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Spatial and Temporal Assessment of Diazotroph Assemblage Composition in Vegetated Salt Marsh Sediments Using Denaturing Gradient Gel Electrophoresis Analysis

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ABSTRACT

Diazotroph assemblage compositions were assessed in rhizosphere sediments from the tall and short form *Spartina alterniflora* growth zones over an annual cycle. Sediment cores were collected for DNA extraction and nitrogenase (acetylene reduction) activity assays, and porewater samples were analyzed for several chemical parameters in March, June, September, and December 1997. These data were collected to determine if within- or between-zone differences in the diazotroph assemblage composition correlated with differences in key environmental variables or acetylene reduction activity. Acetylene reduction rates differed between zones and within a zone over an annual period. Soluble sulfide concentrations were higher in the short form *S. alterniflora* zone on all dates except those in June and differed within both zones on different sample dates. *nifH* sequences were recovered from rhizosphere sediment DNA by PCR amplification using *nifH* specific primers. These amplimers were analyzed using denaturing gradient gel electrophoresis (DGGE), and the resulting patterns were compared by neural network and linear discriminant analyses. Ten prominent amplimers, four of which were apparent heteroduplexes, were observed. DGGE banding profiles showed minor differences among sampling dates and between sample zones, but the overall banding pattern was remarkably consistent. This reflects overall similarity between the amplifiable diazotroph assemblages in the tall and short *S. alterniflora* growth zones and substantial seasonal stability in assemblage composition.

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Introduction

Recent investigations of microbial community structure have demonstrated for various systems that contiguous environments differing in important physicochemical parameters support communities differing in species composition [15, 49]. These communities can be quite sensitive to disturbance [16], and in some cases will change in species composition and/or species diversity with changing environmental conditions [e.g., 9, 16]. However, the impacts of environmental variability on microbial community composition, particularly in physically structured environments where both biotic (i.e., higher organism host) and abiotic conditions vary are not well understood. Environments that differ in both biotic and abiotic characteristics, although complex, can provide insights into the contributions of the different types of environmental variables that shape the microbial community.

Salt marshes provide an excellent setting in which to assess microbial community dynamics along natural abiotic and biotic gradients. *Spartina alterniflora* (salt marsh cord grass) is the dominant macrophyte in salt marshes along the Atlantic coast of temperate North America. *S. alterniflora* has two conspicuous growth forms in these marshes: a tall form ($\geq$1 m) growing along creek banks and a short form ($\leq$30 cm) growing inland from the tall-form zone to higher elevations. The differences in plant height are due to several porewater chemical parameters that form gradients from the tidal creeks to the upper marsh. The most important of these are soluble sulfide concentration, redox potential, and salinity [4, 5, 23]. These edaphic factors, in addition to temperature and porewater ammonium concentration, also are likely to affect the distributions and activities of some key sedimentary microbial functional groups, such as the nitrogen-fixing bacteria.

Nitrogen fixation (diazotrophy) has long been recognized as an important source of new nitrogen for many ecosystems [8, 12, 34]. A particularly significant focus for diazotrophy in terrestrial, shallow freshwater, and coastal marine environments is the rhizosphere of the dominant macrophytes. Plant rhizospheres include the soil directly influenced by plant roots and support elevated levels of many microbial activities, including diazotrophy [10, 57], relative to surrounding unvegetated soils and sediments [7]. Several studies have documented nitrogen transfer from diazotrophs to plants [22, 26, 35, 45], though the significance of this exchange for grasses is still controversial [18, 51]. It is clear, though, that diazotrophic bacteria benefit from a close physical association with the dominant macrophytes in salt marsh systems [17, 33, 55] and have elevated levels of activity in the rhizosphere [10, 30, 48, 57].

Hanson [21] reported differences in potential nitrogen fixation rates between the tall-form and short-form *S. alterniflora* zones. He suggested that these differences were due to physiological or biomass differences between the diazotroph assemblages in the two zones. Differences in diazotroph assemblage composition also may play a role. That is, organisms capable of living in one marsh zone may not be able to live in the other because of differing edaphic conditions. Analyzing assemblage composition *in situ* has been greatly facilitated by molecular biological methods developed over the last decade. Polymerase chain reaction amplification of DNA extracted directly from natural samples and denaturing gradient gel electrophoretic analysis of the products have yielded important insights into community composition and stability in several microbial systems [13–16, 27, 47, 49]. Similar techniques also can be used to study phylogenetically diverse but functionally homologous groups of organisms, such as the diazotrophs. The compositions of rhizosphere diazotrophic assemblages, the stability of these assemblages, the numerical and functional significance of specific diazotrophs in salt marshes, and the effects of the edaphic conditions they experience *in situ* on their distributions are all unknown.

