Analysis of Bacterial Communities in Seagrass Bed Sediments by Double-Gradient Denaturing Gradient Gel Electrophoresis of PCR-Amplified 16S rRNA Genes

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Abstract

Bacterial communities associated with seagrass bed sediments are not well studied. The work presented here investigated several factors and their impact on bacterial community diversity, including the presence or absence of vegetation, depth into sediment, and season. Double-gradient denaturing gradient gel electrophoresis (DG-DGGE) was used to generate banding patterns from the amplification products of 16S rRNA genes in 1-cm sediment depth fractions. Bioinformatics software and other statistical analyses were used to generate similarity scores between sections. Jackknife analyses of these similarity coefficients were used to group banding patterns by depth into sediment, presence or absence of vegetation, and by season. The effects of season and vegetation were strong and consistent, leading to correct grouping of banding patterns. The effects of depth were not consistent enough to correctly group banding patterns using this technique. While it is not argued that bacterial communities in sediment are not influenced by depth in sediment, this study suggests that the differences are too fine and inconsistent to be resolved using 1-cm depth fractions and DG-DGGE. The effects of vegetation and season on bacterial communities in sediment were more consistent than the effects of depth in sediment, suggesting they exert stronger controls on microbial community structure.

Introduction

Seagrasses are submerged flowering plants that grow rooted in coastal sediments of intertidal, subtidal, or shallow coastal zones [12], including the waters contiguous with the Gulf of Mexico in the USA. Seagrasses found in the northern Gulf of Mexico, and in Santa Rosa Sound in northwest Florida in particular, include *Halodule wrightii* (shoal grass) and *Thalassia testudinum* (turtle grass), among others. Seagrass habitats are in decline, both in the USA and around the world [1, 30]. Seagrass bed coverage in Santa Rosa Sound has declined from historical levels, and their preservation has been the subject of study and monitoring, with the hopes of restoring lost habitats [22, 23, 34]. Seagrasses provide several vital estuarine ecosystem functions, and their protection and restoration are important. Seagrasses link sediment and water column nutrient cycles and provide a buffer to erosion by trapping sediment and suspended material [14, 37, 40]. A large variety of micro-, meso-, and macrofauna are found within the margins of the seagrass beds. Many fish and decapod crustaceans (including species recognized for their economic value [25, 35]) are found in seagrass beds at significantly higher densities than in nonvegetated sandy habitats.

A great many bacterial species found in the sediments of seagrass beds are absent in sediment habitats outside of seagrass beds [6, 36]. It has been shown that bacteria inhabit the roots of the seagrasses [18–20, 28] and may form synergistic relationships. While it is known that certain bacterial species are important to seagrass ecosystems, the scope and scale of bacteria that are beneficial to seagrasses are not known. Bacterial communities associated with seagrass bed sediment in affected areas may differ from those associated with sediment in more pristine seagrass habitats, providing an indicator of seagrass health, just as seagrass beds themselves are indicators of water quality [4, 16]. A better understanding of these interactions could help define possible causes of

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seagrass decline and enhance efforts in seagrass restoration. It is therefore important to define the dynamics of bacterial communities associated with seagrasses.

Many strategies for examining microbial diversity entail analysis of ribosomal RNA (rRNA) sequences, including denaturing gradient gel electrophoresis (DGGE) [26, 29]. DGGE is a genetic fingerprinting technique that enables the separation of equally sized DNA fragments obtained by polymerase chain reaction (PCR) according to sequence differences [11]. The separation occurs during electrophoresis as a result of the differential "melting" of the different amplicons in a DNA product as they encounter a linearly increasing concentration of a denaturant mixture (urea and formamide) [26]. Double-gradient DGGE (DG-DGGE) further incorporates a gradient in acrylamide concentration to further sharpen banding patterns [8]. For the work here, we employed DG-DGGE as it was expected to provide the resolution needed to assess the microbial diversity in seagrass bed sediments.

Because the bacterial communities in sediments associated with seagrasses are poorly described, this study was undertaken to investigate some fundamental characteristics of bacterial community composition in relation to seagrasses. This was accomplished by examining differences in bacterial communities based on presence or absence of vegetation, depth into the sediment, and season.

