

# Persistence of Selected *Spartina alterniflora* Rhizoplane Diazotrophs Exposed to Natural and Manipulated Environmental Variability

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**Rhizoplane-rhizosphere nitrogen-fixing microorganisms (diazotrophs) are thought to provide a major source of biologically available nitrogen in salt marshes dominated by *Spartina alterniflora*. Compositional and functional stability has been demonstrated for this important functional group; however, the quantitative responses of specific diazotroph populations to environmental variability have not been assessed. Changes in the relative abundances of selected rhizoplane diazotrophs in response to long-term fertilization were monitored quantitatively by reverse sample genome probing. Fertilization stimulated *Spartina*, with plant height nearly tripling after 1 year. Fertilization also resulted in significant changes in interstitial porewater parameters. Diazotrophic activity (acetylene reduction assay) was sensitive to the fertilization treatments and was inhibited in some plots on several sampling dates. However, inhibition was never consistent across all of the replicates within a treatment and activity always recovered. The rhizoplane diazotrophs were quite responsive to environmental variability and to experimental treatments, but none were displaced by either environmental variability or experimental treatments. All strains were detected consistently throughout this study, and extensive spatial heterogeneity in the distribution patterns of these organisms was observed. The physiological traits that differentiate the diazotroph populations presumably support competitiveness and niche specialization, resulting in the observed resilience of the diazotroph populations in the rhizosphere.**

Microbial communities are often considered to be quite volatile, changing readily in species richness. Such plasticity has been demonstrated in bioreactor systems (25, 64) and in natural bacterioplankton communities (38, 40, 55). This instability presumably reflects either manipulated or natural environmental variability in these systems, which clearly has substantial impacts on community composition. In contrast, microbial communities in highly structured environments (cyanobacterial mats, rhizosphere soils, and sediments) display a much higher degree of compositional stability (24, 26, 27, 56, 57, 58), suggesting that the impact of environmental variability is somewhat reduced or limited. This buffering effect seemingly helps to stabilize species composition against environmental variability, but the activity levels (19, 20) and relative abundances (60) of microorganisms in situ would be expected to be more responsive to environmental changes.

The rhizospheres of salt marsh grasses provide interesting systems in which to test ideas about microbial community plasticity and environmental buffering effects. Salt marsh systems are highly dynamic, and pronounced variability in primary productivity and edaphic environmental conditions is well known (47, 56, 57). The responsiveness of the rhizosphere microbiota within the major vegetated zones of salt marshes to environmental variability is of particular interest due to the importance of these organisms to marsh productivity.

Salt marshes along the Atlantic and northern Gulf coasts of temperate North America are dominated by the smooth cordgrass *Spartina alterniflora* (referred to as *Spartina* hereafter in this report), which grows in a virtual monoculture at low elevations in the marsh (67). High rates of primary production (18, 79) and microbially mediated nutrient cycling (13, 65) make *Spartina* marshes among the most productive ecosystems

known. Numerous studies of salt marsh productivity (e.g., see references 17, 35, 68, and 70) and decomposition (45, 69) suggest that nitrogen is the key nutrient limiting these processes.

Nitrogen fixation has been shown to be a significant source of new nitrogen in salt marshes (33, 53, 78) and is carried out exclusively by certain species of *Bacteria* and *Archaea* (59). Nitrogen-fixing (diazotrophic) microorganisms in vegetated salt marsh sediments are largely distributed along root surfaces (rhizoplane) and within surrounding sediments directly affected by host plant activities (rhizosphere) (12, 46, 66). High rates of nitrogen fixation have been demonstrated in these root microenvironments (33, 51, 52, 66, 77). However, we know relatively little about the diazotrophs involved or their responses to key environmental variables.

Extensive physiological and phylogenetic diversity has been described for the *Spartina* rhizoplane-rhizosphere diazotroph assemblage by classical cultivation (3) and molecular biological approaches (44, 58). This diversity is expected to reflect the microenvironmental heterogeneity of the rhizoplane-rhizosphere (7, 12) and implies a large number of different niches occupied by diazotrophs in situ (2, 7). Phylogenetically related rhizoplane diazotrophs can differ substantially in the ability to utilize different ecologically relevant organic carbon substrates, suggesting that diazotrophs may be differentially adapted to defined physiological niches in situ (2). Physiological niche specialization would provide a competitive advantage to diazotrophs, while the sum of all diazotroph physiological traits would allow for assemblage responsiveness to and resilience (structural and functional) following environmental changes (i.e., functional redundancy). Structural and functional stability has been demonstrated for the diazotroph assemblage associated with *Spartina* over a seasonal cycle of edaphic variability and host plant ontogeny (58) and in response to short-term interstitial nutrient (56) and host plant manipulations (57). These studies provide preliminary evidence for high levels of assemblage stability; however, the techniques used (PCR and denaturing gradient gel electrophoresis) do not allow for quan-

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tification or detailed physiological characterization of the various species detected. In addition, manipulations were only executed for a maximum of 8 weeks and encompassed only a fraction of the *Spartina* ontogenetic cycle. These are important considerations for understanding the connection between assemblage structural and functional stability and environmental variability.

In this study, selected diazotrophs from short- and tall-form *Spartina* and *Juncus roemerianus* (the black needle rush, referred to as *Juncus* hereafter) rhizoplanes were monitored quantitatively in the rhizosphere of short-form *Spartina*. Nitrogen-and-phosphorus (N + P) fertilization was used to stimulate *Spartina* growth and productivity over a period of 13 months. We hypothesized that this treatment would result in changes in the representation of some diazotroph species through alterations in key environmental variables, such as oxygen introduction and carbon supply to the rhizosphere. In addition, nitrogen additions should ultimately alleviate nitrogen limitation within the fertilized plots and, due to the high energetic cost of nitrogen fixation, might select against diazotrophy. By eliminating the presumed selective advantage of diazotrophy, the physiological competitiveness of diazotrophs via niche specialization or other mechanisms can be assessed. Monitoring was also continued after fertilization ceased to evaluate the posttreatment responses of diazotroph populations.

