Vol. 74: 43–57, 2015
doi: 10.3354/ame01725

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online January 12

Microbial community structure and dynamics in restored subtropical seagrass sediments

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ABSTRACT: Microorganisms in seagrass sediments facilitate many key ecosystem processes, yet current knowledge of microbial facilitation of seagrass community recovery following disturbance or restoration is limited. We studied microbial community responses to restoration of a subtropical seagrass meadow disturbed by vessel groundings in south Florida, USA, and relationships between microbial communities and sediment properties at the study sites using terminal restriction fragment length polymorphism. Two restoration methods were evaluated: the installation of bird roosting stakes as a means to provide a nutrient source, and placement of sand fill into excavations to prevent erosion. Both disturbed and restoration sites had less complex microbial community structure than undisturbed reference seagrass sediments. Microbial community structure varied little between disturbed and fertilized sites, but was distinct in filled sites. Sediment bulk density, sediment organic matter and total phosphorus content, porewater ammonium, soluble reactive phosphorus, and dissolved sulfide concentrations were important environmental predictors of microbial community structure across the restoration treatments. We show that community structure and diversity varied with sediment depth, among restoration treatments, and through time. Our results indicate that microbial communities in seagrass meadows are changed by physical disturbance of the rhizosphere, and that common restoration techniques lead to the formation of distinct microbial communities during the first year of recovery.

KEY WORDS: Microbial diversity · Restoration · Disturbance · Soil structure · Biscayne National Park

INTRODUCTION

Microbes remineralize organic matter in marine soils and sediments (Fenchel et al. 1998). Microbially-mediated nitrogen fixation, nitrification, denitrification, iron cycling, and sulfate reduction in seagrass ecosystems support high levels of primary and secondary production, and benthic metabolism (Hemminga & Duarte 2000, Marba et al. 2006). In seagrass ecosystems, photosynthetic activity by the seagrasses themselves creates conditions in the rhizosphere that support varying metabolic pathways in the remineralization process, increasing the availability of nutrients required for seagrass meadow development (Duarte et al. 2005). At the water-sediment interface, and in the rhizosphere of seagrass meadows, oxygen is available to support aerobic metabolism, while in deeper sediments, nitrate, iron, and sulfate become electron acceptors for anaerobic metabolism (Canfield et al. 1993). In tropical seagrass sediments, where iron and nitrate concentrations are typically low (Kristensen et al. 2000), sulfate reduction plays an important role in remineralization and nutrient availability (Holmer et al. 2001). Given this

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spatial distribution of microbial function, structuring factors of microbial communities in seagrass sediments include the presence of seagrass, proximity to seagrass rhizomes, depth in the sediment, and temperature (Danovaro & Fabiano 1995, James et al. 2006, Jensen et al. 2007, Green-García & Engel 2012, Luna et al. 2013). The varying metabolic pathways supporting seagrass ecosystem development indicate that seagrass sediments support complex microbial communities, and suggest that microbial community structure and function are subject to disruption when seagrass sediments are disturbed.

Loss of seagrass resources along the world's coastlines is accelerating (Waycott et al. 2009), and physical disturbance is a key contributor to the global decline (Short & Wyllie-Echeverria 1996, Orth et al. 2006, Grech et al. 2012). Physical disturbances to seagrass meadows that disrupt the rhizosphere and sediment, such as from mussel dragging (Neckles et al. 2005) or vessel groundings (Kenworthy et al. 2002) are some of the most severe types of injuries that can occur in seagrass meadows. Rhizosphere disruption impacts primary production, infaunal and epifaunal communities, sediment physical properties, and organic matter and nutrient pools (Neckles et al. 2005, Hammerstrom et al. 2007, Bourque 2012). Recovery of subtropical and tropical seagrass communities following sediment disturbance may take several years to over a decade (Zieman 1976, Durako & Moffler 1985, Dawes et al. 1997, Kenworthy et al. 2002, Hammerstrom et al. 2007, Uhrin et al. 2011, Hall et al. 2012a).

In the face of global seagrass decline, increased protection for seagrasses by governmental agencies is often accompanied by mandates to restore or otherwise mitigate seagrass impacts. Filling grounding excavations, applying fertilizer, and transplanting seagrasses are commonly-used seagrass restoration techniques (Fonseca et al. 1998, Bourque & Fourqurean 2014). Placing sand fill into excavations is intended to recreate the physical matrix that supports seagrasses and ecosystem functioning (Hammerstrom et al. 2007). Because seagrass ecosystems are often nutrient limited (Short 1987, Fourqurean et al. 1992a), applying fertilizer (via bird roosting stakes, where the feces of roosting seabirds fertilizes the sea floor below) aims to reestablish or augment pools of vital nutrients that may be limiting to seagrass growth (Kenworthy et al. 2000). Seagrasses also may be transplanted to more quickly replace lost plant structure and associated functions than would otherwise be accomplished through natural secondary succession following disturbance (Lewis 1987). Even after restoration has taken place, the seagrass community may take several years to develop, and may differ from the reference community (Zieman 1982, Williams 1990, Rollon et al. 1999, Kenworthy et al. 2002, Whitfield et al. 2002).

As the field of restoration ecology develops, it is critical to ensure that restoration practices are based upon and evaluated in the context of established ecological concepts (Palmer et al. 1997, Young et al. 2005). Knowledge of the soil microbial community (e.g. mass, composition, and activity) may be useful in assessing ecosystem status, particularly of disturbed, degraded, or recovering systems (Harris 2003), and should be considered in the context of energy flow and material cycling when conducting ecological restoration (Heneghan et al. 2008).

Because microbial activity is linked to specific biogeochemical processes, microbial community status is an important consideration for seagrass restoration efforts. For example, disrupted microbial community diversity in seagrass sediments has been linked to high mortality in seagrass transplants (Milbrandt et al. 2008). Changes in microbial community structure may reflect sediment conditions, such as sulfide accumulation, that may be detrimental to seagrass transplants (Christiaen et al. 2013). To our knowledge, microbial communities have only been studied in the context of seagrass transplanting. Our study evaluated microbial community response to 2 other common seagrass restoration methods, and characterized sediment conditions in an effort to explain observed patterns of microbial community structure: relationships that have not previously been demonstrated.

We used microbial community composition and measures of sediment ecosystem structure to evaluate ecosystem status and sediment quality following restoration of disturbed seagrass meadows in south Florida, USA. Two specific seagrass restoration methods were evaluated: installation of bird roosting stakes and placement of sand fill. By examining differences among disturbed, restored, and undisturbed reference sediments, we evaluated whether or not linkages between biotic and abiotic elements of ecosystem structure were being reestablished in these restoration sites (Harris 2003). We hypothesized that microbial community structure and microbially-mediated biogeochemical processes in the sediment would vary among restoration treatments due to differences in environmental variables important to ecosystem metabolism and nutrient storage. We also hypothesized that microbial communities would vary along with the abiotic gradients in the sediments and with time.