We hypothesized that the diazotroph assemblage species composition would differ between the tall and short form *Spartina alterniflora* zones. The premise behind the hypothesis was that the combined differences in edaphic conditions between the zones (reported in numerous studies) and the differences reported in the nitrogen fixation rates between the zones provide and indicate sufficient differences in the microenvironment for the diazotroph assemblage membership to differ. In this study, we have examined the diazotroph assemblage composition in both zones using techniques that sample the native diazotroph assemblages directly (PCR followed by DGGE analysis). We are aware that the chosen methods have certain biases (as all methods for such investigations do) and refer the reader to the following papers for a more complete discussion of these procedures [15, 16, 49, 52, 58]. DGGE analysis produces complex data, and so we included neural network and linear discriminant analyses for evaluating banding pattern similarities as a means of reducing the influence of investigator bias. The goal of this study was to determine if the observable diazotroph assemblage compositions differed between these two marsh zones and whether or not these assemblages were

stable in composition over a seasonal cycle. We found that these assemblages were similar between zones and quite stable within each zone over a 1-year period.

Materials and Methods

Materials and Reference Cultures

The synthetic nucleotides P (6-(β-D-ribofuranosyl)-3,4-dihydro-8*H*-pyrimido[4,5-*c*][1,2]oxazin-7-one) and K (2-amino-9-(2-deoxy-β-ribofuranosyl)-6-methoxyaminopurine) were obtained from Glen Research (Sterling, VA). Polymerase chain reaction (PCR) primers incorporating these nucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Reference cultures of *Azomonas agilis* (ATCC 7494) and *Bacillus licheniformis* (ATCC 14580) were purchased from the American Type Culture Collection (Rockville, MD). *Azospirillum brasilense* Sp7, *A. lipoferum* Sp 59b, *Rhizobium leguminosarum* biovar *viceae* (USDA 2370), and *Sinorhizobium meliloti* (USDA 1025) were provided by Peter van Berkum, United States Department of Agriculture. *Azotobacter chroococcum* (ATCC 9043) and *A. vinlandii* UW were provided by Robert Robson, University of Reading, Reading, U.K. *Klebsiella pneumoniae* M5a1-UN was provided by Gary Roberts, University of Wisconsin. *R. leguminosarum* biovar *trifolii* TA1 was provided by Mike Fair, Nitrogen Fixation Research Group, Murdoch Univ., Western Australia. *Desulfovibrio gigas* (ATCC 19364) and *D. vulgaris* (ATCC 29579) were provided by Harry Peck, University of Georgia. *Rhodospirillum rubrum* Molish S1 was provided by Duane Yoch, University of South Carolina. *Staphylococcus aureus* (ATCC 12600) was provided by Terry Hazen, Lawrence Berkeley National Laboratory. Cultivation media and incubation conditions have been described previously [2, 31].

Sampling Site Description

Samples were collected from an intertidal marsh zone on Goat Island in the North Inlet estuary near Georgetown, SC, USA (33° 20′N: 79° 12′W). At this location a band of tall-form *Spartina alterniflora* surrounded a small tidal creek. Short-form *S. alterniflora* extended inland for several meters from the edge of the tall form *Spartina* zone. See Morris and Haskin [38] for more detailed descriptions of the field site. Sampling transects were established parallel to the creek band (horizontal) or perpendicular to the creek bank (vertical) within each zone. In each zone a transect for collecting cores for recovery of DNA or for nitrogen fixation assay and a separate transect for porewater collection were established about 20 m apart (horizontally). Separate transects were used so the coring activities would not affect the porewater chemistry measurements. Sediment temperatures were taken at the sediment surface and at 5- and 10-cm depth intervals.