Materials and Methods

Study Site.   The area just offshore of the Naval Live Oaks (NLO) Preserve in Gulf Breeze, FL, was chosen for study (Fig. 1). The shoreline in this location is part of the Gulf Islands National Seashore, maintained by the US National Park Service. The 2 mi of adjacent shoreline is free of direct commercial or residential activities and inputs. This site is considered to be pristine for the purposes of this study and most likely contains the highest quality seagrass environment in the Santa Rosa Sound system. A readily accessible mixed bed of seagrasses,

Figure 1. Naval Live Oaks site, Gulf Islands National Seashore, Gulf Breeze, FL.

including *T. testudinum* and *H. wrightii*, was found in the shallow, near-shore waters of this site.

Collection of Sediment Cores.   Sediment cores were collected in February, June, and October of 2001. The depth from the surface of the water to the sediment was approximately 1–1.5 m. Three cores, each approximately 10 cm deep and 3.5 cm in diameter, were taken in vegetated and unvegetated sediment at each sampling time, for a total of 18 cores. The vegetated cores were taken from within the seagrass bed, and unvegetated samples were taken within 10 m of the vegetated sites. Upon retrieval, the cores were sealed at both ends with rubber stoppers and immediately placed on ice.

Cores were processed within 2 h of collection. The top eight 1-cm sections (by depth from the surface) were placed into sterile 50-mL BD Falcon conical centrifuge tubes (BD Biosciences, San Jose, CA, USA). Small amounts of plant tissue were not removed from the sediment. A total of 144 sediment core sections were obtained and stored at −20°C until DNA extraction was performed.

DNA Extraction.   Microbial cell lysis and DNA extraction of the samples were performed using the UltraClean Soil DNA Isolation Kit (MoBio Inc., Solana Beach, CA, USA) according to the manufacturer's protocols with the noted modifications. Frozen sediment samples were thawed, and 0.5 g was added to MoBio Bead Solution tubes. After the addition of MoBio Solution S1, three cycles of freezing/thawing (−70/60°C) were performed. The tubes were transferred to a Mini-BeadBeater-8 (BioSpec Products, Inc., Bartlesville, OK, USA) homogenizer and mixed for 1.5 min at the highest setting. The tubes were centrifuged, and the supernatants were transferred to clean tubes, followed by 250 μL of MoBio Solution S2. When evidence of humic acids or tannins was present, indicated by coloration of the silica spin filter, additional washes with MoBio Solution S4 were performed until the coloration was significantly diminished or eliminated. The DNA was eluted and stored at −20°C. The concentration of the product was determined by electrophoresis and comparison to a standard DNA ladder of known concentrations (Low DNA Mass Ladder, Invitrogen, Carlsbad, CA, USA).

Polymerase Chain Reaction.   Each PCR reaction contained GeneAmp 1× PCR Gold buffer, 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.2 μM GMST-341fGC [forward universal bacterial 16S primer (underlined) with 40-bp GC clamp at the 5′end: CGCCCGCCGCGCCCCGCGCCC GTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAG CAG] [26], 0.2 μM 907r (reverse universal bacterial 16S primer: CCGTCAATTCCTTTGAGTTT) [21], 2.2 U AmpliTaq Gold (Applied Biosystems, Inc., Foster City,
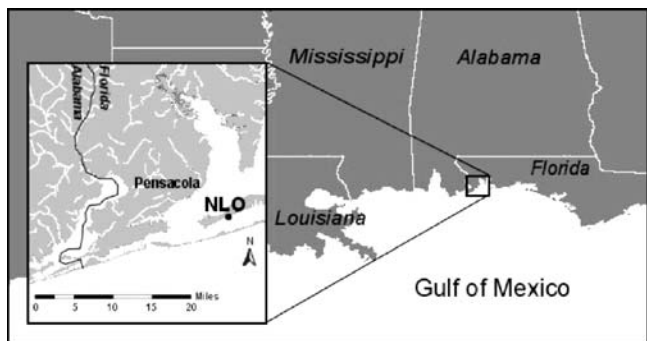
CA, USA), and approximately 120 ng template DNA. Triplicate samples were amplified in using a GeneAmp PCR System 9700 (PE Applied Biosystems) under the following reaction conditions: 94°C for 5 min, 20 cycles of 94°C for 1 min, 65°C for 1 min (decreasing 0.5°C/cycle), 72°C for 3 min, 10 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, 72°C for 7 min, 4°C hold. PCR products were analyzed via agarose gel electrophoresis and stained with ethidium bromide. The gel image was digitized using the Kodak Digital Science Image Station and analysis software as described above. Replicates of sample products were pooled and concentrated to 30-µL total volume using a SpeedVac DNA 110 evaporator (Savant Instruments, Inc., Farmingdale, NY).

