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INCREASED VARIABILITY OF MICROBIAL COMMUNITIES IN RESTORED SALT MARSHES NEARLY 30 YEARS AFTER TIDAL FLOW RESTORATION

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Abstract

We analyzed microbial diversity and community composition from four salt marsh sites that were impounded for 40-50 years and subsequently restored and four unimpounded sites in southeastern Connecticut over one growing season. Community composition and diversity were assessed by terminal restriction fragment length polymorphism (TRFLP) and sequence analysis of 16S ribosomal RNA (rRNA) genes. Our results indicated diverse communities, with sequences representing 14 different bacterial divisions. *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* dominated clone libraries from both restored and unimpounded sites. Multivariate analysis of the TRFLP data suggest significant site, sample date and restoration status effects, but the exact causes of these effects are not clear. Composition of clone libraries and abundance of bacterial 16S rRNA genes were not significantly different between restored sites and unimpounded sites, but restored sites showed greater temporal and spatial variability of bacterial communities based on TRFLP profiles compared to unimpounded sites and variability was greatest at sites more recently restored. In summary, our study suggests there may be long-lasting effects on stability of bacterial communities in restored salt marshes and raises questions about the resilience and ultimate recovery of the communities after chronic disturbance.

Introduction

Many New England salt marshes altered by tidal restrictions and impoundments half a century ago have undergone extensive restoration over the past twenty years. A substantial effort has also gone into monitoring the recovery and evaluating the effectiveness of these restorations (see Warren et al. 2002; Crain et al. 2009). The vast majority of these studies, however, has focused on vegetation (Sinicrope et al. 1990; Roman et al. 2002), macroinvertebrates (Peck et al. 1994; Swamy et al. 2002), birds (Brawley et al. 1998; Benoit and Askins 1999) and fish species (Allen et al. 1994). Very little, if any, attention has been paid to recovery of community structure and diversity of microorganisms.

The high productivity of salt marshes supports an active and diverse microbial community, with microorganisms mediating most major nutrient transformations in the salt marsh. Additionally, salt marsh macrophytes have been shown to enhance microbial colonization and organic matter decomposition (Oliveira et al. 2010; Santos et al. 2007) and influence microbial community structure (Ravit et al. 2006; Ravit et al. 2007). Therefore, changes to the marsh are likely to have significant impacts on the microbial communities and the elemental cycles that they mediate. For example, changes in tidal flow to the marsh may reduce the supply of sulfate, and may cause a shift in the dominant carbon cycling process from sulfate reduction to methanogenesis, thus contributing to the accumulation of greenhouse gases (Valentine 2002). Additionally, changes in drainage from the marsh will effect changes in aeration to the sediment (Portnoy and Giblin 1997; LaRiviere et al. 2004), and may impact anaerobic respiration pathways, including sulfate reduction, methanogenesis, and denitrification. Thus, changes that may be brought about by tidal restriction and subsequent tidal flow restoration would likely be reflected in significant changes in the function of the marsh, brought about, in part, by changes in the microbial community structure and diversity.

Changes in microbial species composition might also be expected as the salinity changes since salinity can influence the abundance and community structure of bacteria (Bordalo 1993; Revilla et al. 2000). This is not surprising as salinity has been identified as one of the primary drivers of microbial community composition (Bouvier and del Giorgio 2002; Crump et al. 2004; Bernhard et al. 2005a; Bernhard et al. 2005b) and function (Bernhard et al. 2007). However, since many other environmental factors covary with salinity (such as nutrient concentrations), it is often difficult to identify a single variable as the effector of community changes.

Because microbes are responsible for much of the nutrient cycling, significant changes in the microbial community may have serious impacts on process rates in an ecosystem (Begon et al. 1990), resulting in changes to nutrient flow through the system. Within in an ecosystem, there are often mulitple populations that carry out the same process, known as metabolic redundancy, providing a level of insurance for the ecosystem in the event that some populations are lost due to changes in the environment. Thus, a loss of a particular population may lead to a loss of metabolic redundancy and may leave the community more vulnerable when challenged with environmental stressors. Empirical studies of microbial community stability and function have generally borne out these predictions (Hashsham et al. 2000; Maoz et al. 2003). Thus, microbial community diversity may serve as an indicator of ecosystem function and stability.

The focus of our research was to evaluate community structure and diversity of bacteria in restored salt marshes and unimpounded marshes. The research was carried out in the Wequetequock-Pawcatuck tidal marshes (locally referred to as Barn Island marshes) and the Cottrell Marsh of southeastern Connecticut. The Barn Island marsh system is composed of a series of valley marshes, each having undergone different management histories (Miller and Egler 1950). The four valley marshes sampled in this study were impounded by a dike in the 1940's to provide habitat for waterfowl. Seawater access to these impoundments has since been restored through a series of culverts, beginning in 1978. Recovery of the vegetation in impounded marshes has been documented, and in most cases, indicates a shift from *Typhus* and *Phragmites*-dominated marshes to *Spartina* spp. (Sinicrope et al. 1990). Many of the macroinvertebrate populations also appear to be recovering and are similar to populations in adjacent unimpounded reference marshes (Swamy et al. 2002). Similar patterns have been reported for fish populations and bird use of the marsh, all indicating a trajectory of recovery for the marshes. Given the data on other communities in the marsh and fast generation times of bacteria, we expected to find little evidence of the impact of the impoundment on the microbial communities. Although our findings suggested little difference in the taxonomic assemblage among restored and unimpounded sites, we detected long-term effects on the stability of the communities, which may have as yet undetermined effects on ecosystem function.