MATERIALS AND METHODS

Study system

This study was conducted on Cutter Bank (25.36715° N, 80.26899° W) in southern Biscayne Bay, a shallow (<3 m) subtropical estuary located at the southeastern tip of the Florida peninsula, USA. Seagrass communities in southern Biscayne Bay are dominated by dense *Thalassia testudinum* meadows typical of oligotrophic tropical seagrass communities throughout the western Atlantic and Caribbean (Zieman 1982). Most shallow seagrass shoals (<1 m) in this area are heavily impacted by vessel grounding injuries, where seagrass has been removed and sediment excavated in discrete areas (Bourque & Fourqurean 2013). Our study sites included multiple vessel grounding injuries, restoration sites, and adjacent undisturbed seagrass meadows on Cutter Bank.

Experimental design

The short-term effects of restoration on seagrass ecosystem structure were evaluated at 12 individual sites at Cutter Bank following implementation of a restoration project in January-February 2010. The maximum distance between sites was approximately 60 m. A factorial design was employed, with restoration status and time since restoration as fixed factors. Restoration status treatments included unrestored vessel grounding injuries ('disturbed' sites), restored grounding injuries that received nutrient input via bird roosting stakes ('fertilized' sites), restored grounding injuries that were returned to grade with sand fill ('filled' sites'), and undisturbed reference seagrass sites ('reference' sites). Sites selected for inclusion in the study (n = 3 per treatment) were an average of 36 m^2 in size, and treatments were randomly assigned to sites. Note that the disturbed sites were not recent grounding injuries, but known to be a minimum of 5 yr old based on knowledge of disturbance features at Cutter Bank (A. S. Bourque unpubl. data). Water depths at study sites averaged 0.5 m. Reference plots were established by delineating 32 m^2 circular plots around randomly selected points in undisturbed seagrass meadows across the shoal. The 12 sites were sampled within 1 mo of restoration implementation ('0 mo' sampling event), and at 3, 6, 9, and 12 mo following restoration (February, May, August, November 2010, and February 2011).

Sediment core collection and processing

To identify environmental predictors of microbial community structure in our treatments, we sampled a suite of sediment properties that define sediment structure and are indicators of microbially-mediated processes in seagrass ecosystems. These variables included sediment particle size and bulk density (microhabitat quality, nutrient exchange); pH, redox potential, organic matter content, and porewater sulfide content (benthic metabolism and remineralization); and nitrogen and phosphorus content in sediment and porewater (nutrient storage). At each sampling event, 3 replicate 7.3 × 40 cm sediment cores were randomly collected from each site using a piston corer with clear butyrate core tubes. Core tubes were immediately plugged at both ends following collection, and temporarily stored in the dark in a vertical position in ambient temperature seawater until processed. Cores were extruded and sectioned into 6 depth horizons at 2, 6, 10, 20, 30, and 40 cm in a nitrogen-filled glove box. The pH and redox potential (Eh) of sediments from each homogenized depth horizon were measured in the glove box. Depth horizons were then subsampled for analysis of sediment properties and porewater constituents (dissolved inorganic nitrogen as ammonium, NH_4^+; phosphorus as soluble reactive phosphorus, SRP; and dissolved hydrogen sulfide, DS). Subsamples of sediment for microbial community analysis were collected from the top 3 depth horizons, placed in sterile Whirl-paks, and stored at −80°C until further analysis. Sediments for porewater extraction were placed into 50 ml centrifuge tubes and capped inside the glove box, centrifuged for 5 min at 3000 rpm, and returned to the glove box. Extracted porewater was filtered through GF-C (in-line syringe filters) and subsampled into aliquots for NH_4^+/SRP and DS (5 ml) analysis. Samples for DS were fixed with 1 M ZnAc in a 1:10 dilution (Holmer et al. 2001) and stored at room temperature; all other sediment and porewater samples were frozen at −20°C until further analysis.

Grain size contributions were determined through sieve analysis (Ingram 1971, Folk 1974) and assigned to Krumbein scale values (ϕ, Krumbein & Sloss 1963). Particle size class contributions were determined for gravel ($\phi < -1$), sand ($-1 \leq \phi < 4$), silt ($4 \leq \phi < 8$), and clay ($\phi \geq 8$). Bulk density was measured as dry mass per unit volume after drying sediments at 75°C for 48 h. Organic matter content was measured as loss on ignition (LOI), or proportional mass loss of dry sediments following combustion at 500°C for 4 h (Gross 1971). Sediment total nitrogen (TN) was de-

termined using a CHN elemental analyzer (Fisons NA1500). Total P (TP) was determined through a dry-oxidation, acid hydrolysis extraction followed by colorimetric analysis of phosphate concentration in the extract (Fourqurean et al. 1992a). Elemental content was calculated on a dry weight basis as mass of element/dry weight of sample × 100%. Porewater samples for NH_4^+ and SRP were acidified to a pH of 2 with 6 N HCl and sparged with nitrogen gas to drive off hydrogen sulfide prior to analysis. Porewater NH_4^+ concentrations were measured colorimetrically with the indo-phenol blue method (Koroleff 1969, Parsons et al. 1984) and SRP concentrations were measured colorimetrically using the ascorbate method (Parsons et al. 1984). Porewater sulfide concentrations were determined spectrophotometrically following the methods of Cline (1969).

Microbial community profiling

Terminal restriction fragment length polymorphism (TRFLP) (Liu et al. 1997) was used to profile microbial community diversity in seagrass sediments. TRFLP may be subject to PCR bias (Lueders & Friedrich 2003) and to limitations in providing phylogenetic inference into the specific taxa altered or variation in the functional composition of those different taxa directly (Torsvik & Øvreås 2002). However, it remains a valuable comparative tool, capable of detecting microbial community changes across large numbers of samples and treatments, particularly in studies involving communities with low to intermediate levels of diversity, such as studies involving colonization and early successional stages (Engebretson & Moyer 2003). We selected the 16S rRNA gene as our gene of interest because it is highly conserved in bacteria and archaea (Leloup et al. 2009). Because terminal restriction fragments (TRFs) can represent both bacterial and archaeal sequences, we described our results in terms of microbial communities. DNA was extracted from 1.5 g sediment samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories). DNA was amplified using the primer sets FAM-Univ 9F and Univ 1509R (Integrated DNA Technologies) in PCR reactions (GoTaq Flexi DNA Polymerase kit (Promega) following the procedures of Thurber et al. (2012). Amplifications were checked for efficiency on 1.2% agarose gels and cleaned using the Wizard SV 96 PCR Clean-Up System (Promega). Multiple restriction enzymes were used to increase the specificity and confidence of our resulting data interpretation (Engebretson & Moyer 2003, Nocker et al. 2007). PCR product (256 ng) was digested in separate 20 µl reactions using the restriction enzymes *Msp*I (Promega) and *Bst*UI (New England Biolabs). Fragments were analyzed at Laragen on an ABI 3730 sequencer (Applied Biosystems), with 9.5 µl Hi-Di formamide and 0.5 µl 1200 LIZ size standard (Applied Biosystems) added to 0.5 µl of each PCR reaction.