Double Gradient Denaturing Gradient Gel Electrophoresis. DG-DGGE analysis of PCR-amplified 16S rRNA gene fragments was performed as described by Cremonesi *et al.* [8] and Muyzer and Smalla [27] with the D-Gene system (Bio-Rad, Hercules, CA, USA). The 1-mm-thick, 6–8% (w/v) polyacrylamide gel with a 20–70% denaturing gradient was poured discontinuously, using a 0.5- to 1-cm stacker of polyacrylamide gel without denaturant [24]. The manufacturer defines a 100% chemical denaturant solution as 7 M urea and 40% formamide. The gels were electrophoresed for 18 h in 1×← Tris–acetate–EDTA (TAE) buffer at 60°C using a constant voltage of 62 V. Gels were stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) and photographed on a UV transillumination table with a Polaroid camera equipped with a SYBR Gold filter (Molecular Probes). The gel photos were then digitized using an HP Scanjet ADF (Palo Alto, CA, USA). Gels containing bands sufficiently bright and clearly discernable from the background were used. PCR-amplified 16S rRNA genes from *Ralstonia* sp., *Bacillus cereus*, *Stenotrophomonas maltophilia*, *Microbacterium* sp., and *Cellulosimicrobium cellulans* provided an external five-band standard for alignment and normalization of band migration between gels [33]. These procedures resulted in banding patterns for 121 1-cm sediment core sections. The 23 remaining samples either gave poor amplification results

or poor band resolution when the PCR products were analyzed by DGGE.

Computer-Assisted Statistical Analysis

Lane and Band Analysis. The digitized image of each usable gel was entered into a BioNumerics (Applied Maths, Austin, TX, USA) database. Lanes were converted to densitometric curves. The curves were analyzed using software-determined thresholds, and bands were identified based on software-determined optimal thresholds. The gels were normalized using the five external standards described earlier and internal standards (bands consistent across several lanes in a gel). Gel photographs were examined with a magnifying glass, and any bands (operational taxonomic units) missed by the software were added and any bands that were clearly artifacts recognized by the software were deleted. Close inspection with the manual insertion and deletion of bands, as appropriate, was considered to result in a more accurate reflection of the community banding pattern than that obtained from the raw computer-generated pattern.

Similarity matrices. The Bionumerics package used the Dice algorithm [9] with the numbers of common and total bands to generate similarity coefficients between banding patterns. For two banding patterns A and B, the Dice similarity coefficient is calculated as,

$$\text{Similarity}_{\text{Dice}} = \left(2 \times \sum AB\right) \Big/ \qquad (1)$$
$$\left[\left(2 \times \sum AB\right) + \left(\sum Ab\right) + \left(\sum aB\right)\right]$$

where AB is the number of bands common to both banding patterns, Ab is the number of bands found in banding pattern A but not banding pattern B, and aB is the number of bands found in banding pattern B but not banding pattern A.

The Bionumerics program was used to calculate the best optimization and tolerance values for the compar-

Table 1. Total number of banding patterns from core sections (121) divided into groups with reference to depth into sediment, month of collection, and presence of vegetation

Depth (1-cm intervals)	No. of sediment core sections	Month (of collection)	No. of sediment core sections	Vegetation	No. of sediment core sections
0–1	15	Feb (2)	43	Vegetated	50
1–2	15	June (6)	46	Unvegetated	71
2–3	17	Oct (10)	33		
3–4	15				
4–5	16				
5–6	16				
6–7	14				
7–8	13				
	121		121		121

Table 2. Assignment of banding patterns to month groups by jackknife analysis (Dice coefficient)