Methods

Site Description. Four sites that were impounded and subsequently restored were located in the Wequetequock-Pawcatuck tidal marshes, which cover 1.4 km^2, bounded on the west by Wequetequock Cove, on the east by the Pawcatuck River near the border of Rhode Island, and to the south by Little Narragansett Bay (Figure 1). The marsh is separated from Long Island Sound by a series of bars and islands. Tidal flow was restored to Impoundments 1 (IP1) and 2 (IP2) in 1978 by the installation of 1.5 m culverts. In 1982, an additional 2.1-m culvert was created in IP1 to more fully restore tidal access. Tidal flow to Impoundment 4 (IP4) was restored in 1987 with a 1.3-m culvert and to Impoundment 3 (IP3) in 1991 with a 1.5-m culvert. Headquarters (HQ) Marsh was selected as one of two reference sites in Barn Island due to its location just seaward of IP1 and IP2. However, HQ has undergone extensive vegetation changes, likely due to sea level rise (Warren and Niering 1993), so Wequetequock Cove (WE) was chosen as an additional reference site within the Barn Island marsh system. Two additional unimpounded sites located in the Cottrell Marsh (CO), 6 km west of the Wequetequock-Pawcatuck marshes, were also selected as reference sites from a nearby marsh that has never been impounded. More extensive descriptions of these marsh sites are found elsewhere (Warren and Niering 1993, Warren et al 2002).

Sample collection. Triplicate sediment cores (6.5 cm diameter) were taken from IP1, IP2, IP3, IP4, HQ, and WE marshes in June, July, and October 2006 and from CO-1 and CO-2 in July and October 2006. Plots in areas dominated (60-100% coverage) by the marsh grass *Spartina patens* were selected at each site. Each plot was designated with a 0.25 m^2 grid sectioned into 25 x 0.1 m squares. A random numbers table was used to select three squares within the grid in which to collect a core. Cores were placed on ice in the dark for transport back to the lab. In the lab, the top 0-2 cm section of each core was homogenized and 0.5 g aliquots were stored at -80°C for DNA processing and an additional 1-2 g of sediment was weighed for dry-weight determination. Wet sediment was dried overnight at 70°C and reweighed. Porewater from each core was obtained from the remaining sediment by centrifugation (5000 x g for 5 minutes) in 50 ml tubes with a 0.45 µm celluose acetate filter insert (Chrom Tech, Inc., Apple Valley, MN).

Physical and chemical sediment characterization. Salinity was measured from the porewater in each core using a hand-held refractometer, except during June when salinity was measured on site (not from individual cores). pH was also measured from each core using a pH100 meter with a piercing probe (YSI, Yellow Springs, OH). Water content of the sediment was determined by the change in weight of sediment after drying at 70°C overnight.

DNA extraction. We extracted DNA from the 0.5 g aliquots using the PowerSoil™ DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's recommendations. The quantity and quality of the DNA was evaluated by measuring the optical density at 260 and 280 nm using a SmartSpec Plus spectrophotometer (BioRad, Hercules, CA) and by gel electrophoresis in a 1% agarose gel with 1 µg/ml of ethidium bromide. Samples that had a 260:280 ratio less than 1.5 were thrown out and another aliquot was extracted.

Real-time PCR of Bacterial 16S rRNA genes. Bacterial 16S rRNA genes were quantified using primers GM3 and 338R as described in Könneke et al. (2005). All reactions were run in an iCycler (BioRad) using SYBR Green I master mix (BioRad), 0.5 µM of each primer, and 0.008% BSA with the following amplification cycle parameters: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 sec, 55°C for 20 sec, 72°C for 30 sec. Melt curve analysis was performed after each experimental run to confirm the product specificity. Sample amplification was compared to a standard curve generated in each experimental run using five standards ranging in DNA concentration from 1 fg/µl to 10 pg/µl, which is equivalent to 1.8 x 10^2 to 1.8 x 10^6 gene copies/µl. Standards were a mix of 29 different purified plasmid DNAs from clones generated from bacterial 16S rRNA genes recovered previously from salt marsh sediments (Nielsen et al. 2004) representing 11 different bacterial divisions. Average amplification efficiency was 91.7 ± 4.5%. Effects of inhibition during PCR were previously tested on the same DNA samples and we determined that a 1:10 dilution was optimal for amplification with minimal inhibition (Moin et al. 2009).

TRFLP analysis. Bacterial 16S rRNA genes were amplified using the Bacterial-specific primer 27F (Lane 1991) and the universal primer 1492R (Lane et al. 1985). The forward primer (27F) was fluorescently labeled with 6-FAM (Operon Technologies, Huntsville, AL). Each 20 µl PCR contained 10 µl of 2X iQ Supermix (BioRad), 0.5 µM each primer and 1 µl of template DNA, diulted 1:10 with water to reduce interference from inhibitors. An iCycler (BioRad) was used for all reactions with the following cycle sequence: 94°C for 10 min., followed by 30 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 2 m, with a final 5 minute extension at 72°C. PCR products were evaluated following electrophoresis on 1% agarose gels and comparison of the band intensities and migration distances to a low DNA mass ladder (Invitrogen, Carlsbad, CA).

PCR products were digested with 10 units of *Msp*I (New England Biolabs, Beverly, MA) overnight at 37°C. Following ethanol precipitation, samples were resuspended in 10µl of deionized H_2O. To prepare samples for analysis, 3 µl of each sample, 0.2 µl of the internal size standard, GS500-ROX (Applied Biosystems Inc., Fremont, CA), and 10 µl of Hi-Di Formamide (ABI) were mixed and sent to the Biotechnology Resource Center at Cornell University (http://cores.lifesciences.cornell.edu/brcinfo/) for analysis on an Applied BioSystems 3730xl DNA Analyzer. Terminal restriction fragment (TRF) sizes and relative abundances were estimated using GeneMarker software, v.1.4 (SoftGenetics, State College, PA). We determined the range of reliable TRF size estimates to range from 67 to 500 bp based on amount of background and reproducibility among replicates. Peak heights were normalized to account for differences in the amount of DNA analyzed using the method from Dunbar et al. (2000).

Clone library construction. Bacterial 16S rRNA genes were amplified from samples collected in June 2006 from sites IP1, IP2, IP3, IP4, WE, and HQ and from all eight sites in July 2006 using the same primers as above, but the 27F primer was unlabeled. PCR products were cloned using the StrataClone PCR Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Transformants were randomly selected and inoculated into 100 µl LB broth with 100 µg ml^{-1} ampicillin in 96-well microtiter plates. All plates were incubated overnight at 37°C. Clones were screened for the presence of correctly-sized inserts by PCR using vector-specific primers T3 and T7. Clones containing the correct insert were sequenced by High Throughput Sequencing Solutions (University of Washington, Department of Genome Sciences, Seattle, WA) using the Bacterial-specific primer 700R (Urbach et al. 2001).