Sizes of TRFs were determined using the Local Southern size-calling algorithm of Peak Scanner Software v1.0 (Applied Biosystems). The peak amplitude threshold was set at 100 fluorescence units. In order to best resolve differences among our samples, to estimate minimum levels of diversity, and to compare differences between the sampled microbial communities, the TRFLP datasets were combined and further processed using the T-REX software (Culman et al. 2009). Peak height was used as a metric of relative microbial abundance. Peaks were retained if height exceeded the standard deviation (assuming zero mean) computed over all peaks (Abdo et al. 2006) and aligned using a clustering threshold of 0.5 base pairs (Smith et al. 2005). The TRFs outside the size range of 40 to 1160 base pairs were omitted to ensure fragments did not exceed the dynamic range of the LIZ-1200 size standard. The TRFs that occurred in less than 1% of samples were omitted. Prior to analysis, TRF heights were standardized within samples to provide relative abundance data, removing some potential effects of differential PCR amplification (Fierer & Jackson 2006).

A total of 925 TRFLP profiles passed quality checks (*Msp*I, n = 462 samples; *Bst*UI, n = 463 samples). These profiles represented 86% of our analyzed sediment samples. Following averaging of site replicates, data analysis was conducted on 169 averaged profiles, representing 94% of our total potential averaged profiles. We did not compare our sample profiles with databases of known TRFLP sequences because of the potential for misidentification of sequences (from sequence similarity between known and unknown taxa, differences in electrophoretic mobility, etc.) during *in silico* analyses (Dickie & FitzJohn 2007, Schütte et al. 2008).

Data analysis

The TRF data were log-transformed to reduce the influence of highly abundant TRFs on the data set. Principal coordinates analysis (PCO) (Anderson et al. 2008) was used to visualize differences in the microbial community on the basis of restoration status.

Permutational analysis of variance (PERMANOVA) (Anderson et al. 2008) was used to test the hypotheses that (1) multivariate microbial community structure varies with sediment depth, (2) restoration affects multivariate microbial community structure, and (3) restoration affects univariate community characteristics including TRF richness (*S,* Chao 2), evenness (Pielou's *J'*), and diversity (Shannon's *H'* and Simpson's 1 − λ). PCO and PERMANOVA analyses of multivariate microbial community data were based on the binomial deviance dissimilarity measure (Anderson & Millar 2004), and analyses of univariate diversity metrics were based on Euclidean distance resemblances. Significance values for PERMANOVA tests were based on 999 permutations of residuals under reduced models. When sediment depth was used as a covariate (i.e. for multivariate community structure and univariate diversity metrics), Type I sums of squares were used in the PERMANOVA analyses. When sediment depth was not a covariate (i.e. particle size composition), Type III sums of squares were used in the PERMOANOVA analysis. Pairwise permutational tests with Bonferroni corrections were conducted on the significant main effects and interactions in the PERMANOVA analyses. The SIMPER procedure (Clarke & Gorley 2006) was used to determine TRF similarity within restoration status groups. SIMPER also identified the contribution of the most abundant TRFs to within-group similarity.

Distance-based linear modeling (DistLM) and distance-based redundancy analyses (dbRDA) (Legendre & Anderson 1999, McArdle & Anderson 2001, Anderson et al. 2008) were used to determine relationships between microbial community relative abundance data and multivariate data on sediment properties. Parameters for the DistLM routine, which is analogous to linear multiple regression, included the Best selection procedure and Akaike's information criteria corrected for small sample sizes (AIC_c) (Akaike 1973, Burnham & Anderson 2002); the procedure was run with 9999 permutations. Environmental data were log-transformed prior to analysis to reduce skewness. Pearson correlations between individual log-transformed environmental variables and diversity metrics were calculated from log-transformed microbial relative abundance data and analyzed for significance.

PCO, PERMANOVA, SIMPER, DistLM, and dbRDA analyses were conducted with the software PERMANOVA+ for PRIMER (Clarke & Gorley 2006, Anderson et al. 2008). Correlations analyses were conducted with SPSS 20.0 (IBM).

RESULTS

Sediment properties

Sediments in undisturbed seagrasses at Cutter Bank were fine grained (6.8 ± 0.2 ϕ, mean ± SE, Fig. 1), and dominated by silt and clay fractions (Fig. 2). These sediments were strongly reduced (−302.6 ± 9.1 mV Eh) with high organic matter content (16.5 ± 0.5% LOI). Nutrient concentrations were low (0.015% ± 0.001% P content; 148.3 ± 41.5 µM NH_4^+; 1.0 ± 0.6 µM SRP). Sediments from disturbed and fertilized sites had similar properties (Fig. 1) and particle size composition (PERMANOVA pairwise test, $\alpha = 0.008$, $p > 0.039$; Fig. 2) as the reference sites. A notable exception is that P content and porewater NH_4^+ concentration increased by 99 and 67%, respectively, in the fertilized sites over the year-long course of the study as a consequence of the deposition of bird feces.

Sediment properties from filled sites differed sharply from reference sites for all variables examined. Filled sites had lower organic matter and dissolved sulfide concentrations, and higher bulk density, pH, Eh, P content, and NH_4^+ and SRP concentrations, than the reference sites (Fig. 1). Filled site sediments were heavily dominated by gravel and sand, and were coarser than reference sediments (PERMANOVA pairwise test, $\alpha = 0.008$, $p < 0.004$; Fig. 2).

Microbial community profiles

Microbial communities were structured across restoration treatments (Fig. 3). Profiles from filled sites clustered tightly and were separated from other treatments along PCO1, which explained 35.0% of variation in the data matrix. Reference profiles also clustered tightly, and were partially overlapped by disturbed profiles. Fertilized profiles showed the least structure, and varied along both PCO axes.