	Banding patterns (%) assigned to groups		
Month collected	Feb	Jun	Oct
Feb	**81.4**	4.7	14.0
June	24.4	**57.8**	17.8
Oct	21.2	6.1	**72.7**

ERCCs of patterns to host class are in boldface. The mean ERCC was 70.7%.

ison. Within Bionumerics, optimization refers an adjustment of bands beyond normalization and was necessary when imperfect normalization resulted in residual shifts. Likewise, tolerance refers to the total distance that bands in different lanes differed by before they were determined to be distinct. The default values were used for optimization and tolerance and were 0.17 and 3.5%, respectively. At these values, bands that differed by more than 3.5% of the total length of the normalized lane would be determined to be distinct. Fuzzy logic was employed when determining similarity scores. Use of fuzzy logic in Bionumerics results in bands being scored as identical only when there was zero distance between them, meaning that with additional distance (in pixels) in band position, the matching score was decreased until the maximal position tolerance (3.5%) was reached, after which the similarity score was zero. This allowed larger tolerances to be chosen while still obtaining meaningful clustering (Del Rosario, Applied Maths, pers. comm.).

Construction of Groups for Jackknife Analysis. The data of the DG-DGGE from 16S rRNA genes amplified from the extracts from the NLO sediment core sections were placed into groups as shown in Table 1. Each group contained any banding pattern from the corresponding descriptor, i.e., month, depth, or vegetation. In this study, month of sampling was used as a proxy for season: February samples represented the sediment bacterial community before the seagrass growing season, June samples represented the sediment bacterial community in the middle of the seagrass

growing season, and October samples represented the bacterial community at the end of the growing season as the seagrasses were dying back. The Month groups were February, June, and October. Likewise, two groups were composed based on vegetation, one each representing vegetated (Veg) and unvegetated (Unveg) sediments. Samples were separated into eight groups composed of 1-cm depth intervals into the sediment (0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–7, and 7–8).

Jackknife analysis [32] tests for the fidelity of a group with its assigned members. This was performed by the removal of a banding pattern from its group and its presentation as a test subject against all groups. The banding pattern was then assigned to the group with which it shared the greatest similarity. This procedure was performed for every sample. Average similarity was used for the assignment of patterns to groups, and ties were spread equally among groups to avoid the bias associated with assigning ties to the originating group. Estimated rates of correct classification (ERCC) for group assignments were determined. ERCC was calculated as described in Ringbauer et al. [33]:

$$\text{ERCC} = 100\% \times (N_{\text{correct}})/(N_{\text{total}}) \quad (2)$$

where N_{correct} was the number of banding patterns from a group that were correctly identified, and N_{total} was the total number of banding patterns from the same group. A correct assignment was the assignment of a sample to the group to which the sample was known to belong.

The random ERCC was conservatively calculated as

$$\text{Random ERCC} = 100\% \times (N_{\text{max}})/(N_{\text{total}}) \quad (3)$$

where N_{max} was the number of banding patterns in the largest group used in an assignment, and N_{total} was the total number of banding patterns in the group. The ERCC was compared with the random ERCC to determine if assignments were significantly better than assignments to groups at random using the binomial table of confidence limits for percentages [39].

Table 3. Assignment of banding patterns to 1-cm depth interval groups by jackknife analysis (Dice coefficient)

Section depth (cm)	Banding patterns (%) assigned to groups							
	0–1	1–2	2–3	3–4	4–5	5–6	6–7	7–8
1	**46.7**	40.0	6.7	6.7	0.0	0.0	0.0	0.0
2	40.0	**26.7**	6.7	20.0	0.0	0.0	6.7	0.0
3	17.6	29.4	**23.5**	11.8	0.0	5.9	11.8	0.0
4	26.7	46.7	6.7	**13.3**	0.0	0.0	6.7	0.0
5	12.5	37.5	18.8	18.8	**0.0**	12.5	0.0	0.0
6	6.3	25.0	25.0	12.5	6.3	**0.0**	18.8	6.3
7	7.1	42.9	14.3	7.1	0.0	7.1	**14.3**	7.1
8	15.4	30.8	15.4	7.7	0.0	0.0	23.1	**7.5**

ERCCs of patterns to host class are in boldface. The mean ERCC was 16.5%.