Sequence analysis. Sequences were aligned using the sequence editor and Fast Align in ARB (Ludwig et al. 2004) and checked manually. Phylogenetic affiliations of individual sequences were based initially on analysis by the Ribosomal Database Project (Wang et al. 2007) and were confirmed by phylogenetic tree construction using the neighbor-joining algorithm in ARB. We compared the placement of each sequence in trees constructed using 256 bases at the 5' end and the 3' end of the sequences to identify potential chimeras. Sequences showing evidence of possible chimeric structure were removed from the analysis. A total of 553 sequences was analyzed, ranging from 48-95 sequences per site. We determined predicted TRF sizes for all sequences in silico.

Statistical analysis. All multivariate analyses were performed using PC-Ord version 6 (McCune and Mefford 1999). The relative abundance data were transformed by an arcsine square root function to reduce skew. Non-metric multidimensional scaling (NMS) (Kruskal 1964) was used to ordinate samples in gene fragment space, using the Sørenson’s distance measure. The autopilot option was set to the slow and thorough level for all ordinations. Dimensionality (the optimal number of dimensions or axes required to explain a sufficient proportion of the variance) was assessed by choosing the number of axes that minimized final stress and maximized interpretability of the results. Monte Carlo tests were run to confirm that results obtained were significantly better than would be obtained from randomized data. Additionally, the proportion of variance explained by each axis and the cumulative variance explained was determined by calculating the coefficient of determination between distances in ordination space and distances in the original p-dimensional space. Correlation coefficients in the ordination space were determined for each environmental variable and TRF by rotating the ordination to maximize the coefficient on one axis (varimax rotation) in order to facilitate detecting clusters of samples (McCune and Grace 2002).

Multi-response perumuation procedure (MRPP), a nonparametric test, was used to test for differences between restored and unimpounded sites, among different sites, and among sampling dates. MRPP is a variant of ANOSIM (Analysis of Similarity) and provides a measure of the effect and p value when testing for differences between two or more groups defined by the user (McCune and Grace 2002).

Coverage of each clone library was calculated with the equation: $C = 1-(n/N)$ where n = number of singleton sequences and N = total number of sequences analyzed. Differences among libraries were evaluated by ∫-Libshuff , AMOVA (analysis of molecular variance), and HOMOVA (homogeneity of molecular variance) analyses using the program mothur (Schloss et al. 2009). These three methods provide a complementary analysis to detect differences among communities (Schloss 2008). AMOVA determines whether the genetic diversity among the communities is greater than their pooled genetic diversity. HOMOVA determines whether the amount of genetic diversity within each community is significantly different (Schloss 2008). ∫-Libshuff is based on the Cramér-von Mises-type statistic and uses a Monte Carlo procedure to compare differences based on pair-wise comparisons (Singleton et al. 2001; Schloss 2008). We ran pair-wise comparisons of clone libraries generated from samples collected in July since we did not sample the Cottrell Marsh in June. To account for experiment-wise error, we applied the Bonferroni correction to the results. For pair-wise comparisons among all 8 libraries, p values less than 0.00092 were considered significant. We also ran a comparison of combined libraries from all four restored sites and all four unimpounded sites. Diversity indices were calculated from TRFLP data (evenness, richness, Shannon-Weiner, and Simpson's) using PC-Ord and from sequence data (Chao1) using mothur.

Nucleotide sequences for Bacterial 16S rRNA genes can be found under the following Genbank accession numbers: JN684211 - JN684753.

Results

Although there were no significant differences between restored and unimpounded sites for salinity, pH, or water content, we identified patterns that corresponded to restoration status (Table 1). For example, among the restored sites, salinity was generally lower at sites more recently restored compared to sites that were restored earlier. Additionally, pH was lower at the unimpounded sites compared to the restored sites in July, but the pattern was not consistent on other sampling dates.

Relationships among the environmental variables suggest some co-variation. Percent water content was positively and significantly correlated with pH (Pearson's correlation coefficient, $r = 0.53$, $p < 0.00001$) and salinity ($r = 0.38$, $p = 0.003$), but no significant correlation was detected between salinity and pH ($r = 0.13$, $p = 0.35$).

Bacterial 16S rRNA gene abundance ranged from 1.6×10^{9} to 2.4×10^{11} copies g sediment (dry weight)$^{-1}$. However, no significant differences were detected between restored sites and unimpounded sites (Figure 2).

Phylogenetic analysis of 553 Bacterial 16S rRNA gene clones revealed sequences affiliated with 14 bacterial divisions (Figure 3, Table 3). Coverage of clone libraries was similar among all libraries (21.6 ± 6.5 and 23.3 ± 6.4% for restored and unimpounded sites, respectively). The most frequently recovered clones were affiliated with the *Gammaproteobacteria*, *Bacteroidetes*, and *Planctomycetes*. Other frequently encountered divisions included *Acidobacteria*, *Deltaproteobacteria*, *Verrucomicrobia*, and *Chloroflexi*. Interestingly, the taxonomic composition in restored and unimpounded sites was very similar (Figure 3). These results were confirmed by the limited number of significant differences among gene clone libraries based on ∫-Libshuff, AMOVA, and HOMOVA analyses (Table 2). Most of the significant differences detected involved comparisons of libraries with IP3 and IP4, which are the most recently restored sites.

TRFLP analysis was performed on all DNA samples to characterize the bacterial communities. A total of 224 TRFs was detected among all the samples, ranging in size from 67 bp to 500 bp. Sequence analysis of bacterial 16S rRNA gene clones was used to identify presumptive phylogenetic affiliations of each TRF. In some cases, sequences of different phylogenetic affiliations represented a single TRF and not all TRFs were represented in the clone libraries. Of the 224 TRFs, 131 (58.4%) matched predicted TRFs from sequenced 16S rRNA gene clones.