Microbial communities were structured by sediment depth across all treatments (PERMANOVA, pseudo-$F = 9.0$, df = 2, $p < 0.001$), and multivariate community structure differed with each depth horizon (PERMANOVA pairwise tests, $p < 0.005$). Community structure differed across the 4 restoration treatments (PERMANOVA, $p < 0.001$; Table 1), with distinct profiles for each treatment ($p < 0.001$). Community profiles also changed with time over the year-long course of the study (PERMANOVA, $p < 0.001$; Table 1), and, overall, differed with each sampling

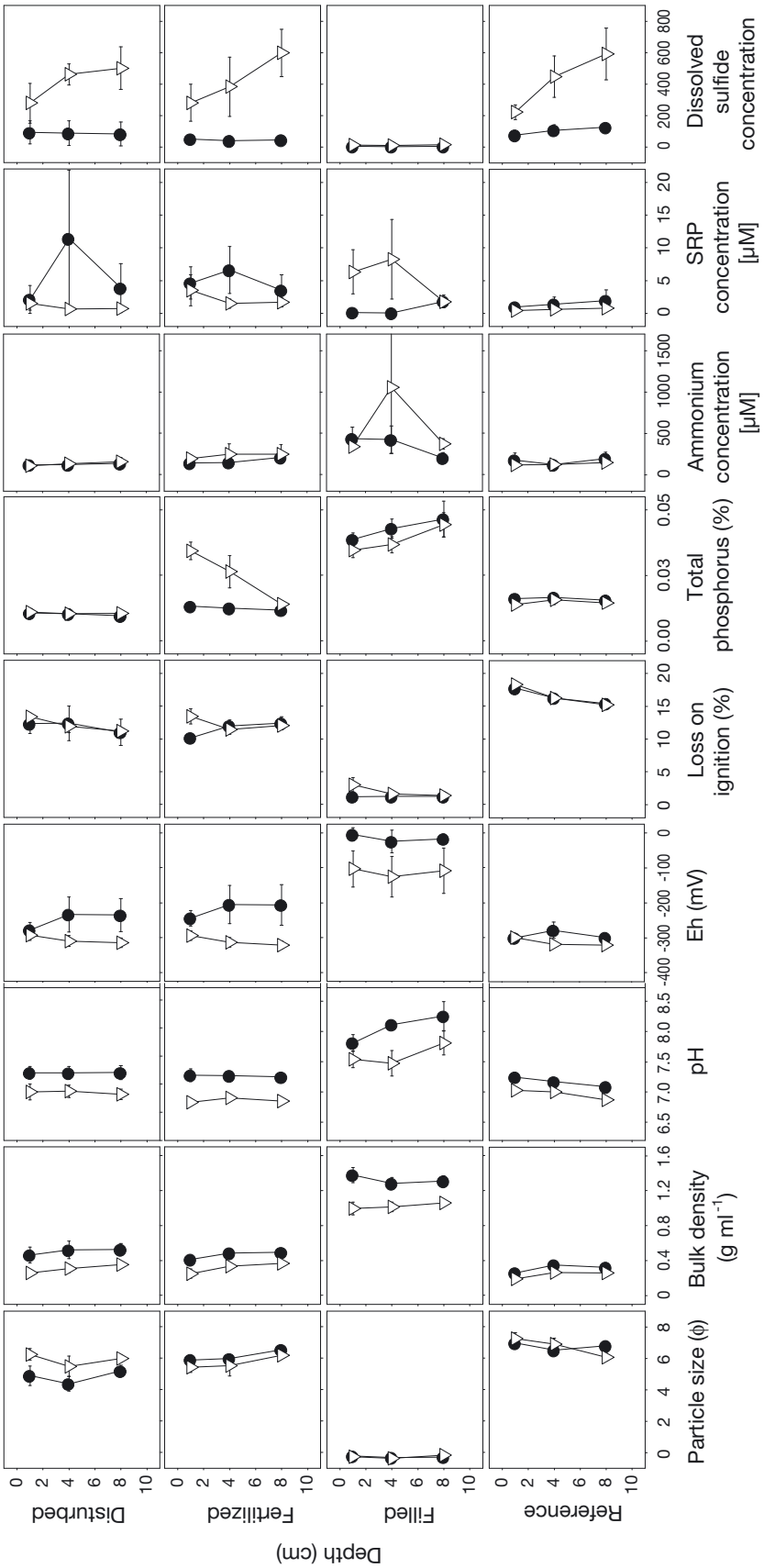

Fig. 1. Depth profiles of sediment environmental variables at disturbed (top row), fertilized (second row), filled (third row), and reference (bottom row) sites, at 0 mo (circles) and 12 mo (triangles) post-restoration. Values are mean ± SE at 3 depth horizons: 0–2, 2–6, and 6–10 cm. Abbreviations: Eh = redox potential; SRP = soluble reactive phosphate

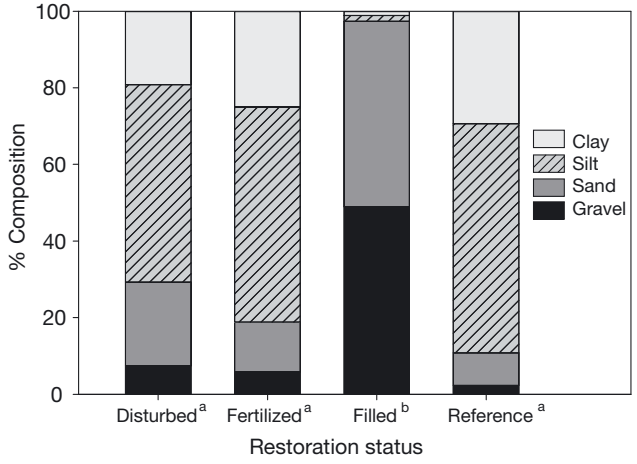

Fig. 2. Sediment particle size class (clay, silt, sand, gravel) contribution in sediment cores collected from disturbed, fertilized, filled, or undisturbed reference seagrass meadows. Data in bars are pooled over 3 depth horizons (0–2, 2–6, 6–10 cm) and 2 sampling events (0 and 12 mo following restoration) within each treatment. Letters indicate significant treatment groupings (α = 0.008), determined through PERMANOVA pairwise tests of multivariate sediment structure between treatments, following PERMANOVA on restoration status (p < 0.001)