Nitrogen Fixation Assay

Acetylene reduction assay (ARA) methods were modified from Whiting and Morris [54]. Sediment cores ($n = 3$ per zone, per date, per treatment) were collected from the short-form *S. alterniflora* zone in March, June, September, and December and from the tall-form *S. alterniflora* zone in June, September, and December of 1997 using 4 cm diameter × 12 cm length corers having self-sealing injection ports at 2-cm vertical intervals. The corers were plugged with solid rubber stoppers, the stoppers bound in place with cable ties, and the cores injected (0.5 ml solution per port) with acetylene- or ethylene-saturated seawater solution after being transferred to the field lab. The gas solutions were prepared by equilibrating 25 ml of seawater (30 ppt salinity) with headspace acetylene or ethylene in sealed 120-ml serum bottles. After injection, ARA cores were incubated on their sides (to reduce pooling of the gas-saturated seawater) for 24 h at room temperature (23–26°C). Cores were rotated 180° once during incubation. The incubations were terminated by freezing the cores on dry ice. For assay, the cores were dipped briefly in hot water to release the sediment core from the corer, and the core was extruded and cut into 2-cm sections. The frozen core sections were placed in 60-ml jars, and 5 ml water was added to each jar. The sediments were dispersed by vigorous shaking on a rotary shaker for 1 h. One-ml headspace samples were analyzed on a Varian 3700 gas chromatograph equipped with a flame ionization detector and a Carbosphere 80/100 column (Alltech, Deerfield, IL). The amounts of ethylene produced from each core section (2 cm) were added to give the total quantity of ethylene produced for each sediment core (10–12 cm). Ethylene values were then standardized to μmol ethylene produced L sediment^{-1} day^{-1}. One outlying value (a June short-form *S. alterniflora* zone sample) was removed from the data set prior to analysis. The rate from that sediment core sample was five times as high as from any other sample and likely did not represent a reliable measurement. Acetylene reduction rate data were analyzed using ANOVA (SAS Institute, Cary, NC) with $\alpha = 0.05$.

Porewater Chemistry

Porewater was collected from each zone using five sippers [5, 24] placed at 1-m intervals along a horizontal transect. Two additional sippers were placed at 1-m vertical distances flanking the middle sipper in each horizontal transect. Porewater was collected within 1 h of low tide from an approximate depth of 10 cm. Samples collected for quantifying soluble sulfide concentrations were fixed immediately with an equal volume of 2 N zinc acetate. Sulfide concentrations were assayed colorimetrically [19]. Ammonium samples were fixed with one drop of concentrated HCl per 2 ml porewater collected. Ammonium concentration was determined using a Technicon ammonium analyzer [20]. pH and salinity were recorded for each sample using a field-portable pH meter and a hand-held refractometer, respectively. Soluble sulfide concentration data were square root transformed and ammonium concentration data were log transformed to normalize the data before statistical analysis. All porewater chemical parameters measured were analyzed by MANOVA (SAS Institute, Cary, NC). The MANOVA procedure adjusts for correlations between the dependent variables (found to exist between several variables) and it reduces the Type I (experimentwise) error rate. Variables were

compared between zones on different dates and among dates within a zone using LS means in which the experimentwise α was held at 0.05 using a Bonferroni correction for multiple comparisons.

DNA Extraction

Six sediment cores (2.4 cm diameter × approx. 5 cm length, 25 ml) were collected along horizontal transects at 1-m intervals in each zone (tall or short form *S. alterniflora*) on each sampling date. Cores collected for DNA extraction were transferred to disposable 50-ml screw-capped tubes and held on ice until processed (<24 h). DNA cores were processed as described previously [32] with the following modifications: (1) the DNA extraction buffer for the field samples was 50 mM Tris, 100 mM EDTA, pH 8.0; (2) a cetyltrimethylethylammonium bromide (CTAB) purification step [1] was included between the two high salt/ethidium bromide/phenol extraction steps; and (3) 0.43 ml 1 M NaCl in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was added to the 1 ml recovered DNA samples prior to loading onto DEAE Sephacel (Sigma, St. Louis, MO) columns. DNA purification from pure cultures followed previously described methods [31].

PCR Primer Design and Rationale

PCR primer design was based on analysis of *nifH* sequences from the NCBI GenBank database [3] using the Wisconsin Genetics Computer Group software [11]. In order to maximize specificity of the primers for amplification of free-living diazotroph and rhizobium-like *nifH* sequences and to limit primer degeneracy as much as possible, *nifH* sequences from cyanobacteria, *Frankia,* and methanogens were excluded from our analysis. To further reduce degeneracy, the primers were synthesized using the artificial nucleotides P [29] and K [6]. P pairs with either purine and K pairs with either pyrimidine. Duplexes formed with primers containing these bases are more stable than would be the case for comparable primers containing a weakly pairing nucleotide, such as inosine [6, 29]. For the primers listed below, one C/T degenerate position was filled with a P and one A/G degenerate position was filled with a K. Positions that were fourfold degenerate (i.e. A, C, G, or T present in different sequences) were filled with a P/K mixture. The forward primer (5′-TACGG(P/K)AAKGG(P/G)GG(P/K)ATPGG-3′) corresponds to *Klebsiella pneumoniae nifH* position 25–44. The reverse primer (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGC-CGCCCCCGCCCG(G/C)ACGATGTAGATPTCCTG-3′) sequence (underlined, the balance is the GC clamp) is more highly conserved among known *nifH* sequences than is the forward primer and corresponds to *K. pneumoniae* position 436–453. Primer degeneracy was eightfold for the forward primer and twofold for the reverse primer. The GC clamp was added to the reverse primer after the melting profiles of the *nifH* amplimer were compared with the GC clamp attached to either primer using MacMelt software (Bio-Rad, Hercules, CA). Primers were tested against DNA from the known diazotrophs and nondiazotrophs listed above (reference cultures) to establish their effectiveness and specificity.