We applied nonmetric multidimensional scaling (NMS) analysis to identify patterns among the bacterial communities based on the TRFLP profiles. The first two axes of the ordination explained 54.1% of the variability among the communities (Figure 4). Although some bacterial communities found at restored and unimpounded sites appeared to be similar, restored sites were more variable compared to unimpounded sites. The average distance between samples among restored sites was significantly higher compared to samples from unimpounded sites (Student's t-test, $p = 0.01$). When the two outlier samples were removed, the effect was still significant ($p = 0.04$).

MRPP (multi-response permutation procedure) confirmed a significant restoration effect, and identified even stronger effects based on site and sampling date (Table 4). Interestingly, at the restored sites, there was a directional and synchronous temporal shift in the communities from June to October, with the exception of two samples from October at sites IP1 and IP4, but no temporal shifts were detected among the unimpounded sites (Figure 5). Within each site, however, there were distinct temporal differences.

Measures of diversity based on TRFLP data revealed very little difference among sites, and there were no significant restoration effects on any of the diversity indices (Table 5). However, there were differences in the variability of diversity within the sites. Average coefficients of variation were always higher at restored sites compared to unimpounded sites. Although the differences were not significant, the trends were consistent. Additionally, variability was highest at IP3 and IP4, the most recently restored marshes. Chao1 estimates of diversity based on sequence data also showed no significant difference between restored and unimpounded sites (104.8 ± 33.9 and 118.5 ± 69.4, respectively).

Discussion

Analysis of bacterial communities in restored and unimpounded salt marsh sediments by DNA fingerprints and gene sequences revealed that although the communities were, in some cases, very similar, communities at the restored sites were more variable compared to communities at unimpounded sites. We hypothesized that because recovery in the marsh has been underway for nearly 30 years and given the relatively fast generation times of bacteria we would not detect any significant differences in microbial communities between restored and unimpounded marshes. However, several other studies of recovery in coastal ecosystems have reported significant differences in hydrologic and edaphic conditions (Onaindia et al. 2001) or phosphorus cycling (Herbert and Fourqurean 2008) after 2 decades of recovery. Our data, in combination with these studies, suggest that although the marsh may appear to be recovered and comparable to pre-disturbance conditions based on vegetation and macroorganism populations, the less visible components, such as nutrient cycles and microbial communities, may not be as resilient. Allison and Martiny (2008) reported that most microbial groups are sensitive to disturbance and not immediately resilient, regardless of taxonomic breadth or type of disturbance.

In support of our initial hypothesis, taxonomic composition of clone libraries suggest little difference between restored and unimpounded sites. Sequence identification of major groups of bacteria indicated diverse bacterial communities, with few differences in the proportion of clones from represented groups among sites. The dominance of *Gammaproteobacteria* and *Bacteroidetes* was not unexpected, as members of these bacterial groups are common in coastal environments (e.g. Bouvier and del Giorgio 2002; Bernhard et al. 2005) and are known to play important roles in carbon mineralization and decomposition. In some cases, members of *Gammaproteobacteria* have been found to account for up to 28% of total sediment DNA (Hardwick et al. 2003). Similarly, *Verrucomicrobia* and *Planctomycetes* are also common members of aquatic communities (Fuerst 1995; Glockner et al. 1999; Urbach et al. 2001). Many of the known sulfate-reducing bacteria are affiliated with *Deltaproteobacteria*, and thus would be expected to be a part of salt marsh bacterial communities where sulfate is often abundant. *Acidobacteria* are common inhabitants of soil communities, so their presence in coastal sediment communities should not be surprising. Unfortunately, most of what is known about *Acidobacteria* comes from molecular studies, which provide little insight into their metabolic function or ecology. In general, results from our clone libraries are consistent with the TRFLP data and salt marsh ecology, but offer little insight into specific microbial populations that might be critical players in the return to communities that are comparable to unimpounded marsh communities.

We were initially surprised by the highly congruent composition of clone libraries from restored and unimpounded sites. However, our clone library results were corroborated by the highly similar diversity indices based on the TRFLP analyses. In fact, at first glance, it would appear that the microbial communities at restored and unimpounded sites are not different until one considers differences in variability.

Increased variability at the restored sites may reflect more variable conditions at these sites, or alternatively, the bacterial communities have not reached a stable state after chronic disturbance (impoundment) and subsequent restoration of tidal inundation. Our data do not indicate significant differences in salinity, pH, or water content of the sediment, but it is certainly possible that the sites may differ in other parameters such as nutrient concentrations or redox potential. Porewater nitrate and ammonium concentrations from some sites and sampling dates suggest no significant differences among sites (Bernhard, unpublished), but the available data on nutrients and redox for these sites are limited.

Variability at the restored sites may also be related to differences in landscape patterns brought about by impoundment. For example, Swamy et al. (2002) state that although IP1 is in an advanced stage of recovery after 21 years of restored tidal inundation, there are still some attributes, such as marsh elevation, that differ from marshes that have never been impounded. Zedler and Kercher (2005) argue further that restoration can reverse some degradation, but other damages may be irreversible, particularly attributes such as marsh elevation and slope that can then impact other abiotic and biotic components. For instance, changes in the elevation can significantly impact hydroperiods in the marsh sediment that would impact important biogeochemical functions mediated primarily by microbes, including denitrification, sulfate reduction, and methanogenesis. The further the community shifted from pre-impoundment community composition, the longer it may take for the community to stabilize once salt water flow was restored. Frequent disturbances have been found to reduce recovery potential and increase the variability of the system (Odum 1985; Collins et al. 2001).

The high variability of bacterial communities at the restored sites in Barn Island may also indicate a more flexible community that can respond to changing conditions and may represent an alternative state, as described by Denslow (1985), in which interactions of species and responses to disturbances are altered from the pre-disturbance condition. If the post-restoration environment is more variable compared to the unimpounded marsh sites, the less stable microbial communities at the restored sites may actually be more resilient (Holling 1973). Fraterrigo and Rusak (2008) suggest that variability, although not typically used as a recovery metric, may actually be a sensitive metric for disturbance. Ayala del Rio et al (2004) also suggest that some microbial communities are dynamic and never reach a climax community, but rather are "shifting mosaics." McCune and Cottam (1985) also reported this phenomenon for forest communities. More dynamic community structures may lead to more functionally stable communities (Fernandez et al. 2000). Our study, however, measured only community composition, and not function.