#### MATERIALS AND METHODS

**Sampling site and experimental design.** This study was conducted in the Bly Creek basin in the North Inlet estuary, near Georgetown, S.C. (79°12' W, 33°20' N). *Spartina* is the dominant macrophyte in this basin and grows largely as a monoculture stand. Two distinct growth forms of *Spartina* can be easily recognized at the study site. Bands of tall ( $\geq 1$  m in height) *Spartina* are found along the banks of tidal creeks, while *Spartina* growth is stunted at higher elevations, resulting in short-form ( $\leq 30$  cm in height) plants. Other common marsh plants (*Juncus roemerianus*, *Spartina patens*, and *Salicornia virginica*) are localized along the terrestrial fringe at elevations above the short-form *Spartina* zone.

In June 1998, a six-by-three-block design was established in the short-form *Spartina* stand. Garden stakes were used to designate 18 4-m<sup>2</sup> plots, each separated by 1 m in all directions from neighboring plots. Previous studies in this system have shown that intrusion of roots from plants outside an experimental plot does not significantly impact the results of treatments within the plot (56, 57). In addition, fertilization within the plots did not have an impact on plants outside the boundaries of experimental plots. A sipper for porewater collection (10, 36) was inserted into the center of each plot to a depth of 10 cm and allowed to equilibrate for 1 week prior to the first fertilization treatment.

Plots were randomly assigned to control and experimental treatments. Each fertilized plot received 144 g of phosphorus/month (as P<sub>2</sub>O<sub>5</sub>) and 130 g of nitrogen/month (as NH<sub>4</sub>NO<sub>3</sub>). We applied phosphate by hand spreading and nitrogen either by ground application of a solution or foliar spraying using a garden sprayer. Control plots received no nutrient additions. All three treatments (control, ground, and foliar) included six replicates each, and all 12 fertilized plots were treated once per month for 1 year. Over the winter period of plant senescence (November to March), foliar spraying was expected to be problematic; consequently, these plots were ground treated to maintain elevated interstitial nutrient levels. Foliar applications were resumed after the emergence of new growth in April 1999. Treatments were stopped following the June 1999 application, resulting in a total of 13 months of N + P additions.

**Field sampling.** All plots were sampled 1 week after the monthly N + P addition from June to August 1998 (active growing season), September 1998 (seed head development and flowering), January 1999 (winter die-back), August 1999 (2 months following termination of N + P additions; only porewater parameters, acetylene reduction potentials, and plant shoot heights and leaf numbers were measured), and October 1999 (4 months following termination of N + P additions). Interstitial porewater and sediment cores were collected from all of the plots on each sampling date for the measurements described below.

**(i) Porewater chemistry.** Interstitial porewater was collected for analysis of salinity, pH, soluble sulfide, and ammonium concentrations. Salinity and pH were measured using a hand-held refractometer (Leica Inc., Buffalo, N.Y.) and a field-portable pH meter (Cole-Parmer Instrument Co., Chicago, Ill.), respectively. Soluble sulfide was fixed immediately upon collection with an equal volume of 2 N zinc acetate and assayed colorimetrically (29). Ammonium was fixed immediately upon collection by adding 1 drop of concentrated HCl per 2 ml of porewater. Prior to analysis, porewater was filtered (0.2- $\mu$ m pore size) and assayed using a Technicon autoanalyzer (31). Porewater parameters were statis-

tically analyzed by multiple analysis of variance (MANOVA) with Bonferroni's correction for type I error in SAS (SAS Institute, Cary, N.C.) for treatment effect, for date effect, and to account for parameter interactions. The utility of the MANOVA procedure for analysis of porewater parameters has been discussed previously (56).

**(ii) Acetylene reduction assay for nitrogen fixation potential.** A small sediment core (approximately 12 g fresh weight) was collected from each plot using a steel coring device (8 by 1.5 cm) to measure nitrogen fixation potential using an acetylene reduction technique described previously (80). Upon collection, cores were immediately packed on ice and transported back to the Belle W. Baruch Institute field lab for processing. Sediments were transferred to sterile 40-ml serum vials, each containing 10 ml of filter-sterilized seawater substitute (pH 7.8) (80). The vials were crimp sealed, and 10% of the headspace gas was replaced with acetylene. All of the samples were vigorously shaken (1 to 2 s) and incubated at room temperature for 24 h. Acetylene and ethylene were analyzed by injecting 50  $\mu$ l of headspace gas into a Varian 3700 gas chromatograph equipped with a flame ionization detector and a Carbosphere 80/100 column (the detection limit for ethylene is on the order of 10<sup>-10</sup> mol; Alltech, Deerfield, Ill.). Acetylene reduction potentials were analyzed for significant differences by *t* test and by MANOVA for assessment of covariance with porewater parameters.

**(iii) DNA extraction from sediments.** A sediment core (approximately 30 g) was collected from each plot using a steel coring device (7.5 by 2.5 cm) and held on ice for processing at the University of South Carolina main campus (Columbia). DNA extractions were started within 24 h of collection and done as previously described (43, 58). DNA quantity and quality were assessed by fluorometry and agarose gel electrophoresis, respectively, and all purified DNA extracts were stored at -20°C as alcohol precipitates until used.

**(iv) Host plant measurements.** Plant shoot heights and leaf numbers were measured in a randomly selected 0.1-m<sup>2</sup> quadrat within each plot in August 1999. Shoot heights were taken as the longest leaf, measured from the sediment surface to the leaf tip, and only live leaves were included in the leaf counts. Shoot heights and leaf numbers were statistically analyzed by *t* test, and covariance between shoot height and leaf number was assessed by regression analysis.

**RSGP.** The reverse sample genome probing (RSGP) technique has been described in detail elsewhere (62, 72-74). Briefly, RSGP utilizes whole-community DNA extracted from environmental samples to probe against dot-blotted DNAs from different reference cultures. Under stringent hybridization conditions and using an appropriate internal standard, the quantity of probe signal detected in each dot reflects the abundance of each reference culture organism in the environmental sample. This technique allows the quantification of many different diazotrophic organisms simultaneously.