Results

Number of Bands in Banding Patterns. DG-DGGE patterns of sediment bacterial communities were composed of between 3 and 31 bands. These numbers only include bands of 16S rRNA gene PCR products amplified from the sediment cores found within the range of the external standards run on every DG-DGGE. Any band found outside the range of standards was not used, as it was not subjected to the normalization process that made the gels comparable to one another. The data were not normally distributed, as determined using the Shapiro–Wilk test ($p < 0.05$), and thus were transformed and analyzed using a nonparametric procedure (Wilcoxon rank sums and Kruskal–Wallis test). Only when the numbers of bands in a profile were sorted by month was a significant effect evident as the number of bands in the June group was significantly different from the number of bands in the February and October groups, which were not significantly different from one another. No significant differences in the number of bands were found when the banding patterns were divided by depth or vegetation presence/absence.

Similarity Matrices and Jackknife Analysis. Matrices were constructed based on maximum similarity coefficients derived by the band-based [Dice: Eq. (1)] correlation methods (data not shown). The similarity coefficients ranged from 65.1% down to 0.0%.

Discriminant analysis of sediment bacterial community banding patterns was performed by cross-validation using jackknife analysis. Assignments of banding patterns to groups based on month are shown in Table 2. It is notable that when incorrect assignments were made for February and October samples, they were made to each other with a higher frequency than they were to the June group. Incorrect assignment of June samples appeared to be spread fairly evenly to both February and October. This is important as conditions (e.g., temperature and seagrass status) in February and October are more alike to one another than conditions in June. Overall, season appeared to have a strong and consistent effect on grouping the microbial communities, as the mean ERCC for the month groups as a whole was 70.7%, significantly higher than the random ERCC for month groups (38.0%).

Table 4. Assignment of banding patterns to vegetation groups by jackknife analysis (Dice coefficient)

Presence of vegetation	Banding patterns (%) assigned to groups	
	Vegetated	Unvegetated
Vegetated	**82.0**	18.0
Unvegetated	31.0	**69.0**

ERCCs of patterns to host class are in boldface. The mean ERCC was 74.4%.

Table 5. Comparison of ERCCs of groups by jackknife analysis (Dice coefficient)

Group type	ERCC (%)	Random ERCC (%)	Statistical difference (P)
Month	70.7	38.0	Yes ($p < 0.01$)
Depth	16.5	14.0	No
Vegetation	74.4	58.7	Yes ($p < 0.01$)

Assignments of banding patterns to groups based on 1-cm depth intervals into sediment were not evenly spread among groups. Using 1-cm depth intervals to divide groups did not provide evidence of consistent and significant effects of depth into sediment on microbial communities. A full 68.0% of assignments were to the 0- to 1-cm depth and 1- to 2-cm depth groups, despite the fact that these groups only contained 24.8% of the total banding patterns. The mean ERCC for group assignment by 1-cm depth was 16.5%, and the random ERCC was 14.0% (Table 3).

The presence or absence of vegetation had a consistent and significant effect on the assignment of banding patterns (Table 4). The mean ERCC for group assignment by the presence or absence of vegetation was 74.4%, whereas the random ERCC was 58.7%.

All the ERCCs for the different groups as determined by jackknife analysis were compared with the random ERCC (Table 5). Significant differences between the ERCCs were determined using the binomial table of confidence limits for percentages [36]. The ERCC for the month groups and the ERCC for the vegetation groups were significantly higher than random, with 99% confidence. These groups also appeared to have a fairly even assignment of banding patterns to groups, as compared to the uneven assignments in the depth group.

Discussion

The microbial communities of seagrass vegetated and unvegetated sediments were successfully investigated using DG-DGGE analysis of PCR amplicons of 16S rRNA genes. This method provided a rapid overview of the community profile when jackknife analysis was used to assign banding patterns to groups using similarity coefficients generated with the band-based Dice coefficient. The method cannot afford quantitative analysis of microbial communities on the basis of band intensities because of possible PCR bias and the sensitivity of the software to localized gel backgrounds associated with bands. The microbial communities of seagrass vegetated and unvegetated sediment at the NLO site in the Santa Rosa Sound could be consistently separated by this method, based on season as well as the presence or absence of vegetation. On the other hand, the communities could not be distinguished by depth intervals into

sediment. Although this study does not refute the fact that depth into sediment greatly influences the microbial community, it highlights the fact that such influences may not be consistent enough over the seasons or in the presence or absence of vegetation as to be elucidated using this community DNA fingerprinting technique. It is also possible that the DGGE method is insufficiently sensitive to detect differences with depth.