PCR

The PCR was preformed using rTth DNA polymerase, XL (Perkin-Elmer, Foster City, CA), which has proofreading capability. The reaction mixture consisted of the following: 1 ng template DNA μl^{-1}, 1.1 mM magnesium acetate, 200 μM each deoxynucleotide triphosphate, 0.5 pmol μl^{-1} each primer, 400 ng BSA μl^{-1} reaction. Addition of BSA to the PCR reaction mix [28] improved amplification at least two- to fourfold from field samples (data not shown), but was not required for amplification from pure cultures. Following an initial denaturation step at 94°C for 2 min, a touchdown thermocycling program was used: 94°C for 45 sec, 58°C for 30 sec, decreasing 0.5°C $cycle^{-1}$, 70°C for 30 sec for 20 cycles. This was followed by an additional 15 cycles (10 cycles for pure culture DNA and DNA recovered from gel plugs) of standard thermocycling PCR using a 48°C annealing temperature for 30 sec. A final extension step at 70°C for 2 min was used. The products from two 50-μl reactions were combined, ethanol precipitated at −20°C overnight, and resuspended in 10 μl TE.

Denaturing Gradient Gel Electrophoresis (DGGE)

All reagents were prepared as described in the Bio-Rad D Gene Instruction Manual and Applications Guide (Bio-Rad, Hercules, CA). DGGE gels were run using a Protean II electrophoresis unit (Bio-Rad, Hercules, CA). Samples were electrophoresed for 9 h at 200 V on denaturing gradient gels (1 mm thick, 6.5% polyacrylamide, 72.5–95% denaturant). The running buffer (1× TAE buffer; 40 mM Tris, 20 mM acetic acid, 1 mM EDTA) temperature was maintained at 48°C, and the Protean II core temperature was maintained at 54°C. Gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) and gel images were recorded with an Alpha Imager 2000 (Alpha Innotech Corp., San Leandro, CA) gel documentation system. Gel plugs were taken from several bands from each gel using a wide-orifice micropipette tip and stored in 100 μl TE at −20°C until used for PCR. A 1 μl sample of the TE from each plug was reamplified using the primers and amplification conditions described earlier and the amplimer(s) resolved using the DGGE conditions described above. A single band from a reamplification indicated that the parent band was a homoduplex; three bands indicated a heteroduplex parent band. Heteroduplex bands form during PCR when two similar sequences anneal to one another [14]. Note that *nifH* sequences from cyanobacteria, *Frankia,* and the methanogens were specifically excluded during the design of the PCR primers in order to reduce primer redundancy. Sequences from these and from any other organisms that could not be recovered through PCR and resolved via DGGE could not be included in our examination of the diazotroph assemblages. Whenever the term "diazotroph assemblage" appears in this text we are, of course, referring only to those organisms we detected using PCR and DGGE.

Computer Assisted Analysis of DGGE Profiles

Denaturing gradient gel images were digitized and the images processed using the PPC MA Fingerprinting software (Bio-Rad, Her-

Table 1. Nitrogen fixation assay (acetylene reduction) rates for each *Spartina alterniflora* growth form zone by sample date[a]

Spartina zone	Sample date			
	5 March 1997	4 June 1997	16 September 1997	11 December 1997
Tall-form	N.D.[b]	2.63 ± 0.24	1.51 ± 1.08	1.27 ± 0.38
Short-form	1.50 ± 0.39	2.12 ± 0.35[c]	0.35 ± 0.44	0.47 ± 0.30

[a] Units are μmol ethylene produced L sediment^{-1} day^{-1}. Values are the mean ± SD (n = 3).
[b] N.D. = not determined.
[c] n = 2.

cules, CA). One lane (S3) from the June sample date gel was designated as a standard to which all gels were aligned. We did not exclude known heteroduplex bands from the data since their appearance is still characteristic of the samples and thus informative [14]. Neural network (NN) and linear discriminant analysis (LDA, SAS Institute, Cary, NC) were employed to find patterns in these complex data sets and to eliminate operator bias in pattern analysis. Neural networks and their applications are described in greater detail in Noble et al. [40] and Weinstein et al. [53]. The back-propagating NN software used in this study has been described previously [40]. A binhex and binary version of this application is available through an anonymous ftp at inlet.geol.sc.edu.