**(i) Diazotroph master blot.** Isolation and characterization of rhizoplane diazotrophs from *Spartina* and *Juncus* has been described previously (3). Physiologically different diazotroph strains were evaluated by quantitative whole-genome DNA-DNA hybridization to eliminate redundancy within the strain collection. The details of these experiments are presented elsewhere (2). The master blot used in the RSGP experiments consisted of 27 physiologically and genetically different rhizoplane diazotroph strains (Table 1) and 15 additional organisms from other sources. The latter consisted primarily of diazotrophic and nondiazotrophic oxygen-utilizing and anaerobic strains that are known to associate with grasses or are considered common in intertidal salt marsh sediments (i.e., *Azotobacteriaceae*, *Enterobacteriaceae*, *Vibrio* sp., sulfate reducers, and *Clostridium* spp.) but were not represented among the diazotrophs isolated from *Spartina* or *Juncus*. This collection is not intended to be inclusive of all *Spartina*-associated diazotrophs but does represent several significant diazotroph groups.

**(ii) Master blot preparation.** DNA (30 ng) from each reference strain and from bacteriophage lambda (5-, 15-, 20-, 25-, and 30-ng standards) was chemically denatured in a 96-well microtiter plate (39, 42) and loaded onto Duralose-UV membranes (Stratagene, La Jolla, Calif.) using a dot blot apparatus. Prior to blotting of the denatured DNA, each dot on the membrane was marked with 5  $\mu$ l of India ink solution (1:150 dilution in distilled H<sub>2</sub>O). Membranes were air dried and baked at 80°C under a vacuum for 2 h and then stored in a desiccator at 4°C until used. All dot blots were used within 2 to 3 weeks of DNA loading.

**(iii) Probe preparation.** Field DNA (1  $\mu$ g) spiked with 0.1 ng of lambda phage DNA (internal standard) was digested with *Sau3A1* (New England BioLabs Inc., Beverly, Mass.) for 30 min at 37°C, and the reaction was stopped by heating at 65°C for 10 min. The digests were radiolabeled using a random priming procedure (23) with [ $\alpha$ -<sup>35</sup>S]dATP (ICN Biomedicals, Inc., Costa Mesa, Calif.), and the labeled products were purified using Sephadex G-50 spun columns (Boehringer Mannheim, Indianapolis, Ind.). The specific activity of the radiolabeled DNA was typically 10<sup>8</sup> cpm  $\mu$ g of DNA<sup>-1</sup>.

**(iv) Master blot hybridization.** Membranes were prehybridized at 40°C for 6 h in 30 ml of prehybridization solution (10 $\times$  NET, 0.1% sodium dodecyl sulfate [SDS], 3 $\times$  Denhardt's solution [1 $\times$  NET is 150 mM NaCl, 15 mM Tris at pH 7.5, and 1 mM EDTA, and 1 $\times$  Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone]) containing denatured salmon sperm DNA (50  $\mu$ g/ml) and hybridized at 40°C overnight in 30 ml of hybridization solution (50% formamide, 5 $\times$  SSC [1 $\times$  SSC is 150 mM NaCl plus 15 mM sodium citrate], 25 mM potassium phosphate buffer at pH 7.4, 5 $\times$  Denhardt's solution), denatured salmon sperm DNA (50  $\mu$ g/ml), and denatured probe (approximately 10<sup>8</sup> cpm). Membranes were washed twice in 2 $\times$  SSC-0.1% SDS at room tem-

TABLE 1. Key physiological features and preliminary taxonomic affiliations of selected tall- and short-form *Spartina* and *Juncus* rhizoplane diazotrophs used on the master blot<sup>a</sup>

Strain	Key physiological features	Closest taxonomic group
S-M1-1	M, ON, L	Unknown
S-C1-6	F, OP, L	Unknown
S-G2-4	M, OP, C	Unknown
S-M1-8	F, OP, C	Unknown
S-C2-8	F, ON, C, AA	<i>Enterobacteriaceae</i>
S-C2-6	F, OP, C, AA	<i>Vibrionaceae</i>
S-G2-2	M, ON, C	<i>Pseudomonadaceae</i>
S-G2-1	M, ON, C	<i>Azotobacteriaceae</i>
S-C2-7	M, ON, B	<i>Vibrionaceae</i>
S-M2-12	M, OP, C	Unknown
T-M2-1	F, ON, L	<i>Enterobacteriaceae</i>
T-M2-14	M, ON, L	Unknown
T-G2-7	F, ON, L	<i>Enterobacteriaceae</i>
T-G2-5	F, ON, C	<i>Enterobacteriaceae</i>
T-S2-12	A, OP, C, AA	<i>Spirillaceae</i>
T-C2-11	F, OP, B	<i>Vibrionaceae</i>
T-G2-3	F, ON, C	Unknown
J-C1-18	M, OP, L	<i>Spirillaceae</i>
J-G1-8	M, ON, L	<i>Pseudomonadaceae</i>
J-S2-16	M, ON, CA, AA	<i>Rhizobiaceae</i>
J-C1-10	M, OP, CA, AA	Unknown
J-C1-1A	F, OP, C	<i>Vibrionaceae</i>
J-G1-1	M, ON, C	Unknown
J-S1-10	M, ON, C	Unknown
J-M2-1	F, OP, C, AA	<i>Vibrionaceae</i>
J-S2-2	F, ON, C	<i>Enterobacteriaceae</i>
J-M2-11	M, ON, L	Unknown

<sup>a</sup> Strains are designated by the host plant origin (S, short-form *Spartina*; T, tall-form *Spartina*; J, *Juncus*), the carbon source used for isolation (C, citrate; M, malate; S, sucrose; G, glucose), the pH of the isolation medium (1, pH 7.0; 2, pH 7.5); and the strain number. All of the strains are gram-negative motile rods. Physiological characteristics include oxygen requirements (A, obligate aerobe; M, microaerophile; F, facultative anaerobe), cytochrome oxidase production (OP, oxidase positive; ON, oxidase negative), and preferred substrate classes as determined by BIOLOG testing (C, carbohydrates; CA, carboxylic acids; AA, amino acids; L, low utilization of all substrate classes; B, broad substrate range).