It has been suggested that regular disturbances can increase community stability (Ayala-Del-Rio et al. 2004; Grman et al. 2010). Therefore, it is possible that the regular disturbance caused by tidal inundation helps to maintain the stable communities observed at the unimpounded sites. Because tidal inundation was disrupted for 40-50 years in the impounded sites, community stability may have been significantly disrupted, and may take much longer to return. Although tidal inundation and impoundment may both be considered disturbances, they operate on very different spatial and temporal scales, so their effects may be reflected in very different community dynamics.

Given the significant differences in stability of microbial communities between restored and unimpounded sites in our study, one might predict differences in diversity as well. Studies have reported positive links between microbial diversity and stability (Naeem and Li 1997; Grman et al. 2010). Our results, however, suggest that diversity may recover more quickly than stability, leading to an uncoupling of diversity and stability. Finlay et al. (1997) argue that microbial diversity is unimportant because communities harbor many rare or cryptic species that are just waiting for new niches to open as conditions change. However, it is also possible that community composition is more important than diversity alone in driving community stability after perturbation (Griffiths et al. 2004).

Many studies of microbial communities have identified salinity as a major factor in driving microbial diversity and community composition in estuaries (Bouvier and del Giorgio 2002; Crump et al. 2004; Bernhard et al. 2005; Bernhard et al. 2005), so we expected salinity to be important in the community composition in the restored marshes. However, salinity patterns did not correlate with restoration status, suggesting that other factors exert greater influence on microbial community structure in these marshes. Swamy et al. (2002) identified salinity as one of the key factors driving the biology of restoration in Barn Island, but found that it did not account for all the differences in recovery rates detected in different marshes in Long Island Sound. Multivariate analysis of bacterial communities in our study indicated differences among the sites, but no single environmental variable was identified as a major driver of the observed differences. Additionally, significant effects of site, sampling date, and restoration status on bacterial community composition were detected by MRPP analysis, making it difficult to isolate specific environmental factors that may contribute to these effects.

We find it intriguing that the temporal shifts appear to be directional and synchronous among the restored sites while communities at unimpounded sites appear more temporally stable. The causes of the temporal patterns in restored sites and the lack of such patterns in unimpounded sites are not clear. It is possible that the microbial communities are more sensitive than communities in the unimpounded sites to changes in environmental conditions, such as salinity, pH, or temperature. We did find differences in salinity and pH that correlated with sampling dates. Temporal shifts in communities at restored sites may also help to explain increased variability among samples from these sites compared to those from unimpounded sites. However, in another New England salt marsh, distinct temporal patterns were not detected in control sites or fertilized sites (Bowen et al. 2009), suggesting that salt marsh microbial communities may not generally show pronounced temporal shifts.

In conclusion, our results indicate that after nearly 30 years of restored tidal flow, microbial community composition in restored sites was not significantly different from undisturbed sites, but there were significant differences in community stability. Because our study focused on surface sediments from one impounded marsh system, it is uncertain how broadly the effects we detected may be distributed in other marshes. Future studies on marshes undergoing restoration should include a microbial community component to determine the extent of restoration impacts. Furthermore, whether more variable microbial communities lead to changes in microbial processes in the marsh, such as nutrient cycling, has yet to be investigated. We believe this is a critical next step.

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Table 1. Average values (± standard deviation) of sediment chemistry parameters from Barn Island and Cottrell salt marsh sites.

Site	Month	Salinity (psu)	pH	% water content
CO1	Jun	nd	nd	nd
	Jul	21.3 ± 1.8	5.97 ± 0.01	0.73 ± 0.03
	Oct	25.0 ± 0.0	6.84 ± 0.05	0.52 ± 0.09
CO2	Jun	nd	nd	nd
	Jul	30.8 ± 3.4	5.78 ± 0.18	0.72 ± 0.04
	Oct	33.2 ± 0.3	6.43 ± 0.07	0.51 ± 0.01
HQ	Jun	nd	5.66 ± 0.30	0.67 ± 0.02
	Jul	23.0 ± 1.4	5.54 ± 0.42	0.65 ± 0.03
	Oct	30.0 ± 0.0	6.28 ± 0.02	0.57 ± 0.02
WE	Jun	10.0[a]	6.37 ± 0.02	0.54 ± 0.10
	Jul	16.7 ± 1.5	5.50 ± 0.49	0.75 ± 0.02
	Oct	25.0 ± 0.0	6.49 ± 0.01	0.57 ± 0.04
IP1	Jun	18.0[a]	6.22 ± 0.18	0.57 ± 0.07
	Jul	24.8 ± 0.4	5.86 ± 0.08	0.53 ± 0.01
	Oct	32.0 ± 0.7	6.51 ± 0.04	0.53 ± 0.04
IP2	Jun	13.0[a]	6.42 ± 0.11	0.67 ± 0.08
	Jul	22.7 ± 0.6	6.16 ± 0.58	0.68 ± 0.03
	Oct	30.8 ± 1.0	6.43 ± 0.09	0.49 ± 0.01
IP3	Jun	10.0[a]	6.44 ± 0.02	0.67 ± 0.02
	Jul	20.7 ± 1.4	6.15 ± 0.11	0.73 ± 0.07
	Oct	25.3 ± 0.7	6.65 ± 0.01	0.56 ± 0.05
IP4	Jun	11.0[a]	6.10 ± 0.00	0.60 ± 0.02
	Jul	16.3 ± 1.5	6.07 ± 0.02	0.66 ± 0.03
	Oct	25.0 ± 1.0	6.47 ± 0.04	0.39 ± 0.10

[a] Salinity values in June were taken on site from a single reading in situ.

Table 2. Results from AMOVA, ∫-Libshuff, and HOMOVA analyses of Bacterial 16S rRNA gene clone libraries. All pairwise comparisons for individual sites were conducted, but only pairs of individual sites that were significantly different are shown.

