more refined analysis of DGGE band patterns, our analysis included a much larger number of bands and demonstrates seasonal changes in the bacterial community. This analysis was further supported by recovery of DGGE band *nifH* sequences that included clades representing seasonally variable diazotroph taxa. These findings expand on previous work [32, 35] by showing that seasonal changes cause shifts in the presence and/or activity of certain organisms.

Seasonal changes in edaphic parameters could affect the diazotroph community composition. The productive summer months would result in increased plant exudates, increased oxygen diffusion into the rhizosphere, and increased organic matter availability. Clearly, seasonal aspects of *Spartina* growth heavily impact the rhizosphere bacterial community. Increased activity was measured during the summer months, corresponding to an increase in DGGE band numbers, some of which are representative of season. Further studies are needed to elucidate the influence of specific abiotic and biotic variables on the diazotrophic assemblage.

Acknowledgements We acknowledge the Belle W. Baruch Institute for Coastal and Marine Sciences for access to sampling sites and for logistical support and Mike Friez for assistance with DNA sequencing. This research was supported by the National Science Foundation awards DEB-0108412 and MCB-0237854 to C.R.L. Contribution 1578 to the Belle W. Baruch Institute for Coastal and Marine Sciences.

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