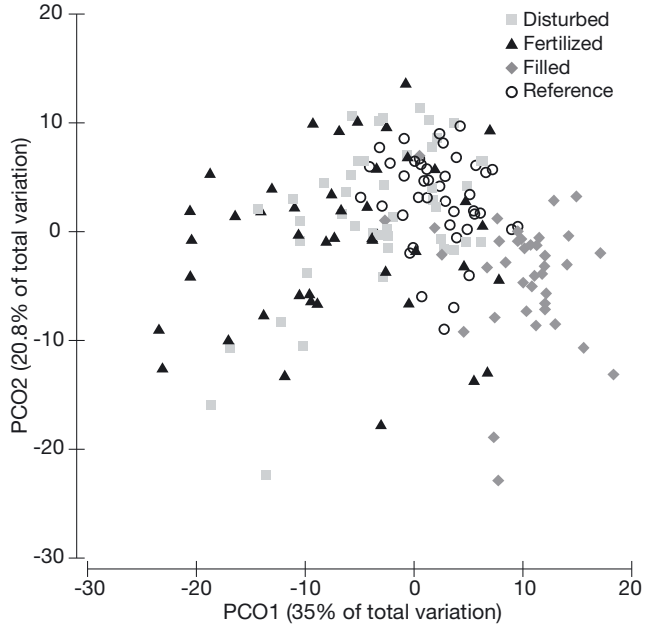

Fig. 3. Principal coordinates analysis ordinations of TRFLP profiles from sediment samples at disturbed, fertilized, filled, and reference sites

event (PERMANOVA pairwise tests, p < 0.001). There was a significant Time × Treatment interaction in the community analysis. Disturbed and fertilized sites had similar community profiles for all time steps (PERMANOVA pairwise tests, p > 0.064; Table 1) except at the 0 mo sampling event. Filled site profiles were different from the reference profiles and from the disturbed and fertilized profiles at every time step (PERMANOVA pairwise tests, p < 0.002; Table 1) except at the 0 mo sampling event, when profiles between filled and reference sites were similar. Within restoration treatments, community profiles at the 12 mo sampling event differed from the 0 mo sampling event for the disturbed, fertilized, and filled treatments (PERMANOVA pairwise tests, p < 0.002; Table 1), but not for the reference treatment.

Table 1. PERMANOVA analysis of the effects of restoration status (disturbed, fertilized, filled, reference) and time since restoration (0, 3, 6, 9, 12 mo) on multivariate microbial community structure, with sediment depth as a covariate. p-values in bold text indicate statistical significance at α = 0.05. Results are also included for PERMANOVA pairwise tests on the Treatment × Time interaction, for levels of the treatment factor within each time step (middle panel) and of the time factor within each treatment (bottom panel). Different letters indicate significant differences between levels within each column, at an adjusted α

Source	df	MS	Pseudo-*F*	p
Depth	1	3391.8	29.8	**0.001**
Treatment	3	2977.5	26.1	**0.001**
Time	4	810.8	7.1	**0.001**
Treatment × Time	12	325.5	2.9	**0.001**
Residual	149	575.0		

Treatment (α = 0.008)	0 mo	3 mo	6 mo	9 mo	12 mo
Disturbed	a	ac	a	a	ac
Fertilized	b	a	a	a	a
Filled	c	b	b	b	b
Reference	cd	c	c	c	c

Time (α = 0.005)	Disturbed	Fertilized	Filled	Reference
0 mo	a	a	a	ad
3 mo	b	ac	abcd	abd
6 mo	ab	a	bd	b
9 mo	b	b	cd	bd
12 mo	b	bc	c	ac

Microbial community diversity

In total, 122 and 95 TRFs were detected with the *Msp*I and *Bst*UI digests, respectively, and 166 unique TRFs were found in the combined dataset. Maximum TRF richness across restoration treatments was 122 TRFs, with an average of 59.6 ± 1.8 TRFs per sample. For clarity of presentation, microbial community diversity results are included for the 0 and 12 mo

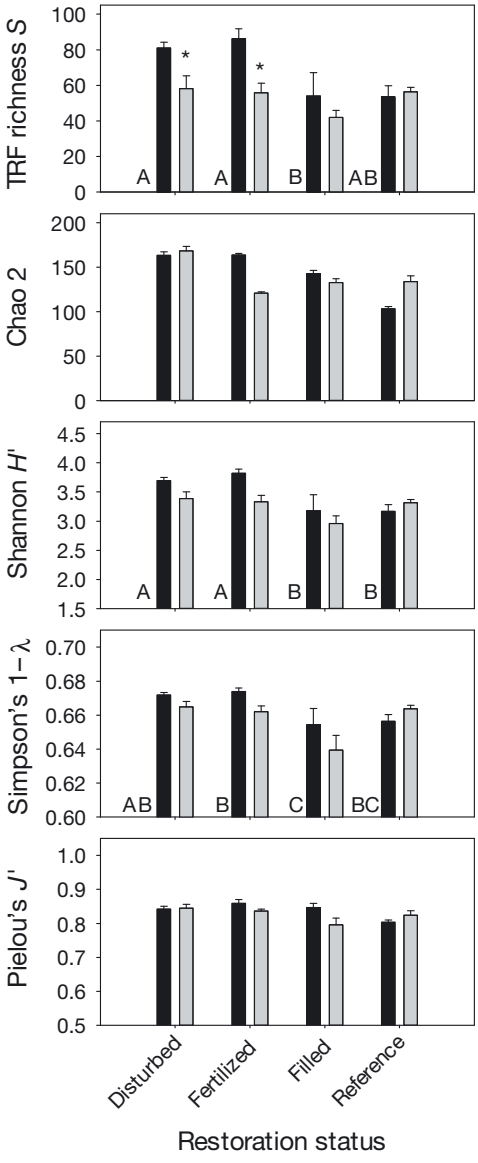

Fig. 4. TRF richness (*S*, Chao 2), diversity (Shannon's *H′* and Simpson's 1 – λ), and evenness (Pielou's *J′*), by restoration treatment and time since restoration (0 mo, dark bars and 12 mo, light bars) calculated from TRFLP abundance data. Values are mean ± SE. Where the treatment main effect was significant (α = 0.05), significance (α = 0.008) of pairwise tests of the treatment levels is indicated by letters at the base of each pair of bars. Significance of time since restoration within a treatment is indicated by an asterisk (α = 0.05)

sampling events. TRF richness (Fig. 4), averaged over all time steps for each treatment, was highest for the fertilized treatment (71.1 ± 5.4 TRF) and lowest for the filled treatment (48.0 ± 8.5 TRF). TRF richness in disturbed and fertilized treatments was nearly a third greater than in reference treatment. Chao 2 estimates (Fig. 4) were highest for the disturbed

Table 2. PERMANOVA analysis of the effects of restoration status (disturbed, fertilized, filled, reference) and time since restoration on microbial community richness, evenness, diversity, and dominance at the 0 and 12 mo sampling events, with sediment depth as a covariate. p-values in bold text indicate statistical significance at α = 0.05