A cross-validation strategy was used to evaluate the NN and LDA analyses. Although both NN and LDA can be used to determine which variables (e.g., bands in the case of DGGE profiles) discriminate between two or more naturally occurring groups, we included LDA primarily as a classical statistical tool for comparative purposes. LDA uses a linear function to maximize the distance between members of different groups, whereas a NN uses a non-linear function. Differences between these functions can significantly influence which patterns are important for recognizing differences between groups in complex data [53].

The following cross-validation scheme was employed. The order of the samples in the data set was randomized; 75% of the data were used to train the NN and, in the case of the LDA, were used to develop a classification criterion. The remaining 25% of the data were employed to test the NN or the LDA. Control data were included in the DGGE data set to check the validity of the NN and LDA predictions of the gel profiles. Eight randomly generated dummy "gel lanes" were included, with densitometry values limited by the maximum and minimum values of the raw data. These samples served as out-groups. For all data, the densitometry values were converted to a two-point binary scale (1 for the presence of a band, 0 for the absence of a band). This avoids undue influence of differential PCR amplification on the DGGE profile analysis. NN performance was optimized by using a range of hidden layer neurons (5–40) and error tolerances (0.5–0.0001) [43]. Correct and incorrect classifications were recorded. The above scheme was repeated 10 times and the output was used to generate a cross-validation table.

The distributions of individual bands in the denaturing gradient gels were determined by visual inspection of the gels. A band was considered present in a zone if it could be detected in four of the six lanes for a given sample date and zone.

Results

Nitrogen Fixation Assay

The means and standard deviations for acetylene reduction rates are given in Table 1. There was a significant difference among dates (ANOVA, P = 0.0027) and a marginally significant difference between zones (P = 0.034). Rates in June were significantly higher than rates in September or December (Tukey HSD test, α = 0.05). Acetylene reduction rates were significantly higher in the tall-form than in the short-form *S. alterniflora* zone (Tukey HSD test, α = 0.05).

Porewater Chemistry and Sediment Temperature

The means and standard deviations for the porewater parameters measured are given in Table 2. Porewater chemistry differed significantly by season in both zones. The differences were attributed to soluble sulfide concentrations and, to a lesser extent, to salinity and pH. Table 2, however, shows that pH and salinity differences are not pronounced and likely are not biologically significant. Soluble sulfide concentrations differed significantly between zones for all sample dates except June (LS Means, Bonferroni inequality adjusted α = 0.006). Ammonium values were variable in both zones. The sediment temperatures at 5 cm depth ranged from 23.6 to 26.6°C for samples collected March through September. The temperature at 5 cm was 14.5°C in December.

nifH *Amplification*

The primers designed for this study were tested against DNA purified from a number of known diazotrophs (*A. chroococcum, A. vinlandii, A. agilis, A. brasilense, A. lipoferum, D. gigas, D. vulgaris, K. pneumoniae, R. rubrum, R. leguminosarum* biovar *viceae, R. leguminosarum* biovar *trifolii,* and *Sinorhizobium meliloti*) and known nondiazotrophs (*B. licheniformis* and *S. aureus*). All of the known diazotroph

Table 2. Porewater results by date and *Spartina alterniflora* growth form zone at 10 cm depth[a]

Parameter	*Spartina* zone	Sample date			
		5 March 1997	4 June 1997	16 September 1997	11 December 1997
Sulfide	Tall-form	74 ± 61[b]	744 ± 350	3554 ± 1164[b]	964 ± 453[b]
(μM)	Short-form	842 ± 809[b]	746 ± 560	5266 ± 691[b]	3092 ± 617[b]
Ammonium	Tall-form	17.0 ± 1.4	14.8 ± 4.6	31.9 ± 10.6	34.9 ± 13.4
(μmol N L^{-1})	Short-form	22.4 ± 12.8	17.7 ± 3.7	42.3 ± 39.6	43.6 ± 31.8
pH	Tall-form	7.0 ± 0.2[c]	6.2 ± 0.5	7.4 ± 0.1	7.0 ± 0.1
	Short-form	7.1 ± 0.1[c]	6.7 ± 0.2	7.5 ± 0.1	7.1 ± 0.1
Salinity	Tall-form	26.1 ± 1.5[c]	29.7 ± 0.5	33.9 ± 1.2	31.3 ± 0.8
(ppt)	Short-form	30.0 ± 0.6[c]	30.4 ± 1.1	33.1 ± 2.0	31.3 ± 1.3

[a] Values are the mean ± SD (n = 7).
[b] Soluble sulfide concentrations differed significantly (at Bonferroni inequality adjusted α = 0.006) between zones on these dates.
[c] Samples taken on 26 March 1997.

DNA amplifications yielded a single amplimer of the appropriate size (approx. 469 bp, data not shown). No amplimers were obtained from the nondiazotrophs tested. Previous studies employing different *nifH* PCR primers also have shown successful and specific amplification of *nifH* sequence segments from a variety of organisms and natural samples [44, 50, 56, 59].