Statistical Test	Comparison	P value
AMOVA [a]	IP3 vs IP4	0.001
	IP3 vs WE	<0.001
	IP1 vs IP4	<0.001
	unimpounded vs restored[b]	0.279
∫-Libshuff [c]	WE-CO1	0.0005 (XY), 0.0036 (YX)
	IP3-WE	0.0002 (XY), 0.0117 (YX)
	unimpounded vs restored	0.7825 (XY), 0.0009 (YX)
HOMOVA	unimpounded vs restored	0.882

[a] $P \leq 0.00178$ were considered significant based on the Bonferroni correction for multiple comparisons.

[b] Sequences from the four unimpounded sites and the four restored sites were combined.

[c] Only when both comparisons (XY and YX) are significant are the two clone libraries considered to be significantly different in composition. To be considered signifcant, $p \leq 0.00092$ based on the Bonferroni correction for mulitple comparisons (this is lower than the cutoff for AMOVA or HOMOVA because ∫-Libshuff makes two comparisons for each pair of libraries).

Table 3. Percentage of sequences in each taxonomic group recovered from each site.

Taxonomic Group	CO1	CO2	HQ	WE	IP1	IP2	IP3	IP4
Acidobacteria	0.06	0.06	0.06	0.08	0.10	0.07	0.09	0.14
Actinobacteria	0.02	0.00	0.04	0.01	0.00	0.00	0.03	0.06
Alphaproteobacteria	0.06	0.00	0.06	0.07	0.03	0.03	0.04	0.08
Bacteroidetes	0.19	0.08	0.16	0.25	0.17	0.19	0.14	0.14
Betaproteobacteria	0.00	0.00	0.00	0.01	0.03	0.01	0.01	0.04
Chloroflexi	0.06	0.13	0.04	0.04	0.12	0.07	0.09	0.02
Cyanobacteria	0.02	0.15	0.01	0.03	0.06	0.12	0.06	0.04
Deinococcus	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.00
Deltaproteobacteria	0.15	0.04	0.01	0.17	0.07	0.09	0.08	0.12
Gammaprotebacteria	0.19	0.25	0.26	0.18	0.16	0.23	0.13	0.20
Gemmamondales	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00
Nitrospira	0.00	0.00	0.01	0.00	0.03	0.01	0.03	0.01
OP11	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
Planctomycetes	0.19	0.25	0.19	0.10	0.19	0.12	0.24	0.08
Plastid	0.02	0.00	0.01	0.00	0.01	0.05	0.03	0.00
TM7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Verrucomicrobia	0.04	0.00	0.07	0.03	0.03	0.01	0.05	0.06
Number of clones	48	48	68	71	69	75	79	95

Table 4. Results from multiresponse permutation procedure (MRPP) based on TRFLP fingerprints. Grouping variables were used to group the samples according to site, sample date and restoration status (either restored or unimpounded).

Grouping variable	A[a]	T[b]	Observed δ[c]	Expected δ	P value[d]
Site	0.030	-5.42	0.571	0.588	<0.0001
Sample date	0.014	-5.25	0.579	0.588	<0.0001
Restoration status	0.004	-2.14	0.585	0.588	0.032

[a] A is the intragroup average distance; when all items are identical within groups, A=1

[b] T = (δ-m)/s = (observed – expected)/s. dev. of expected, where m and s are the mean and standard deviation of δ under the null hypothesis

[c] Observed δ is the average of the observed intragroup distances weighted by relative group size

[d] P is the probability of a smaller or equal δ

Table 5. Average diversity indices ± one standard deviation for restored and unimpounded sites. Average coefficients of variation ± one standard deviation are shown parenthetically. Indices were based on TRFLP data and are averaged over all dates and sites.

Diversity index	**Restored sites**	**Unimpounded sites**
Richness[a]	63.6 ± 1.7 (9.2 ± 2.2)	63.4 ± 1.1 (6.8 ± 1.7)
Evenness[b]	0.984 ± 0.001 (0.24 ± 0.08)	0.985 ± 0.001 (0.22 ± 0.09)
Shannon-Weiner (H)	4.083 ± 0.03 (2.4 ± 0.7)	4.085 ± 0.02 (1.7 ± 0.4)
Simpson's (D)	0.982 ± 0.0007 (0.20 ± 0.08)	0.982 ± 0.0005 (0.14 ± 0.04)

[a] the number of terminal restriction fragments detected in a sample

[b] H/ln(richness), where $H = \sum P_i (\ln P_i)$, where P_i = the proportion of each TRF in a sample

Figure Captions

Figure 1. Map of sampling sites in the Cottrell (a) and Wequetequock-Pawcatuck (b) marshes in Southeastern Connecticut. IP = Impoundments (numbered 1-4); WE = Wequetequock Marsh; HQ = Headquarters Marsh; CO = Cottrell Marsh (numbered 1-2).

Figure 2. Average abundance (± standard error) of Bacterial 16S rRNA genes in marsh sediment samples. Site abbreviations are the same as in Figure 1.

Figure 3. Distribution of Bacterial 16S rRNA gene clones from restored and unimpounded marshes. A total of 235 sequences was analyzed from the unimpounded sites and 318 sequences from the restored sites.

Figure 4. Non-metric multidimensional scaling ordination of samples based on TRFLP analysis of the Bacterial 16S rRNA genes amplified from restored and unimpounded marshes. Site abbreviations are the same as in Figure 1. The ordination was rotated to maximize separation based on restoration status. Open symbols represent restored sites, closed symbols represent unimpounded sites.

Figure 5. Non-metric multidimensional scaling ordination of samples from restored (panel a) and unimpounded (panel b) marshes. The ordination has been rotated slightly from that depicted in Figure 4 to maximize the separation based on sampling date. Samples collected from each sampling date are circled. Numbers next to the symbols in panel A indicate the Impoundment from which the samples were collected.