perature for 5 min each time and twice in 0.5× SSC–0.1% SDS at 45°C for 20 min each time. Dots were punched from the membrane using a hole punch, and the radioactivity in each dot was quantified using a Packard 1500 TR liquid scintillation analyzer (Packard Instrument Co., Downers Grove, Ill.). Quench correction was done by the internal-standard method. Background radioactivity from control (no target DNA) dots was subtracted from that of experimental dots, and the quantity of DNA from each strain was calculated from regression analysis of the internal standard. RSGP profiles were generated using samples from three randomly selected treatment plots for each sampling date. Profiles were compared by PROC MIXED in SAS for a robust assessment of profile differences by treatment and sample date. This analysis procedure averages across individual populations within a profile and determines a representative profile description value for cross comparisons. RSGP profiles were analyzed in greater detail by principal-components analysis (PCA) to assess the contributions of each population to profile variability.

## RESULTS

**Porewater chemistry.** The means and standard deviations of the porewater parameters across all sampling dates are provided in Table 2. Fertilization had a significant effect on the porewater parameters measured (MANOVA, Wilk's lambda,  $P < 0.05$ ). Interstitial concentrations of ammonium and sulfide and salinity were the major parameters contributing to the treatment effect. Soluble sulfide was significantly lower in the treatment plots than in the controls ( $P < 0.05$ ) on the September 1998 and January 1999 sampling dates, while salinity was significantly higher ( $P < 0.05$ ) in September 1998, January 1999, and October 1999. As expected, monthly nutrient addi-

tions maintained significantly higher ammonium concentrations in the treatment plots relative to the controls for the entire year of fertilization ( $P < 0.05$  for the June 1998 to the January 1999 sampling dates). All porewater parameters contributed significant seasonal variability (MANOVA, Wilk's lambda,  $P < 0.05$ ). However, sulfide and salinity accounted for the majority of this variability, particularly in the final months of the growing season, when both parameters reach seasonal high values (Table 2). Ammonium concentrations returned to background levels, taken as the concentrations measured in the control plots, within 2 months following the termination of N + P application (June 1999). In October 1999, a significant ( $P < 0.05$ ) pulse of ammonium was measured in all of the plots relative to the previous sampling date (August 1999); however, ammonium did not differ significantly among treatments.

**Host plant response.** *Spartina* was very responsive to monthly N + P additions. By the end of the first growing season (September 1998), all treatment plots were noticeably greener and taller than the control plots (personal observation). Fertilized plots remained green for the duration of this study, while winter die-back was observed in the control plots. The appearance and life cycle of *Spartina* in the control plots were typical of this site in the Bly Creek basin.

Plant shoot heights and live-leaf numbers were measured in August 1999 (2 months after the final N + P addition) for all plots. *Spartina* was significantly taller in the ground-treated and foliar spray-treated plots relative to the controls ( $P < 0.05$ ). Shoots in fertilized plots more than doubled in height after 1 year of fertilization, with shoot heights averaging  $65.5 \pm 21.2$ ,  $70.5 \pm 13.7$ , and  $28.7 \pm 12.0$  cm for ground-treated, foliar spray-treated, and control plots, respectively. Shoot heights did not differ significantly between fertilization treatments. *Spartina* in the ground-treated and foliar spray-treated plots also had significantly more leaves per shoot compared to the controls ( $P < 0.05$ ). Leaf numbers did not differ significantly between the two fertilization treatments. Shoot height and leaf numbers were only weakly correlated by regression:  $R^2 = 0.39$  for ground-treated plots,  $R^2 = 0.20$  for foliar spray-treated plots, and  $R^2 = 0.42$  for control plots.

**Diazotrophic activity.** Acetylene reduction measurements were scored as detectable activity or no detectable activity (1 or 0, respectively) to make the trends in activity more apparent. Acetylene reduction activities (ARA), reported as the fraction of replicate plots with detectable ethylene production, by treatment and sampling date are provided in Table 3. The handling of the data in this manner, as opposed to production rates or product yields, had no effect on the outcome of the statistical analyses performed (data not shown) and was preferred since slurry incubations can yield rate measurements that are substantially different from in situ rates (76). ARA contributed significantly (MANOVA, Wilk's lambda,  $P < 0.05$ ) to the variability between treatments and across sample dates. However, a significant difference ( $P < 0.05$ ) in ARA between fertilization treatments was found only in January 1999. At that time, all of the control plots had detectable activity while only one out of six replicate ground-treated and foliar spray-treated plots had detectable activity. Seasonally, there was significantly ( $P < 0.05$ ) higher ARA in August 1999 compared to August 1998 and October 1999; all other sampling dates were not significantly different.

**RSGP.** The reverse sample genome probing profiles for all treatments and sample dates are provided in Fig. 1. On average,  $10^8$  ng of labeled probe DNA was used in each hybridization experiment. Based on the RSGP profiles (Fig. 1), it is clear that the rhizoplane diazotrophs and other reference strains on the master blot did not bind a large fraction of the total probe.