Diversity metric	Source	df	MS	Pseudo-*F*	p
TRF richness	Depth	1	25	0.09	0.770
	Treatment (Tr)	3	2233.4	7.7	**0.001**
	Time (Ti)	1	4522	15.6	**0.003**
	Tr × Ti	3	829.9	2.9	**0.040**
	Residual	57	0.253		
Pielou's *J′*	Depth	1	0.000	0.034	0.851
	Treatment	3	0.005	3.832	**0.008**
	Time	1	0.002	1.964	0.171
	Tr × Ti	3	0.004	2.823	**0.037**
	Residual	57	0.001		
Shannon's *H′*	Depth	1	0.035	0.310	0.577
	Treatment	3	1.030	9.232	**0.001**
	Time	1	0.892	8.000	**0.007**
	Tr × Ti	3	0.278	2.489	0.067
	Residual	57	0.112		
Simpson's 1 – λ	Depth	1	0.000	0.502	0.473
	Treatment	3	0.007	9.915	**0.001**
	Time	1	0.003	4.016	0.050
	Tr × Ti	3	0.001	1.874	0.151
	Residual	57	0.001		

treatment (165.7 ± 4.6 TRF), and lowest for the reference treatment (118.3 ± 4.7 TRF).

Microbial community richness, evenness, and diversity (Shannon's *H′* and Simpson's 1 – λ) varied among restoration treatments (PERMANOVA, p < 0.008; Table 2; Fig. 4), though with complex patterns among metrics. Values for all 4 diversity metrics were similar in samples from disturbed and fertilized treatments (Fig. 4). Diversity (*H′*) was lower in reference sites than in disturbed and fertilized sites (PERMANOVA pairwise tests, p < 0.003; Fig. 4). Filled sites had similar richness, evenness, and diversity (*H′* and 1 – λ) as reference sites (Fig. 4).

Time was a significant factor explaining variation in microbial richness and diversity (*H′*; PERMANOVA, p < 0.007; Table 2, Fig. 4). Values for both metrics were lower at 12 mo than at 0 mo following restoration for the disturbed and fertilized treatments. No temporal changes were observed for richness and diversity (*H′*) in filled or reference treatments, or for evenness or diversity (1 – λ) in any treatment (Fig. 4).

Within treatments, reference sites had the most similar microbial communities (68.3%), while the least similar communities (46.4%) were found in

Table 3. SIMPER analysis of microbial TRF similarity across restoration treatments

Treatment	Within treatment mean similarity (%)	Most abundant TRF (bp) Size (bp)	Contribution (%)
Disturbed	65.5	106	7.1
Fertilized	57.1	106	7.6
Filled	46.4	504	9.6
Reference	68.3	504	7.4
	Among treatment mean dissimilarity (%)		
	Disturbed	Fertilized	Filled
Fertilized	40.2		
Filled	54.2	58.1	
Reference	35.0	43.3	52.3

filled sites (SIMPER, Table 3). The most abundant TRF in each treatment was shared between the disturbed and fertilized communities (106 bp), and also between the filled and reference communities (504 bp; SIMPER, Table 3). Dissimilarity between communities in treatment pairs was lowest between the disturbed and reference treatments (35.0%). Among all treatment pairs, filled site communities shared the highest dissimilarity percentages (>50%) with each of the other 3 treatments (SIMPER, Table 3).

Environmental predictors of microbial community structure and diversity

Total nitrogen, mean phi size, and water content were excluded from the DistLM analysis due to high correlation ($|r| > 0.95$) with organic matter content and bulk density. When constrained by environmental variables, microbial community structure among restoration treatments became even more pronounced, as seen in the dbRDA ordination (Fig. 5).

Microbial community profiles from filled sites distinctly separated from the other restoration treatment profiles along dbRDA1. Reference site profiles clustered tightly, sharing little space with disturbed and fertilized profiles, and the reference, disturbed, and fertilized profiles reflect an apparent gradient of organic matter and bulk density (Fig. 5).

There were strong negative correlations between organic matter content and dbRDA1 (DistLM, r = –0.998; Table 4), between bulk density and dbRDA2 (r = –0.804; Table 4),

Table 4. Multiple partial correlations between dbRDA coordinate axes and environmental variables

Variable	dbRDA1	dbRDA2	dbRDA3
Organic matter	–1.00	–0.04	–0.06
Bulk density	0.01	–0.80	0.59
NH_4^+ concentration	–0.07	–0.59	–0.80

and between NH_4^+ concentration and dbRDA3 (r = –0.802; Table 4; not plotted in Fig. 5). We interpret these correlations to indicate that high organic matter content in reference, disturbed, and fertilized sediments, high NH_4^+ concentration in fertilized sediments, and high bulk density in fill sediments are important drivers of the microbial community structure across the restoration treatments.

The DistLM marginal tests that fit each environmental variable individually to the microbial community data, showed that every variable except SRP concentration had a significant relationship with microbial community structure (DistLM marginal tests, $p < 0.001$). The DistLM procedure selected bulk density, organic matter content, and NH_4^+ concentration for inclusion in the best multivariate predictor model explaining microbial community structure

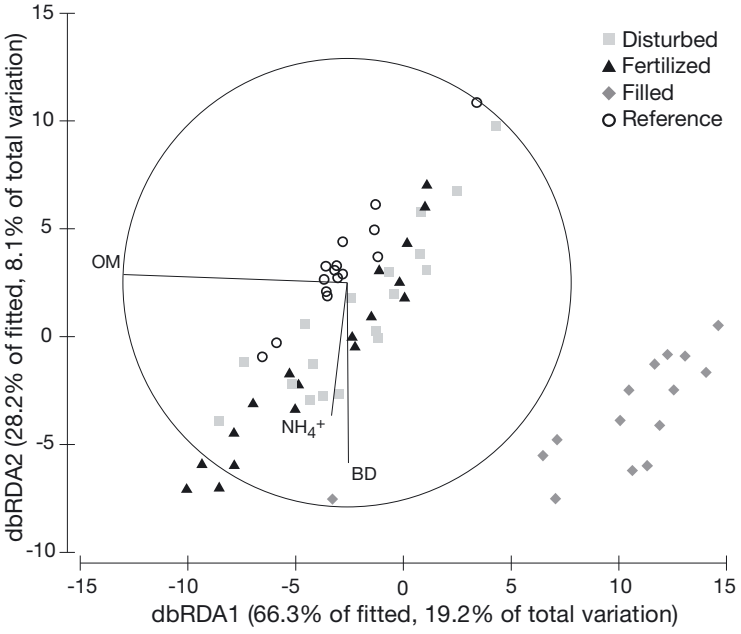

Fig. 5. dbRDA ordination of microbial community data (binomial deviance resemblance matrix calculated from log transformed relative abundance data) fitted to log transformed environmental variables. Data are from 0 and 12 mo post restoration sampling events. Ordination is based on best-fit DistLM model with 3 variables visualized in the encircled vector overlay: log(bulk density, BD), log(ammonium, NH_4^+), and log(organic matter, OM))