Diazotroph Assemblage Composition

DGGE profiles contained 10 prominent bands and a few faint bands. Band designations, including presumed heteroduplex bands, are shown in Fig. 1. Some bands that were well separated in these gels appeared to be the same or a very similar amplimer according to band locations of reamplified products. Bands in the area designated "a" generally migrated to the same location on the gel and formed a tight doublet when well resolved. The broad bands observed (Fig. 1) might result from primer degeneracy or less than optimal resolution in the upper portion of the gradient. Tight doublets were seen frequently and might be caused by the GC clamp used. The artifact band (Art) noted in Fig. 1 may result from DNA complexing with denatured BSA during the precipitation step after PCR. The migration of this band is independent of the denaturing gradient, and PCR amplification of gel plugs taken from this band reproduce the original amplimer (when taken from a pure culture or a homoduplex band gel plug).

DGGE profiles were highly reproducible, as indicated by the striking similarity of replicate profiles within a given sample set (Fig. 1). It is also clear that the tall and short *S. alterniflora* zone profiles are quite similar overall (Fig. 1). The neural network cross-validation results underscore the band profile similarities between the tall and short form *Spartina* zones (Table 3). Several of the incorrect classifications by the NNs, particularly the incorrect predictions of tall zone samples as short zone samples, were likely due to profile similarities between the zones. Table 3 shows the number of correctly and incorrectly predicted sample group (either date or zone) affiliations as a function of 10 training and testing validation runs. Two additional sets of 10 training and testing validation runs gave very similar results (data not shown). Comparison of the cross-validation outputs indicated that the LDA results supported the NN results. The short *Spartina* zone samples were correctly identified by zone 71% of the time. The tall *Spartina* zone samples were correctly identified 59% of the time (data not shown). Overall, the samples were placed into the correct zone classification 66% of the time by LDA and 65% of the time by the NN.

For a more detailed comparison of which bands were present in which zones on the various dates, we used the denaturing gradient gel images directly. In the denaturing gradient gel images, band intensity varied within and between zones on any given date. We relied on the presence or absence of a band in four of the six samples from a group for identifying trends in diazotroph assemblage composition. Four bands were common among the samples: a, b, d, and f (Fig. 1). Bands in the "a" region were much less prevalent in March short-form samples than in other sample sets. The "c" band was more prevalent in the tall-form than in the short-form *S. alterniflora* zone, though it was clearly present in September short-form samples. The "d" band was more prevalent in the short-form than in the tall-form *S. alterni-*

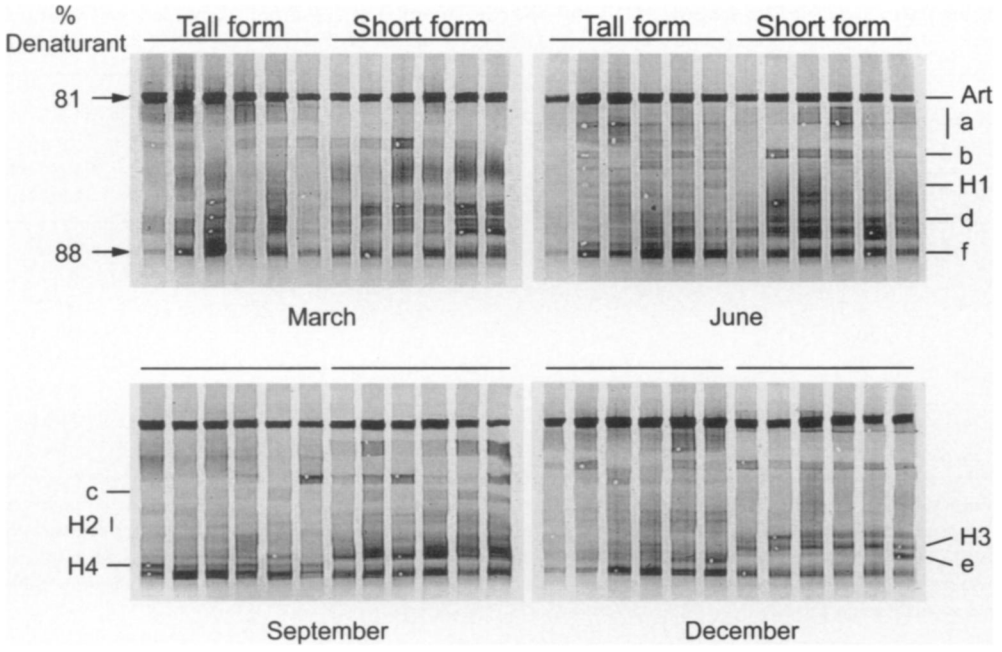

Fig. 1. Denaturing gradient gel electrophoresis images of *nifH* amplimers from seasonal samples collected from tall-form and short-form *Spartina alterniflora* zones. The original images were cropped, inverted, and unwarped. The percent denaturant concentrations are approximate. Prominent bands are designated with lowercase letters if they are homoduplex bands and H1–H4 if they are heteroduplex bands. "Art" designates an artifact band.