TABLE 2. Porewater parameters measured at a 10-cm depth in each sample plot<sup>a</sup>

Parameter and treatment	1998				1999		
	June	July	August	September	January	August	October
pH							
C	7.5 ± 0.1	7.5 ± 0.3	7.3 ± 0.3	7.2 ± 0.2	7.3 ± 0.1	7.7 ± 0.2	7.4 ± 0.1
G	7.4 ± 0.2	7.6 ± 0.3	7.3 ± 0.4	7.3 ± 0.5	6.9 ± 0.4	7.5 ± 0.2	7.3 ± 0.2
F	7.4 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	7.1 ± 0.1	6.9 ± 0.2	7.5 ± 0.2	7.5 ± 0.4
Salinity (ppt)							
C	33.5 ± 2.8	34.8 ± 3.3	36.2 ± 1.5	35.8 ± 1.7	33.2 ± 1.0	39.3 ± 1.0	31.3 ± 1.5
G	33.2 ± 3.1	35.0 ± 2.4	38.8 ± 2.7	38.8 ± 2.0	36.2 ± 1.8	47.7 ± 2.9	32.0 ± 4.4
F	31.3 ± 1.6	36.3 ± 1.2	37.2 ± 1.8	37.8 ± 1.0	36.0 ± 1.3	46.8 ± 2.1	33.0 ± 3.7
H <sub>2</sub> S (mM)							
C	3.9 ± 0.6	3.6 ± 0.5	3.1 ± 1.1	4.8 ± 1.2	1.0 ± 0.7	4.9 ± 0.4	4.7 ± 0.7
G	3.0 ± 0.9	2.4 ± 0.8	2.2 ± 1.4	2.3 ± 1.8	0.1 ± 0.1	4.3 ± 0.8	4.2 ± 0.3
F	4.2 ± 0.6	3.2 ± 0.9	3.1 ± 1.0	3.2 ± 1.0	0.0 ± 0.0	4.9 ± 0.3	4.7 ± 0.5
NH <sub>4</sub> (μM)							
C	5.9 ± 3.3	6.1 ± 4.7	7.8 ± 5.7	9.3 ± 3.2	4.9 ± 3.8	9.1 ± 2.3	21.8 ± 17.3
G	24.9 ± 0.0	15.5 ± 8.8	43.4 ± 24.7	19.6 ± 17.8	29.7 ± 13.2	9.9 ± 5.3	41.3 ± 47.1
F	24.9 ± 0.0	21.4 ± 4.2	42.6 ± 16.2	33.8 ± 21.9	27.8 ± 13.3	10.9 ± 4.9	27.9 ± 11.1

<sup>a</sup> C, control; G, ground treatment; F, foliar spray treatment. Values are the means of six results ± the standard deviations.

Pervasive treatment effects were not supported by these profiles, primarily due to variability among replicates for each treatment and date. However, a general trend toward decreasing diazotroph abundance in the ground-treated and foliar spray-treated plots in June 1998 and July 1998 presumably reflects the impact of fertilization. This impact is clearly delayed by ground treatment compared to the foliar spray treatment, which had an immediate negative impact on the majority of the populations. A similar trend was also noted in January 1999, when overall abundances were lower in the treatment plots relative to the controls. Seasonal effects were statistically ( $P < 0.05$ ) supported for all treatments. Control plot profiles differed significantly ( $P < 0.05$ ) among June 1998 to July 1998, July 1998 to September 1998, January 1999, and October 1999. The ground-treated plot profiles differed significantly ( $P < 0.05$ ) among June 1998 to September 1998 and January 1999, July 1998 to September 1998, September 1998 to October 1999, and January 1999 to October 1999. In the foliar spray treatment plot profiles, significant differences ( $P < 0.05$ ) occurred among sample dates June 1998 to October 1999, July 1998 to October 1999, September 1998 to October 1999, and January 1999 to October 1999.

All strains accounted for roughly the same low percentages of the variability among RSGP profiles as assessed by PCA (data not shown). Up to 10 PC values were necessary to explain at least 50% of the variability among profiles when they were analyzed in toto by treatment or sample date.

## DISCUSSION

Previous studies employing PCR-based methods and the acetylene reduction assay have demonstrated substantial stability in species composition and ecological function (diazotrophy) of the *Spartina* rhizoplane-rhizosphere diazotroph assemblage (56, 57, 58). In this study, selected rhizoplane diazotrophs were monitored to examine this stability at the level of population representation by quantitative RSGP. Under the hybridization conditions described, we estimated our lower limit for quantification of a given diazotroph to be 5 ng of DNA, approximately  $5 \times 10^6$  cells (assuming 5 fg of DNA/cell). As expected, this technique is less sensitive than PCR-

based methods (approximately 0.01 ng of DNA or  $10^4$  cells when using *nifH* primers; Y. M. Piceno and C. R. Lovell, unpublished data) but avoids amplification biases, allowing selected species (or ecotypes) to be quantified accurately. In addition, quantification of physiologically characterized diazotroph species can provide new insights into how populations of these microorganisms interact with and are controlled by their environment.

Obviously, the utility of the RSGP technique is contingent on the ability to isolate reference organisms into pure culture, the biases of which are well known and documented (1, 11, 63), or to select environmentally relevant type cultures for the master blot. All of the rhizoplane diazotrophs and other reference organisms included on the master blot (or at least their highly homologous close relatives) were detected at all dates during this study. These organisms also responded to environmental variability (natural and experimental) by increasing or decreasing in abundance. Consequently, it is expected that the diazotrophs selected for these experiments provided a suitable though clearly not inclusive group for examining responses of *Spartina* rhizoplane diazotrophs to environmental variability. Reference strains from non-*Spartina* sources were included to provide some representation of groups of anaerobes known to occur in vegetated salt marsh sediments (sulfate reducers and *Clostridium* spp.) (21, 22, 28, 34, 37) and additional diazotrophs that have been shown previously to be associated with *Spartina* or other grasses (3, 21). However, the average population sizes of these strains were not significantly different from those of

TABLE 3. ARA of each sample plot<sup>a</sup>

Treatment	1998				1999		
	June	July	August	September	January	August	October
C	4/6	5/6	2/6	5/6	6/6	5/6	4/6
G	4/6	4/6	1/6	4/6	1/6	4/6	1/6
F	1/6	4/6	2/6	4/6	1/6	5/6	2/6

<sup>a</sup> C, control; G, ground treatment; F, foliar spray treatment. ARA results are presented by treatment as number of plots with detectable ethylene production (i.e., nitrogenase activity)/total number of replicate plots examined.

*Spartina* rhizoplane diazotrophs (data not shown). The lack of numerical dominance of any of these diazotrophic strains may reflect the great extent of diazotroph diversity in this system (44).