Table 5. Pearson correlations between log-transformed diversity metrics calculated from TRFLP abundance data and environmental variables, sampled at 0 and 12 mo post-restoration. Correlations in bold text with 1 and 2 asterisks indicate significance at the $\alpha = 0.05$ and $\alpha = 0.01$ level, respectively. phi = particle size class, BD = bulk density, Eh = redox potential, OM = organic matter, P = total phosphorus, NH_4^+ = ammonium, SRP = soluble reactive phosphorus, and DS = dissolved hydrogen sulfide

Diversity metric	phi	BD	pH	Eh	OM	P	NH_4^+	SRP	DS
Richness *S*	**0.34****	−0.16	−0.23	−0.18	**0.32****	**−0.51****	−0.16	0.06	−0.01
Shannon-Wiener's *H′*	**0.34****	−0.17	−0.21	−0.22	**0.31***	**−0.51****	−0.14	−0.01	0.02
Simpson's 1 − λ	**0.40****	**−0.30***	−0.24	**−0.33****	**0.40****	**−0.52****	−0.22	−0.05	0.15
Pielou's *J′*	0.16	−0.06	0.04	−0.13	0.09	−0.23	−0.01	−0.19	0.00

across the restoration treatments (DistLM, $r^2 = 0.29$). However, the solutions for the 10 best models all had AIC_c values within 2 units of each other, indicating that all models may be considered viable (Burnham & Anderson 2002). The 10 best models included between 3 and 6 variables; all included bulk density, organic matter content, and NH_4^+ concentration. Dissolved sulfide was included in 6 models, and SRP and P were included in 5 and 4 models, respectively. Two models included Eh, and none of the best models included pH as a predictor variable.

Univariate measures of microbial community diversity and evenness showed slightly different relationships with environmental predictor variables. TRF richness and diversity (*H′*, 1 − λ) were correlated with particle size and with organic matter and phosphorus content (Pearson correlations, $p < 0.05$; Table 5). Diversity (1 − λ) was correlated with bulk density and redox potential ($p < 0.05$; Table 5). However, pH, NH_4^+, SRP, and dissolved sulfide concentrations were not correlated with microbial community diversity or evenness. No significant correlations were found between microbial community evenness and any of the measured environmental variables.

DISCUSSION

By exploring microbial community structure and diversity in seagrass sediments that were intact, disturbed, or restored using 2 different methods, we were able to show that community structure varied with sediment depth, among restoration treatments, and through time. We also identified environmental variables important to sediment structure, ecosystem metabolism, and nutrient storage that are predictors of microbial community structure. During the course of our study, we observed very little recolonization of seagrasses in our restoration or disturbed sites (Bourque & Fourqurean 2014), so the changes among treatments and with time were not influenced by the structuring role of vascular plants in these sites that formerly supported well-developed seagrass sediments.

Sediment depth was a significant factor in our analyses of relative microbial community structure, but not for community diversity metrics. Electron acceptors available for microbial use in mineralization vary with depth and the presence of belowground plant biomass. It follows that microbial communities will differ with depth, reflecting the different metabolic processes taking place throughout the seagrass rhizosphere. For example, microbial community differences have been detected between oxidized and reduced sediments in seagrass ecosystems, and in the presence of root zone sediments (Jensen et al. 2007, Sørensen et al. 2007), although community changes with depth are not always evident (James et al. 2006, García-Martínez et al. 2009).

The most distinct differences in microbial community structure across our restoration treatments were seen when comparing filled sites to other treatments. Microbial communities from filled and reference sites had similar diversity metrics but separated on the PCO ordination, suggesting communities of similar complexity but different composition. Filled sites were characterized by a near absence of organic matter in the top 10 cm, which is not surprising given that the fill material was created from mined limestone. Organic matter content is an important determinant of microbial community structure in terrestrial systems (Sessitsch et al. 2001, Girvan et al. 2003, Blum et al. 2004), and our results support this relationship for seagrass ecosystems. Organic matter supplying benthic remineralization processes is provided by (1) dead roots and rhizomes, (2) root exudates, (3) organic particles and litter buried by sedimentation and bioturbation, and (4) benthic microalgal exudates (Pedersen et al. 1997, Holmer et al. 2001).

Because organic matter content in developing seagrass meadows is driven by the accumulation of plant biomass, and is a slow process (Pedersen et al. 1997,

McGlathery et al. 2012), organic matter content in filled sites is expected to remain low until these sites support dense, climax seagrass communities. In the initial stages of seagrass community development, a lack of organic matter in the sediments as substrate for microbial remineralization may lead to persistently low nutrient pools available to support seagrass and macroalgae colonization. Low organic matter content may also slow some microbially-mediated metabolic pathways in sediments, like sulfate reduction, and the subsequent accumulation of DS in the porewater (Ruiz-Halpern et al. 2008). Incorporation of organic material into fill used in restoration sites may help to accelerate development of the microbial community, and, in turn, the seagrass community.

Microbial communities can persist through spatial relocation of soils especially when environmental conditions between sites are similar (Lazzaro et al. 2011, Christiaen et al. 2013). The carbonate sand used for fill at our restoration sites was locally sourced from lake mines in south Florida. Any microbial community present in the fill material prior to placement at our restoration sites was likely strongly impacted following inundation by sea water. Organic matter content at all of our study sites was also measured at deeper sediment horizons, down to 40 cm (data not shown). In the deeper horizons, organic matter content increased and particle size decreased (Bourque & Fourqurean 2014), presumably due to mixing between the fill layer and underlying sediments. It is possible that the microbial community in this mixing layer can stimulate remineralization using the organic matter present as a substrate. However, in sites where the fill layer is thicker, or if organic matter is not available as a substrate, this may not be possible.