The ERCC for assignments based on month (as a proxy for season) was high, and a likely explanation is that the bacterial communities for a given month were likely responding to the seasonal status of the seagrasses. Microbial activities in seagrass bed sediments show strong seasonality and are highest when the plants are actively growing [10, 38].

The ERCC for assignments based on the presence of vegetation was also significantly higher than random. Seagrass bed sediments support higher numbers of bacteria and greater bacterial activities than nonvegetated sediments because of enrichment with organic carbon [10, 19, 20, 38]. Bacterial communities in unvegetated sediment do not experience these inputs and would be expected to differ at some level from the seagrass bed sediment communities. In contrast, Bagwell et al. [2] obtained one reproducible DGGE banding pattern for *nif*H sequences amplified from seagrass bed sediments in an oligotrophic environment and nearby nonvegetated sediments. Similarly, Smith et al. [38] concluded that the community composition of sulfate-reducing bacteria, based on comparisons of dissimilatory sulfite reductase genes, at the same site used in the present study did not vary substantially between vegetated and unvegetated sediments. Smith et al. [38] did find some small groups of sulfate-reducing bacteria unique to either the vegetated or nonvegetated sediment and suggested they could represent responses to available carbon sources, either root exudates or benthic algae, respectively. Similarities in microbial communities between vegetated and nonvegetated sediment are therefore to be expected, as indicated by Bagwell et al. [2], Smith et al. [38], and by the consistent grouping found in this study based on month. Yet, the microbial community in each type of sediment, particularly in temperate environments, is likely to have its own distinct characteristics.

Depth into sediment had a less consistent effect on the bacterial communities. This can be explained in part by the great influence of oxic conditions on bacterial communities. Oxygen is rapidly depleted with depth into the sediment, typically within millimeters [5, 13]. However, seagrasses exude oxygen into the sediment, creating an oxygen gradient into the rhizosphere [3, 7, 5, 15, 31] extending to (and upwards of, during daylight) 50 μm from the root surface outward into the sediment [5]. As a result, bacteria in the immediate rhizosphere may have some similarity to bacteria found in the oxic zone at the sediment surface,

regardless of the latter's condition with respect to vegetation. The vertical shift toward the surface by the oxic zone in the summer may also occur in the zones where other specialized metabolizers would be found. Sulfate reduction rates (SRR) of vegetated and unvegetated sediments were measured in the winter and summer of 2001 by Smith et al. [38] at the same study site. When they looked at SRR by depth, it was possible to differentiate unvegetated sediments from vegetated sediments within a season. Also, it was possible to differentiate one season from another for a particular plot of sediment. However, when both season and vegetation were considered, it was more difficult to differentiate by SRR, as the SRR for unvegetated sediment in summer was similar to that of vegetated sediment in winter, especially in the upper 2 cm. Taking this into account, it is apparent that there were depths where different types of communities existed in different months, especially depths near the surface and the transition from aerobic metabolism to anaerobic metabolism. These shallow depths tended to draw more assignments that would have been expected. This was demonstrated in the large number of assignments to the 0–1, 1–2, and 0–2 cm depth intervals. Although relative depth within a sediment core may be useful for separation of bacterial communities, the variability was too great for depth to be used reliably when comparing communities from different sediment cores. Whereas it was expected that the effects of depth would sort the microbial communities with greater resolution, these results are consistent with the results of similar studies [17].

Jackknife analysis sorted complex DG-DGGE patterns into groups based on vegetation and month. The differences in the communities were likely because of small percentages of the bacteria that changed in abundance, depending on season or vegetation. There have been few studies on microbial community diversity interactions with seagrasses. Our results provide additional evidence for interactions of sediment microbial communities with seagrasses.