flora zone. Band "e" was often too close to one of the heteroduplex bands (H3) to allow its distribution among groups to be definitely stated. Bands "b" and "f" were present in all groups. Preliminary data obtained by reamplification of the DGGE gel plugs, followed by cloning and restriction fragment length polymorphism (RFLP) analysis using HaeIII and MspI (unpublished data), shows that some homoduplex bands (b and f) contained multiple sequences.

Four of the 10 distinct bands in the DGGE profiles are known or suspected heteroduplexes (as assessed by reamplification of DNA in plugs taken from the DGGE gels) and are indicated in Fig. 1. H1 was clearly present in JT and possibly in DT samples. H2 was especially prevalent in JT, JS, SS, and DT samples. H3 was always present. H4 was distinct only in JT, ST, and DT samples. H1 and H4 were the only two heteroduplex bands that showed a clearly identifiable pattern associated with either plant growth form zone or sample date. RFLP analysis of clones indicated that these heteroduplexes each contained only two sequences.

Discussion

Obtaining information regarding community composition across various spatial and temporal scales is an important first step in understanding microbial community structure–function relationships. The combination of the polymerase chain reaction, denaturing gradient gel electrophoresis (DGGE) analysis, and cross-validation strategy provides a powerful tool for examining the assemblage compositions of key bacterial functional groups in microbial communities. This approach permits the amplifiable species compositions of bacterial assemblages to be analyzed and important issues in community dynamics, such as stability of the assemblages and responses to seasonal changes in key environmental variables, to be addressed. A recent study of microbial community composition in grassland soil showed remarkable consistency over a large spatial scale [13]. Our results also showed consistency in the composition of an important rhizosphere functional group, the diazotrophic bacteria, over moderate spatial scales. We also observed surprising stability of the diazotroph assemblage composition over an annual cycle of change in host plant productivity and edaphic conditions in a dynamic environment.

The diazotroph assemblage compositions in the tall-form and short-form *Spartina alterniflora* growth zones were quite stable over a moderate spatial scale (30 m) during a 1-year period. Visual examination of the DGGE gels highlighted the strong similarity of the assemblages in the two zones on all sampling dates. In addition, the NN analysis of DGGE banding patterns showed overall similarity between the two *S. alterniflora* growth zones, and greater consistency in the short-form than in the tall-form *S. alterniflora* zone. Samples from the short-form *S. alterniflora* zone were almost always classified correctly, but samples from the tall-form zones

Table 3. Prediction of diazotroph assemblages using back-propagating NNs with the configuration of 126-input, 20-hidden, and 5-output neurons and an error tolerance of 0.1[a]

Actual sample	Predicted samples: Date						Zone				# Correct/ # tested
	March	June	Sept.	Dec.	Random	Other	Tall	Short	Random	Other	
March	**34**					1					34/35
June	1	**17**		1		1					17/20
Sept.			**31**	2							31/33
Dec.				**30**							30/30
Random		11		2	**7**	2					7/22
Tall							**33**	21	3	1	33/58
Short							9	**50**	1		50/60
Random							13	1	**8**		8/22

[a] The left column gives the actual samples; numbers in bold are correct predictions, numbers not in bold are incorrect predictions. Actual and predicted random samples represent eight possible nonsense samples that were based on random data (see Methods). The total of correct predictions for date was 119 (85.0%); the total of incorrect predictions was 21 (15.0%). The total of correct predictions for zone was 91 (65.0%); the total of incorrect predictions was 49 (35.0%). The category "Other" was included so the NN had the option of placing an unknown into a category other than the training categories if that sample did not fit in any known categories as analyzed by the NN.

were misclassified fairly frequently (Table 3). Greater variability in the tall-form zone may reflect greater exchange between porewater and tidal flood water in this zone [23, 41], though we cannot offer a clearly defined mechanism for the interaction between porewater exchange and diazotroph assemblage variability at present. It is clear, however, that the diazotroph assemblages in both zones are remarkably similar and quite stable over a seasonal cycle.