Nutrient addition can stimulate microbial remineralization of organic matter (Lopez et al. 1998), and we expected to see evidence and products of metabolism in the fertilized treatment. We predicted that bird stakes would provide nutrient input that would affect diversity as the microbial community responded to N and P inputs in this nutrient-limited system (Danovaro & Fabiano 1995). Phosphorus content was elevated at the fertilized sites after 1 yr, and the microbial community did change in the fertilized treatment over the course of the study, though microbial communities in the fertilized sites differed from those in disturbed sites only at the initial sampling event. Redox potential was lower and DS was elevated in fertilized sites after 1 yr compared to the start of our study, though this pattern was also seen in the disturbed and reference sites.

Particle size composition is important in shaping microbial communities. In agricultural systems, underlying soil type affects microbial communities, and microbial diversity is negatively correlated with particle size (Sessitsch et al. 2001, Girvan et al. 2003). Different particle size fractions are thought to act as microhabitats with different organic matter content and redox conditions that accordingly support different microbial communities (Miller & Dick 1995, Zhang et al. 2007). Our results also indicated that microbial diversity was negatively correlated with particle size. Disturbed, fertilized, and reference sites generally had more complex communities than filled sites, and bulk density and particle size were important predictors of variability in microbial community structure.

In seagrass ecosystems, the importance of particle size and porosity in seagrass bed sediments is linked to exchange of porewater with overlying waters (Koch 2001). Particle size is correlated with porewater exchange (Fourqurean et al. 1992b), and thus nutrients and also toxic compounds such as sulfide may accumulate in fine-grained sediments. We did see elevated NH_4^+, SRP, and DS in the porewaters from the fines-dominated disturbed, fertilized, and reference sites, when compared to the filled treatments. However, we attribute those differences to lower benthic metabolism in the newly-placed fill material, rather than differences in porewater constituent retention related to sediment particle size.

The carbonate sand used in our filled treatment was far coarser than ambient sediments. Turbidity created during fill placement can be difficult to control with fine-grained materials, and there is also concern that the fill may wash away from the site with tides and wave energy. The silt/clay fraction of fill material used in this restoration project (1 to 6%) was within the range of particle size composition known to support *Thalassia testudinum* growth (Koch 2001), but far lower than ambient sediments at Cutter Bank. Despite dramatic differences in particle size distributions, TRF richness was similar in filled and reference sites. However, TRFs of similar sizes may be derived from different microbial taxa, and thus richness alone is not a good indicator of community similarity with the TRFLP method (Engebretson & Moyer 2003). Fine sediments are expected to increase in the fill sites as the seagrass community develops with time and seagrass blades trap particles from the water column (Terrados & Duarte 2000), but these sites will likely always remain coarser than the surrounding

sediments. Filled sites, then, may continue to support a distinct microbial community on the basis of sediment structure.

In our study, TRF richness and diversity were negatively correlated with sediment Eh. Relatively higher Eh values were recorded for filled sites, which had little to no vegetative cover during the study period, whereas reference seagrass sediments were strongly reduced. Microbial community diversity in seagrass beds has been shown to differ in the presence vs. absence of seagrass (James et al. 2006, Green-García & Engel 2012, Luna et al. 2013) and between the root zone and bulk sediments (Jensen et al. 2007). Seagrasses can modify redox conditions in the rhizosphere (Enríquez et al. 2001, Marbà & Duarte 2001). This capability, linked to photosynthetic activity and leaching of O_2 from seagrass roots (Pedersen et al. 1997, Connell et al. 1999, Terrados et al. 1999, Jensen et al. 2007), may also influence microbial activity in the rhizosphere. However, Eh in the surface (<10 cm) layer of vegetated sediments can be lower than in unvegetated sediments in the presence of elevated organic matter subject to microbial metabolism (Pedersen et al. 1997), or because the photosynthetic activity of benthic microphytobenthos may be reduced by seagrass canopy shading (Enríquez et al. 2001). We suggest that the differences in redox potential we observed between filled and reference sites can be explained by the high organic matter content in the reference sediments and its near absence in the filled sites.

We found that time was a significant factor in altering the microbial community. However, in the multivariate analysis, patterns of change through time were not clear within treatments. Further, the direction of change in the univariate metrics was unexpected, as diversity values were often lower at the 12 mo mark within restoration treatments. A clear cause for these patterns is elusive. One potential explanation is that the 0 mo sampling event occurred within weeks of a rare extreme cold event in south Florida during January 2010. The average water temperature in January and February at this location ranges between 20° and 21°C (Biscayne National Park unpubl. data). During the cold snap, water temperatures remained below 15°C for the 12 d period 4–16 January 2010, and reached a low of 9.2°C on 11 January 2010. Water temperatures during the 12 mo sampling event were between 19.2° and 19.7°C, nearly back to the normal range. Temperature can affect microbial development in seagrass ecosystems (Danovaro & Fabiano 1995, James et al. 2006), and it is plausible that the microbial community was impacted by the cold snap. Microbial diversity can increase following disturbance (Hall et al. 2012b), and the community may have been in a period of recovery when we sampled it.

CONCLUSION

Current knowledge of microbial facilitation of seagrass community recovery following disturbance or restoration is limited. Our study is among the first to examine sediment microbial communities in the context of ecosystem effects of seagrass restoration (see also Milbrandt et al. 2008, Christiaen et al. 2013). We found that microbial community structure varied with physical and biogeochemical sediment properties that had been manipulated by restoration practices, and that microbial communities changed in parallel with temporal changes in biogeochemical conditions, as ecosystem function developed in the restoration sites. The presence or absence of sediment organic matter and sediment particle size composition were important drivers of microbial community structure in restoration sites. Our study of microbial community status using rapid comparative measures of relative abundance, when evaluated in the context of relevant environmental variables, provides insight on the status of restoration sites in the early stages of macrophyte community development, relative to the intact ecosystem.

These analyses demonstrate that abiotic and microbial community structure are altered during restoration. Future research using next generation sequencing technology is a clear next step and would more quantitatively evaluate measures of microbial relative abundance and functional genes analysis. Knowledge of changing microbial communities and the composition of those communities will help clarify the reciprocal relationships of microbes and the biogeochemical environment in developing (or declining) seagrass meadows.

Acknowledgements. The work was supported by Biscayne National Park and Florida International University. We thank D. Mills, S. Rosales, A. Simoes-Correa, M. Cuvelier, R. Welsh, M. Tongue, E. McGrath, D. Crossett, C. Johnson, K. Slattery, J. Martens, P. Parker, J. Drake, A. Perez, G. Puig-Santana, and R. Decker for assistance and advice. Funding was also provided by an NSF grant # OCE-0960937 to R.V.T. This is contribution # 693 of the Southeast Environmental Research Center, Florida International University.

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Editorial responsibility: Tom Battin, Vienna, Austria

Submitted: March 6, 2014; Accepted: October 6, 2014
Proofs received from author(s): December 5, 2014