Acknowledgments

References

1. Abal, EG, Dennison, WC (1996) Seagrass depth range and water quality in southern Moreton Bay, Queensland, Australia. Mar Freshw Res 47: 763–771

2. Bagwell, CE, La Rocque, JR, Smith, GW, Polson, SW, Friez, MJ, Longshore, JW, Lovell, CR (2002) Molecular diversity of diazotrophs in oligotrophic seagrass bed communities. FEMS Microbiol Ecol 39: 113–119

3. Borum, J, Pedersen, O, Grave, TM, Frankovich, TA, Zieman, JC, Fourqurean, JW, Madden, CJ (2005) The potential role of plant oxygen and sulfide dynamics in die-off events of the tropical seagrass, *Thalassia testudinum*. J Ecol 93: 148–158

4. Bulthuis, DA (1994) Light environments/implications for management. In: Wyllie-Echeverria, S, Olson, AM, Hershman, MJ (Eds.) EPA 910/r-94-004. Seagrass Science and Policy in the Pacific Northwest: Proceedings of a Seminar Series, pp 23–27

5. Caffrey, JM, Kemp, WM (1991) Seasonal and spatial patterns of oxygen production, respiration and root-rhizome release in *Potamogeton perfoliatus* L. and *Zostera marina* L. Aquat Bot 40: 109–128

6. Campbell, R, Greaves, MP (1990) Anatomy and community structure of the rhizosphere. In: Lynch, JM (Ed.) The Rhizosphere. John Wiley & Sons, Chichester, England, pp 11–34

7. Carlson, PR, Yarbro, LA, Peterson, BJ, Ketron, A, Arnold, H, Madley, KA (2002) The influence of sediment sulfide on the structure of South Florida seagrass communities. In: Greening, HS (Ed.) Seagrass Management: It's Not Just Nutrients! August 22–24, 2000, St. Petersburg, FL. Tampa Bay Estuary Program, pp 246. St. Petersburg, FL

8. Cremonesi, L, Firpo, S, Ferrari, M, Righetti, PG, Gelfi, C (1997) Double-gradient DGGE for optimized detection of DNA point mutations. BioTechniques 22: 326–330

9. Dice, LR (1945) Measures of the amount of ecologic association between species. Ecology 26: 297–302

10. Donnelly, AP, Herbert, RA (1999) Bacterial interactions in the rhizosphere of seagrass communities in shallow coastal lagoons. J Appl Microbiol Symp Suppl 85: 151S–160S

11. Fischer, SG, Lerman, LS (1979) Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis. Cell 16: 191–200

12. Fonseca, MS, Julius, BE, Kenworthy, WJ (1998) Integrating biology and economics in seagrass restoration: how much is enough and why. NOAA Conf. on goal setting and success criteria for coastal habitat restoration. Jan. 12–14, 1998, Charleston, SC

13. Herbert, AB, Morse, JW (2003) Microscale effects of light on H2S and Fe^{2+} in vegetated (*Zostera marina*) sediments. Mar Chem 81: 1–9

14. Harlin, MM, Thorne-Miller, B (1981) Nutrient enrichment of seagrass beds in a Rhode Island coastal lagoon. Mar Biol 65: 221–229

15. Kantrud, HA (1991) Wigeongrass (*Ruppia maritima* L.): a literature review. US Fish and Wildlife Service, Fish and Wildlife Research 10. Jamestown, ND: Northern Prairie Wildlife Research Center Home Page. http://www.npwrc.usgs.gov/resource/literatr/ruppia/ruppia.htm (Version 16JUL97)

16. Kenworthy, WJ, Haunert, DE (1991) The Light Requirements of Seagrasses: Proceedings of a Workshop to Examine the Capability of Water Quality Criteria, Standards and Monitoring Programs to Protect Seagrasses. National Oceanic and Atmospheric Administration, Beaufort, NC, p 181 (Tech. Memo. NMFS-SEFC-287)

17. Koizumi, Y, Takii, S, Fukui, M (2004) Depth-related change in archaeal community structure in a freshwater lake sediment as determined with denaturing gradient gel electrophoresis of amplified 16S rRNA genes and reversely transcribed rRNA fragments. FEMS Microbiol Ecol 48: 285–292