Figure 1

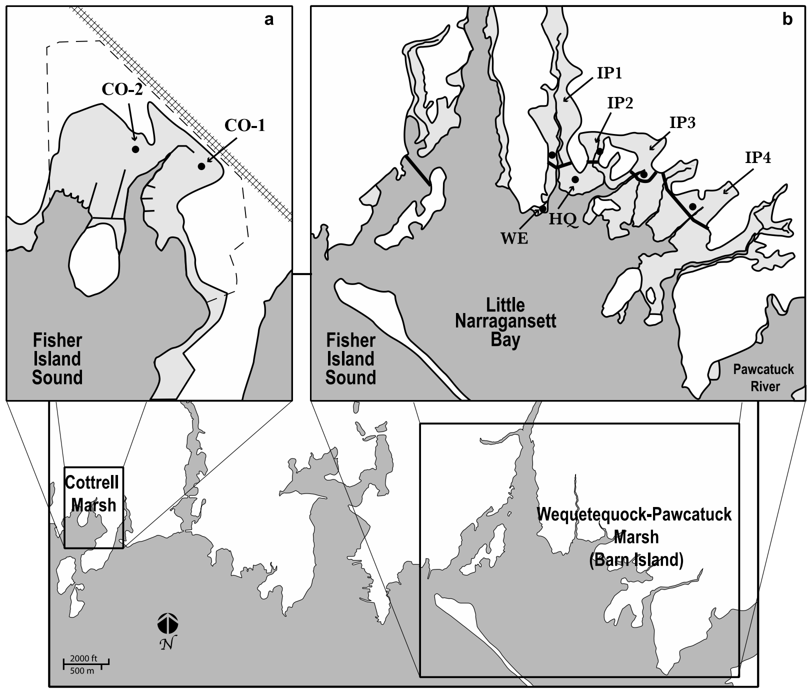
a
CO-2
CO-1
Fisher Island Sound
b
IP1
IP2
IP3
IP4
HQ
WE
Little Narragansett Bay
Fisher Island Sound
Pawcatuck River
Cottrell Marsh
Wequetequock-Pawcatuck Marsh (Barn Island)
N
2000 ft
500 m

Figure 2

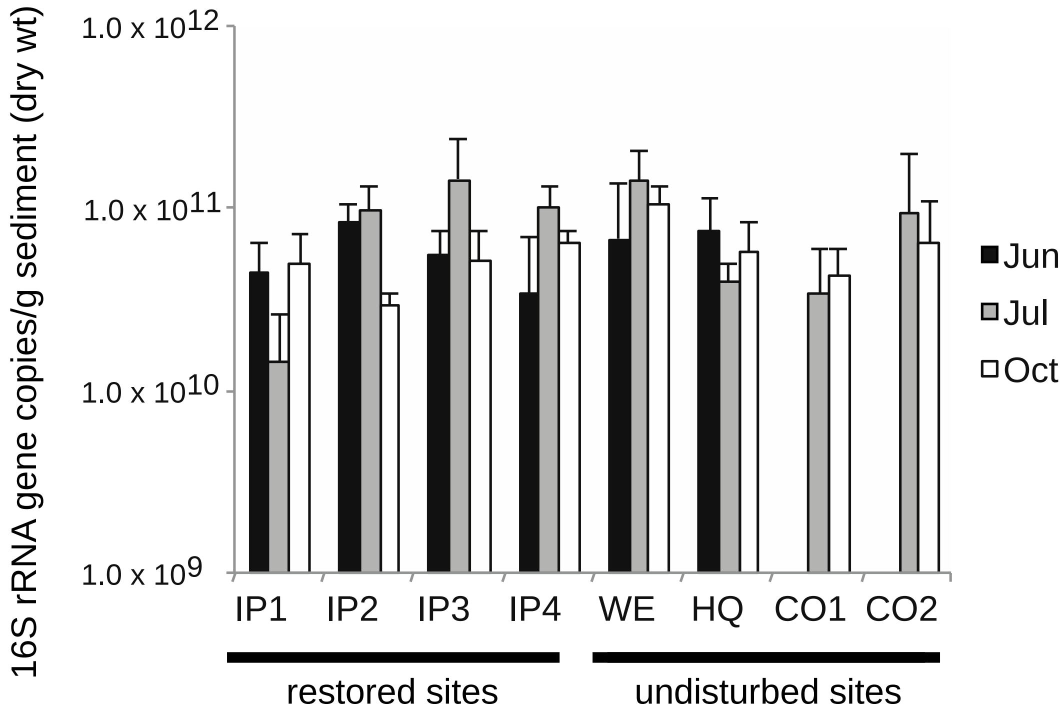
16S rRNA gene copies/g sediment (dry wt)
1.0 x 10^12
1.0 x 10^11
1.0 x 10^10
1.0 x 10^9
IP1
IP2
IP3
IP4
WE
HQ
CO1
CO2
restored sites
undisturbed sites
Jun
Jul
Oct