Seasonal stability in diazotroph assemblage composition within a zone was not particularly surprising. Each growth zone presents a characteristic set of microenvironmental conditions conducive to the development of a homogeneous population of a specific *S. alterniflora* growth form. The close association of at least some of the rhizosphere diazotrophs with the plant roots [2, 17, 33] and the general responsiveness of diazotrophy in this microenvironment to changes in plant photosynthesis [55] imply strong selection for a specific diazotroph assemblage. Numerical representation or activity of particular assemblage members may be more variable over the spatial scales examined, but the species composition of the assemblage would not be expected to vary greatly within a zone [13].

The striking DGGE pattern similarity between zones, however, was not anticipated. Previous studies have shown changes in microbial community structure in response to changing environmental conditions [e.g., 9, 15, 42, 49]. In salt marshes, numerous studies of this and similar sites have shown significant differences in a number of sediment geochemical parameters between the tall and short form *S. alterniflora* growth zones [5, 25, 36, 38, 41]. There is interannual variability, though, in the magnitude of these differences [37, 38], and this may contribute to the similarities between zones observed during this study. With the exception of soluble sulfide concentration, porewater chemical parameter differences between zones in this study were minor, and thus would not be expected to strongly influence diazotroph assemblage composition. The consistent presence of four to five bands in the DGGE profiles (a, b, d, e (?), and f) from both *S. alterniflora* growth form zones supports the conclusion that the diazotroph assemblage compositions of the two zones are substantially similar, given that RFLP and preliminary sequence information shows the same or nearly identical sequences from bands in the same position in different gels (unpublished data).

The relatively subtle differences were observed in DGGE profiles may reflect functionally important differences in the diazotroph assemblage structure if the organisms represented by the variable bands are numerically significant or highly active. Because of PCR biases, we cannot deduce numerical representation of different diazotrophs from the quantity of an amplimer [14, 49, 58]. Also, DGGE resolution of very similar sequences may have been hindered by a GC-rich domain in the amplimer sequence located approximately 240–290 nucleotides from the 5′ end. An open loop structure could form between this GC-rich domain and the GC clamp. Since the exact sequences in this domain and thus the stabilities of the dimers vary, this loop structure could alter the migration of some of the amplimers, poten-

tially reducing the number of resolvable bands we could recover. However, the DGGE profiles do not support the idea that there are substantial differences in diazotroph assemblage compositions between the two zones.

It is important to note that we do not consider detection of a diazotroph to indicate that it is actively fixing nitrogen. We did, however, measure diazotrophic activity during the study to examine possible correlations between diazotroph assemblage composition changes and changes in diazotrophic activity. The intact core nitrogen fixation assay method we used was chosen to minimize disturbance effects (mainly stimulation) on acetylene reduction rates. The ARA rates reported here differed seasonally within each zone and differed between zones, though the rates are low in comparison to other such estimates derived from sediment slurry measurements [21]. As there were no substantial differences in diazotroph assemblage compositions or diazotrophic activity within or between zones during this study, it is not possible to propose specific links between these variables. The DGGE patterns, however, do suggest that the small differences observed in acetylene reduction rates between zones were not the result of differences in the diazotroph assemblage composition. Relative abundances and/or activities of diazotroph species are more likely to be responsible for such variations in activity.

Under some circumstances, DGGE may not detect all of the assemblage compositional changes among physiologically distinct organisms having nearly identical DNA sequences in the segment of the gene amplified [27, 47]. In a related study, numerous diazotrophs were isolated from both growth forms of *Spartina alterniflora* [2]. This physiological diversity was not reflected in the DGGE profiles, but may be important *in situ*. As has been demonstrated for bacteria from other environments, however, culture-based techniques can select for organisms without regard to their numerical or functional significance *in situ* [46]. The large sample sizes required for adequate DNA recovery and purification using our protocol and the small amount of template used in PCR may inadvertently select for abundantly represented sequences in the samples. Nevertheless, the molecular and culture-based studies on community composition do provide information regarding which organisms do not overlap in their distributions, further providing a basis for generating testable hypotheses on environmentally determined differences in diazotroph distributions and activities.

This study assessed the spatial and temporal variability of diazotroph assemblage composition in a complex and dynamic environment. Variability within a zone on a given date was very low (Fig. 1). Within-zone variability across seasons was often low as well, though this was more characteristic of the short-form *Spartina alterniflora* zone. Between-zone variability also was low, but still was higher than within-zone variability. There were no clear correlations between the minor variations in the DGGE profiles and either the edaphic conditions or acetylene reduction activity. The stability of the diazotroph assemblages in the two *S. alterniflora* growth zones and the similarity between these assemblages was unanticipated, but clear. Physiological groupings of culturable diazotrophs from the two zones were also similar [2], supporting our DGGE results. Our findings and those of other workers [13] indicate that the "rhizosphere effect" may not just support locally elevated microbial biomass and activity, but also promote microbial community stability and persistence within this important microenvironment.

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