18. Kuo, J, McComb, AJ, Cambridge, ML (1981) Ultrastructure of the seagrass rhizosphere. New Phytol 89: 139–143

19. Kurtz, JC, Yates, DF, Macauley, JM, Quarles, RL, Genthner, FL, Chancy, CA, Devereux, R (2003) Effects of light reduction on growth of the submerged macrophyte *Vallisneria Americana* and the community of heterotrophic root-associated bacterial. J Exp Mar Biol Ecol 291: 199–218

20. Küsel, K, Pinkart, HC, Drake, HL, Devereux, R (1999) Acetogenic and sulfate-reducing bacteria inhabiting the rhizoplane and deep cortex cells of the sea grass *Halodule wrightii*. Appl Environ Microbiol 65: 5117–5123

21. Lane, DJ, Pace, B, Olsen, GJ, Stahl, DA, Sogin, ML, Pace, NR (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc Natl Acad Sci USA 82: 6955–6959

22. Lores, EM, Pasko, E, Patrick, JM, Quarles, RL, Campbell, J, Macauley, J (2000) Mapping and monitoring of submerged aquatic vegetation in Escambia–Pensacola Bay System, Florida. Gulf Mex Sci 18(1): 1–14

23. Lores, EM, Specht, DT (2001) Drought-induced decline of submerged aquatic vegetation in Escambia Bay, Florida. Gulf Mex Sci 19(2): 161–164

24. Moeseneder, MM, Arrieta, JM, Muyzer, G, Winter, C, Herndl, GJ (1999) Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol 65: 3518–3525

25. Murphey, PL, Fonseca, MS (1995) Role of high and low energy seagrass beds as nursery areas for *Penaeus duoraru* in North Carolina. Mar Ecol Prog Ser 121: 91–98

26. Muyzer, G, de Waal, EC, Uitterlinden, AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695–700

27. Muyzer, G, Smalla, K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73: 127–141

28. Nielsen, JT, Liesack, W, Finster, K (1999) *Desulfovibrio zosterae* sp. nov., a new sulfate reducer isolated from surface-sterilized roots of the seagrass *Zostera marina*. Int J Syst Bacteriol 2: 859–865

29. Nübel, U, Engelen, B, Felske, A, Snaidr, J, Wieshuber, A, Amann, RI, Ludwig, W, Backhaus, H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J Bacteriol 174: 5636–5643

30. Orth, RJ, Moore, KA (1983) Chesapeake Bay: An unprecedented decline in submerged aquatic vegetation. Science 222: 51–53

31. Pederson, O, Binzer, T, Borum, J (2004) Sulphide intrusion in eelgrass (*Zostera marina* L.). Plant Cell Environ 27: 595–602

32. Quenouille, MH (1956) Notes on bias in estimation. Biometrika 61: 353–360

33. Ringbauer, JA, James, JB, Genthner, FJ (2006) Impact of large-scale poultry farms on aquatic microbial communities: a molecular investigation. J Water Health 04: 77–86

34. Schwenning, LM (2001) Seagrass Management Plan for Big Lagoon and Santa Rosa Sound. Florida Department of Environmental Protection, Pensacola, FL

35. Sheridan, P, Henderson, C, McMahan, G (2003) Fauna of natural seagrass and transplanted *Halodule wrightii* (shoal grass) beds in Galveston Bay, Texas. Restor Ecol 11: 139–154

36. Shieh, WY, Yang, JT (1997) Denitrification in the rhizosphere of the two seagrasses *Thalassia hemprichii* (Ehrenb.) Aschers and *Halodule uninervis* (Forsk.) Aschers. J Exp Mar Biol Ecol 218: 229–241

37. Short, FT, Short, CA (1984) The seagrass filter: purification of estuarine and coastal waters. In: Kennedy, VS (Ed.) The Estuary as a Filter. Academic Press, Orlando, pp 395–413

38. Smith, AC, Kostka, JE, Devereux, R, Yates, DF (2004) Seasonal composition and activity of sulfate-reducing prokaryotic communities in seagrass bed sediments. Aquat Microb Ecol 37: 183–195

39. Sokal, RR, Rohlf, FJ (1987) Introduction to Biostatistics. W.H. Freeman and Company, New York, pp 333–337

40. Ward, LG, Kemp, WM, Boynton, WR (1984) The influence of waves and seagrass communities on suspended particulates in an estuarine embayment. Mar Geol 59: 85–103