The fertilization treatments clearly had a substantial impact on *Spartina* production and above-ground biomass. Increased evapotranspiration and oxygenation of the root zone microenvironment, likely resulting from this stimulation, also had an effect on interstitial porewater chemistry in these plots. Nitrogen additions and increased host plant production have been shown in other fertilization studies to result in elevated interstitial salinities (15, 16). Foliar spraying appears to provide a more direct route to the rhizoplane-rhizosphere microenvironment (4, 5, 75) compared to ground treatment, but both methods were effective in increasing interstitial nitrogen levels. The rhizoplane diazotrophs and other reference strains were clearly physiologically responsive (as assessed by the amount of variability in population size) to these rather dramatic changes in the abiotic and biotic environment; however, relative population size was particularly stable. A tight coupling between rhizoplane-rhizosphere diazotrophic activity and *Spartina* production has been demonstrated previously (9, 80), presumably reflecting a dependence of diazotrophs on host plant-derived carbon-energy resources (2, 9, 32, 33, 41). The uncoupling of this stimulatory effect on diazotrophic function (Table 3) or species richness (Fig. 1) of the strains examined suggests that other factors are limiting to growth and activity in the root microenvironments. However, it is clear that while environmental variability can be extreme, these organisms are not readily displaced by such variability.

Changes in edaphic variables are expected to have lesser impacts on rhizoplane diazotrophs relative to that of the host plant. Several oxygen-utilizing rhizoplane diazotrophs have been shown to be exceptionally tolerant of soluble sulfide exposure despite differing optimal sulfide concentration ranges for nitrogen fixation (J. R. LaRocque and C. R. Lovell, unpublished data). Presumably, similar tolerance for broad ranges of other important edaphic variables (pH, salinity, redox potential, etc.) may also occur. These findings suggest that edaphic factors are important regulators of diazotrophic activity but have relatively little impact on diazotroph abundance. However, our ability to interpret these experiments is somewhat limited by the fact that *Spartina* exudation (6, 14, 30, 71) and interstitial porewater parameters can change rapidly and simultaneously in intertidal sediments (14, 49, 54, 56–58). Consequently, a combination of host plant and edaphic variables may be more realistic determinants of diazotroph abundance and activity in situ than either in isolation.

High variability among replicate profiles likely reflects the extent of microenvironmental heterogeneity in these sediments (48) and along the *Spartina* root surfaces (8). Physiological specialization of rhizoplane diazotrophs implies niche separation (2), and presumably the distribution patterns of diazotrophs and the niches they occupy can vary over relatively small spatial scales. Spatial variability of sulfate reducers over such small scales has been demonstrated in salt marsh sediments (22). In addition, microscopic examinations of sediment particles (8, 48) and root surfaces (8, 50, 61) have shown microorganisms distributed as discontinuous aggregates with a major portion of the available surface area uncolonized. Extensive spatial heterogeneity would clearly make population level comparisons intrinsically difficult to interpret. Valid trends in RSGP profiles may be masked by variability between samples; consequently, it may be more realistic to treat each profile independently. However, there were no gross differ-

ences in averaged RSGP profiles in response to the treatments used.

All of the strains on the master blot were detected throughout this study, suggesting that even under highly enriched conditions (both combined nitrogen and host plant-derived carbon), competition between populations may be either low or simply insufficient to displace any species. Diazotroph niche specialization should prevent any strain or ecotype from numerically dominating a highly heterogeneous habitat like the rhizoplane-rhizosphere. This hypothesis is supported by the findings of Lovell et al. (44), who reported little overlap of diazotroph *nifH* sequences recovered from different wetland plants. If these findings are consistent for diazotrophs associated with *Spartina* and other wetland grasses, then it could be hypothesized that r species (fast-growing, opportunistic species) would not be successful competitors in these assemblages unless the assemblages were drastically disturbed in some way. Consequently, all of the diazotrophs we examined might behave as k species (slower-growing, resilient species). Alternatively, physiological niche adaptation may allow diazotrophs to function as both r- and k-selected species. This capability would permit responsiveness, by rapid growth and/or increased levels of activity, to favorable conditions, while slow growth or even dormancy could be maintained under limiting conditions. Minimal competition between populations and high levels of environmental heterogeneity would promote compositional stability, while differences in the physiological responses of each population would allow the maintenance of environmental function (i.e., functional redundancy) across a wide range of environmental conditions. The physiological adaptations that differentiate these rhizoplane diazotrophs clearly provide competitive advantages for diazotrophs in their specific niches.

The maintenance of diazotrophic activity in the majority of the control plots throughout this study clearly demonstrates that some fraction of the diazotroph assemblage is consistently fixing nitrogen, and is presumably abundant, despite host plant ontogeny and changes in edaphic factors. Nitrogen fixation was inhibited in many of the fertilized plots on several dates, likely due to the high interstitial concentrations of nitrogen in these plots. Despite the elevated interstitial nitrogen levels maintained in the treatment plots, inhibition of diazotrophy was never consistent across all of the replicate plots for either fertilization treatment and activity always recovered to levels comparable to that of the controls by the following sample date. Similar results showing substantial short-term resilience of diazotrophic activity have been presented previously (21, 80). In contrast, Hanson (32) found that nitrogen fixation rates were stimulated by nitrogen additions but substantially less nitrogen was added in that study relative to this and other studies. Clearly, the quantity of nitrogen added to the experimental plots in this study exceeded the requirements of the host plant; however, microenvironments suitable for nitrogen fixation were maintained despite this excess.