Figure 3

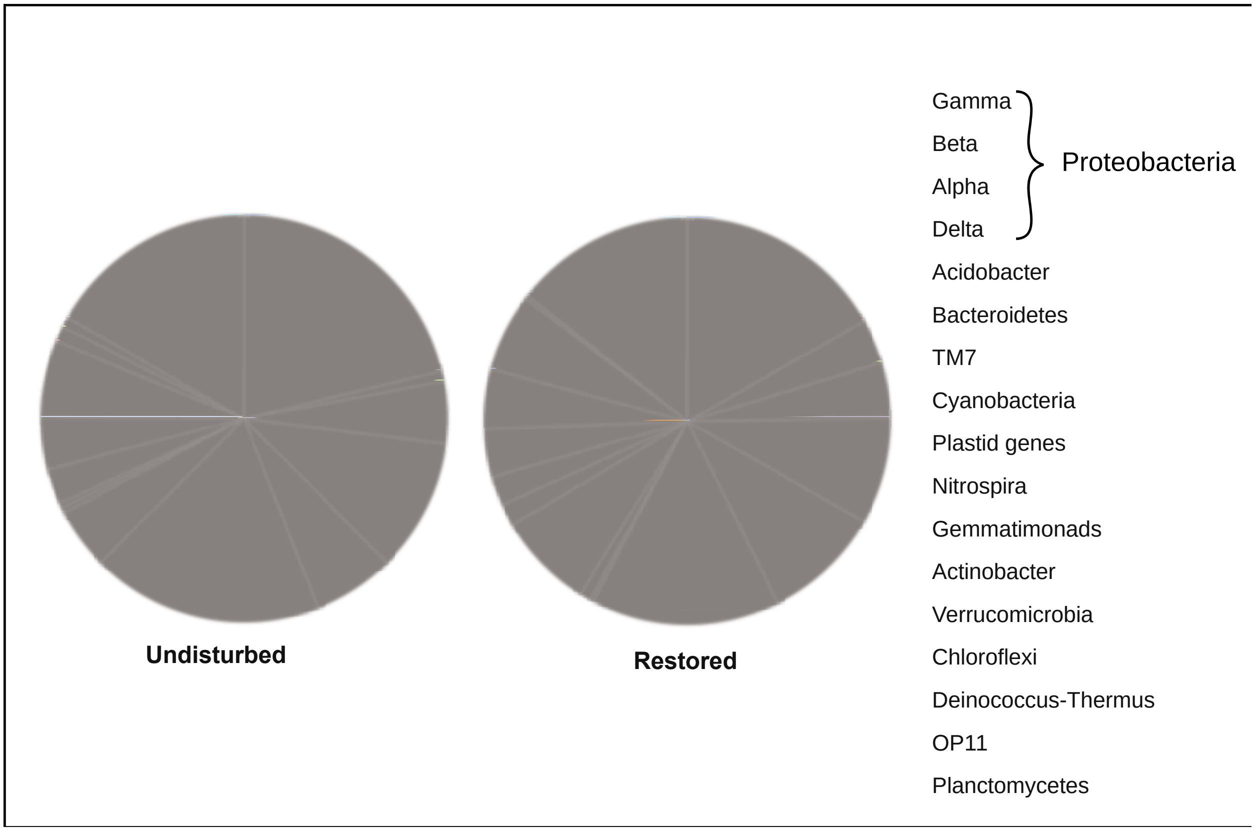
Gamma
Beta
Alpha
Delta
Proteobacteria
Acidobacter
Bacteroidetes
TM7
Cyanobacteria
Plastid genes
Nitrospira
Gemmatimonads
Actinobacter
Verrucomicrobia
Chloroflexi
Deinococcus-Thermus
OP11
Planctomycetes
Undisturbed
Restored

Figure 4

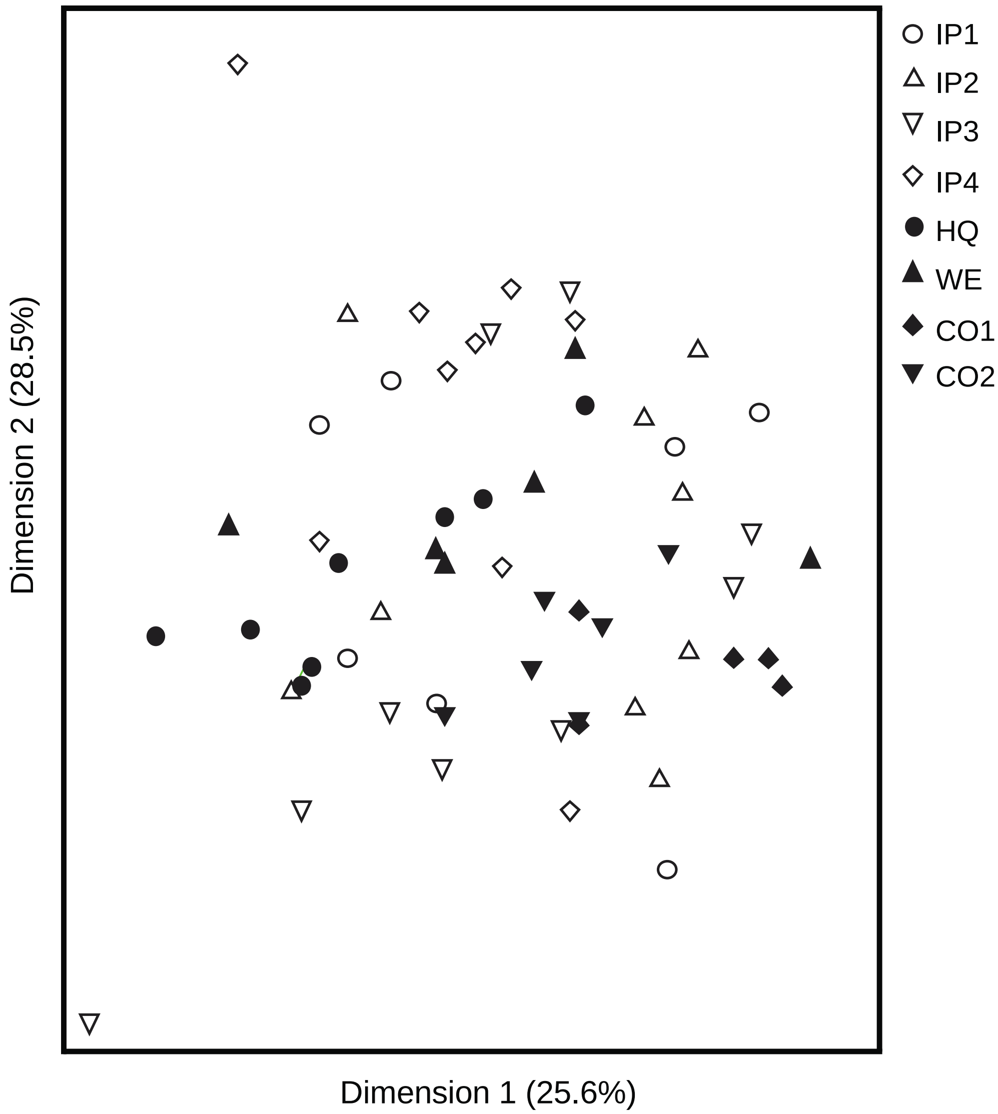

Figure 5