Previous nonquantitative studies demonstrated remarkable stability of the *Spartina* rhizoplane-rhizosphere diazotroph assemblage in response to natural and experimentally manipulated (short-term) environmental variability (56–58). Here, both selected rhizoplane diazotrophs and diazotrophic activity were persistent and resilient despite dramatic shifts in the abiotic and biotic environments resulting from long-term fertilization. We credit these features of the diazotroph assemblage to the physiological adaptations of diazotrophs to defined niches in situ and to the heterogeneity of the rhizosphere microenvironment. These physiological and environmental features seemingly support the maintenance of diazotroph assemblage structure and environmental function over a wide

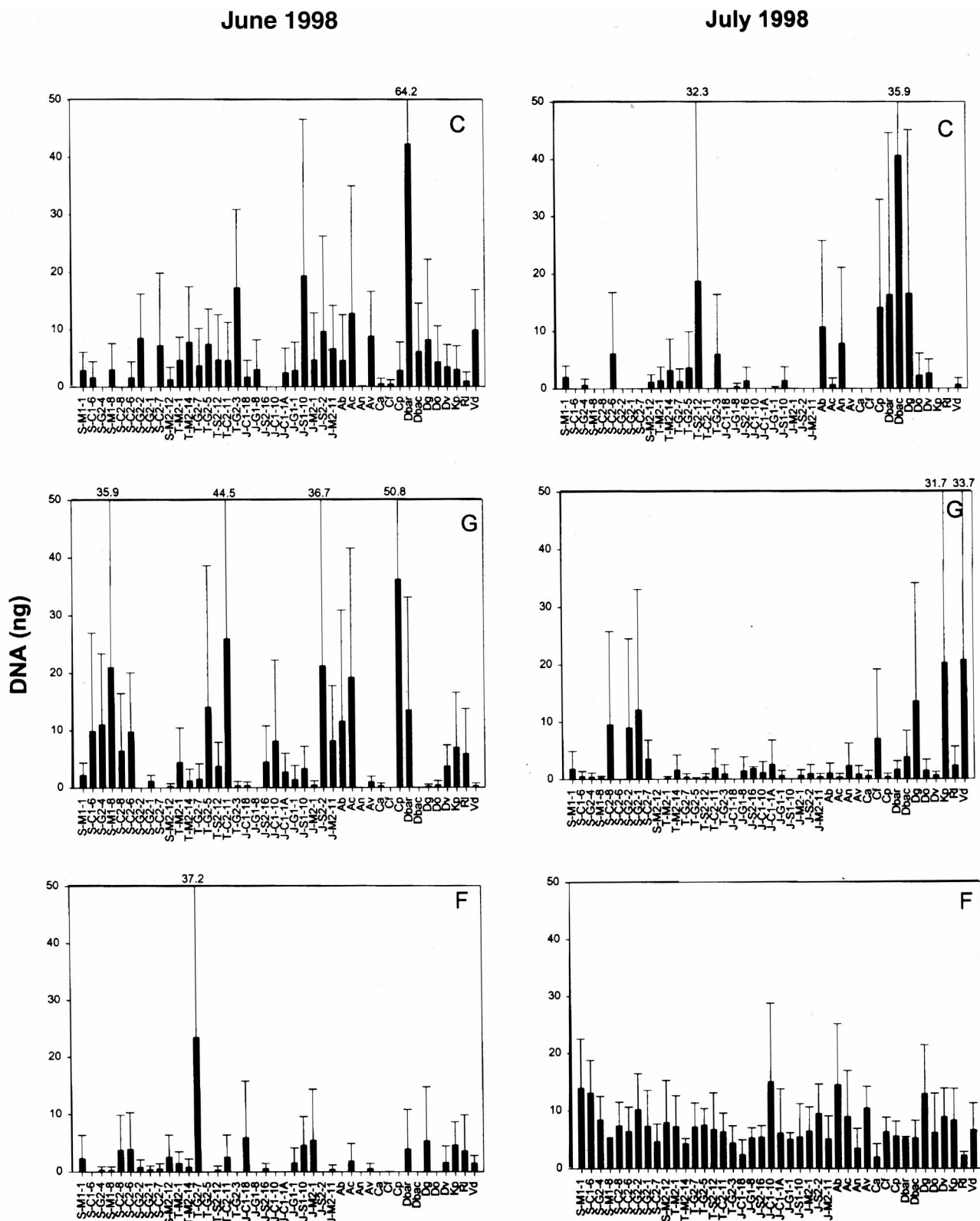


FIG. 1. RSGP profiles for each sample date by treatment ( $n = 3$ ). In July,  $n = 2$  for the foliar spray treatment profile. Values are mean nanograms of DNA  $\pm$  the standard deviation for each rhizoplane diazotroph and reference strain. Standard deviations exceeding the y-axis scale are provided across the top of each panel. Strains are designated by the host plant origin (S, short-form *Spartina*; T, tall-form *Spartina*; J, *Juncus*), the carbon source used for isolation (C, citrate; M, malate; S, sucrose; G, glucose), the pH of the isolation medium (1, pH 7.0; 2, pH 7.5), and the strain number. Reference strains are abbreviated as follows: Ab, *Azospirillum brasilense*; Ac, *Azotobacter chroococcum*; An, *Arcobacter nitrofigilis*; Av, *Azotobacter vinelandii*; Ca, *Clostridium acidurici*; Cf, *Clostridium formicoaceticum*; Cp, *Clostridium perfringens*; Dbar, *Desulfovibrio baarsii*; Dbac, *Desulfovibrio bacculatus*; Dg, *Desulfovibrio gigas*; Do, *Desulfotomaculum orientis*; Dv, *Desulfovibrio vulgaris*; Kp, *Klebsiella pneumoniae*; Rl, *Rhizobium leguminosarum*; Vd, *Vibrio diazotrophicus*. C, control; G, ground treatment; F, foliar spray treatment.

September 1998

January 1999

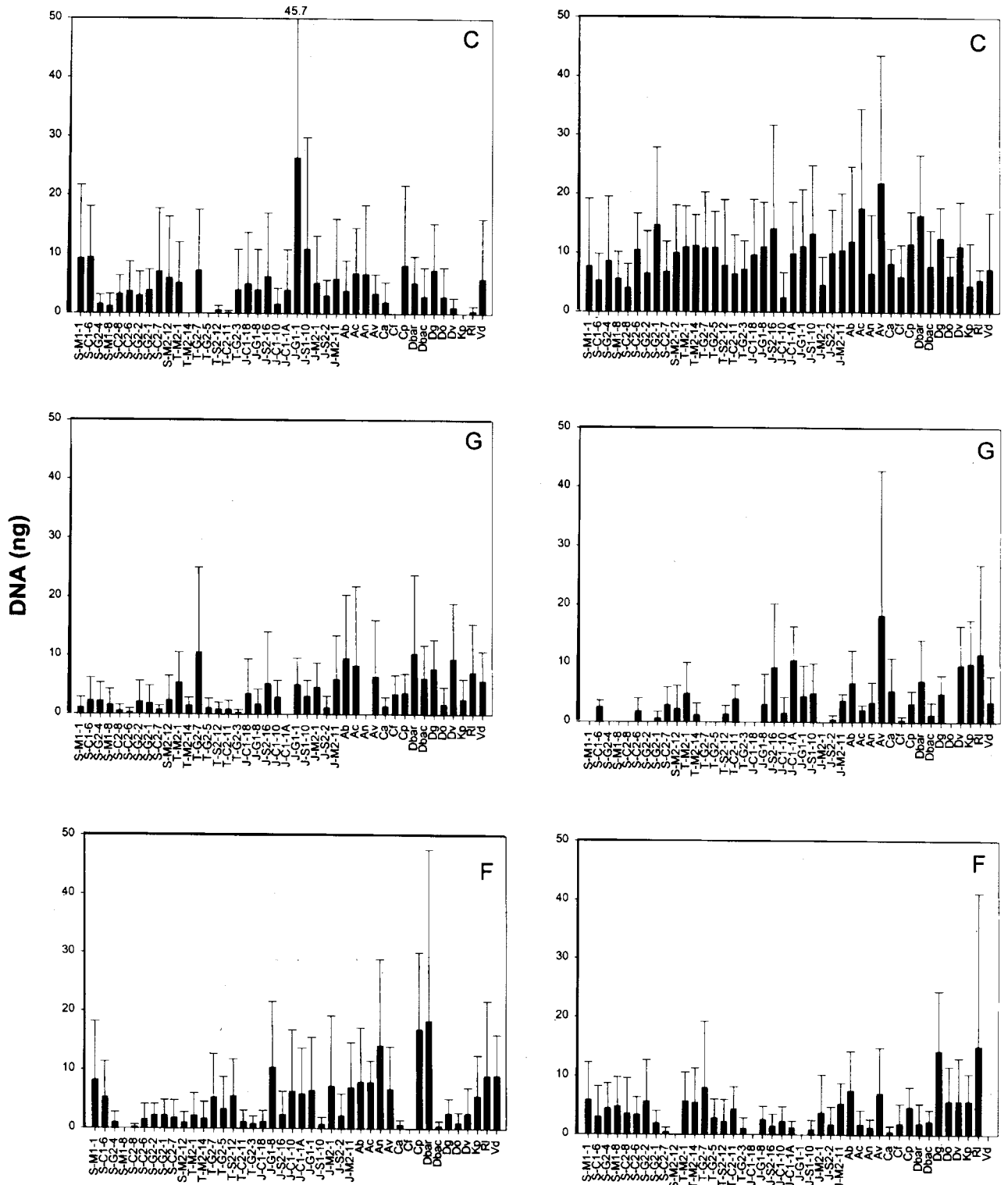


FIG. 1—Continued.

October 1999

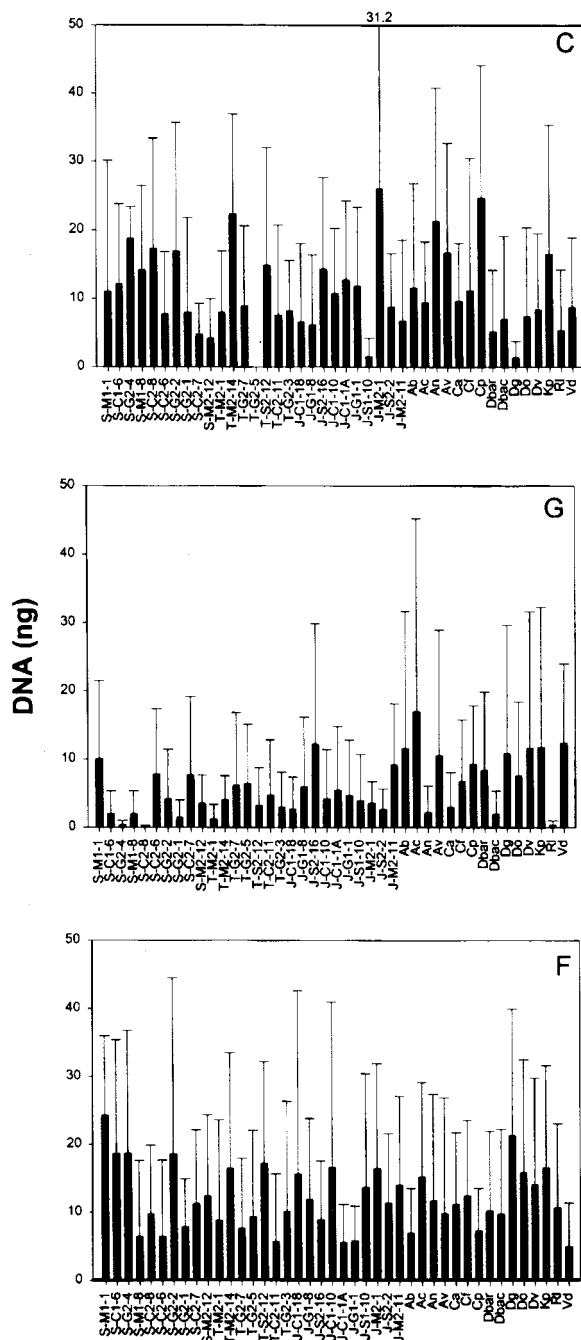


FIG. 1—Continued.

range of environmental conditions (functional redundancy) (56–58).

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