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RESEARCH

Estuarine Sediment Microbiomes from a Chronosequence of Restored Urban Salt Marshes

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Abstract
Salt marshes play an important role in the global nutrient cycle. The sediments in these systems harbor diverse and complex bacterial communities possessing metabolic capacities that provide ecosystem services such as nutrient cycling and removal. On the East Coast of the USA, salt marshes have been experiencing degradation due to anthropogenic stressors. Salt marsh islands within Jamaica Bay, New York City (USA), are surrounded by a large highly urbanized watershed and have declined in area. Restoration efforts have been enacted to reduce further loss, but little is known about how microbial communities develop following restoration activities, or how processes such as nitrogen cycling are impacted. Sediment samples were collected at two sampling depths from five salt marsh islands to characterize the bacterial communities found in marsh sediment including a post-restoration chronosequence of 3–12 years. We used 16s rRNA amplicon sequencing to define alpha and beta diversity, taxonomic composition, and predicted metabolic profile of each sediment sample. We found significant differences in alpha diversity between sampling depths, and significant differences in beta diversity, taxonomic composition, and predicted metabolic capacity among the five sampling locations. The youngest restored site and the degraded natural sampling site exhibited the most distinct communities among the five sites. Our findings suggest that while the salt marsh islands are located in close proximity to each other, they harbor distinct bacterial communities that can be correlated with post-restoration age, marsh health, and other environmental factors such as availability of organic carbon.
Importance Salt marshes play a critical role in the global nutrient cycle due to sediment bacteria and their metabolic capacities. Many East Coast salt marshes have experienced significant degradation over recent decades, thought largely to be due to anthropogenic stressors such as nitrogen loading, urban development, and sea-level rise. Salt marsh islands in Jamaica Bay (Queens/Brooklyn NY) are exposed to high water column nitrogen due to wastewater effluent. Several receding marsh islands have been subjected to restoration efforts to mitigate this loss. Little is known about the effect marsh restoration has on bacterial communities, their metabolic capacity, or how they develop post-restoration. Here, we describe the bacterial communities found in marsh islands including a post-restoration chronosequence of 3–12 years and one degraded marsh island that remains unrestored. We found distinct communities at marsh sites, despite their geographic proximity. Differences in diversity and community composition were consistent with changes in organic carbon availability that occur during marsh development, and may result in differences in ecosystem function among sites.

Keywords 16s rRNA · Microbial community · Restoration · 16s barcoding · Nitrogen cycling

Introduction

Microbial communities drive essential ecosystem services in estuaries and salt marshes, most significantly via nitrogen recycling and removal. For example, microbes carry out denitrification and anammox pathways, using nitrate (NO_3^-) or nitrite (NO_2^-) to produce di-nitrogen gas (N_2) [1–4]. These nitrogen (N) removal processes are particularly important in urban estuaries, which frequently suffer from eutrophication due to runoff and incomplete treatment of sewage [5]. Conversely, N recycling can sustain eutrophic conditions through mineralization of sediment organic matter as well as dissimilatory nitrate reduction to ammonium (DNRA), a microbial metabolic pathway that reduces nitrate and nitrite to ammonium (NH_4^+). Denitrification is an important nitrogen pathway in salt marshes [6], but eutrophic environments

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may alter sediment microbial communities to favor N recycling over N removal [7–10].

The loss and degradation of salt marshes in estuaries has resulted in long-term restoration efforts to supplement or replace sediments and vegetation. A major goal of these restoration efforts is to maximize ecosystem services such as N retention and removal provided by microbial communities [11, 12]. Despite this critically important objective, relatively little is known about the diversity of microbes in restored marshes, or the extent to which different clades are responsible for the metabolic processes involved in N cycling. Studies performed on natural marshes have shown that eutrophic conditions can alter microbial community structure and function [12–17]. Studies performed on salt marsh chronosequences have also demonstrated changes in microbial community composition [18] and N cycling processes [19, 20]; however these studies were performed on a marsh chronosequence that had developed naturally over time, not due to restoration efforts. We know less about microbial community composition and diversity in restored estuarine sediments within urban ecosystems, and how these communities may change with increased age of the restored habitat. Constructed marshes may differ from natural marshes in microbial community composition due to the lack of accumulated organic carbon, fixed N, and differences in succession timelines [21]. Urban constructed marshes are of particular interest because of their role in N removal in these highly eutrophic systems.

Jamaica Bay is an urban estuary surrounded by Brooklyn, Queens, and part of Nassau County (NY). The watershed of Jamaica Bay is ~ 37,000 ha, and almost all of this area is highly urbanized [22]. Approximately 15,785 kg of total dissolved nitrogen is exported to Jamaica Bay every day, primarily derived from wastewater treatment plant effluent [22]. Current estimates of salt marsh loss in Jamaica Bay are roughly 13 ha $year^{-1}$ [23, 24], and most of this loss is from inland marsh islands, which have shrunk from 950 ha in 1951 [25] to 344 ha in 2013 [26]. In an attempt to combat this loss, government entities including the National Park Service (NPS) and Army Corps of Engineers began marsh restoration projects in 2003 [26, 27]. Since these projects have been carried out in different locations every few years (from 2003 through 2012), a chronosequence of restored salt marsh islands now exists in Jamaica Bay. In addition, because Jamaica Bay is well mixed, these locations experience similar water-column chemistry, providing an ideal system to investigate how microbial communities may change with age within eutrophic estuaries.

Management plans and ecosystem models for eutrophic estuaries require a better understanding of the composition and metabolic capacity of sediment microbial communities within restored marshes and how they change during marsh development. Here, we investigate the diversity of microbial communities found in sediments across the chronosequence of restored salt marsh islands in Jamaica Bay. We used high-throughput sequencing of the 16S rRNA gene to describe and compare the diversity, taxonomic composition, and predicted functional capacity of these communities at two sediment depths. We predicted that (1) microbial communities would differ between sampling depths and among sites along the chronosequence; (2) younger salt marshes would harbor more taxa related to autotrophic N fixation; and (3) as plant biomass and sediment organic content increased the microbial communities would shift to taxa with the metabolic capacity to break down organic material and remove N. By examining the bacterial assemblages found in marsh sediment in various stages of development, and at sites that have been historically affected by intense anthropogenic pressure, our results provide novel insights into estuarine bacterial communities that may be used as indicators of marsh health and ecosystem function.

Results and Discussion

Sampling Locations

Sediment was collected from five salt marsh islands in July 2015, including four sites that at time of sampling were 3–12 years post-restoration (Fig. 1). Yellow Bar Marsh (YB), Elders West Marsh (EW), Elders East Marsh (EE), and Big Egg Marsh (BE) represent 3, 5, 9, and 12 years post-restoration, respectively. All of the sites were restored with the addition of dredged fill material to the desired elevation and then planted with native vegetation using a variety of methods [28]. A fifth site, Black Bank Marsh (BB), > 200 years old [29, 30], has not yet been restored and is considered to be a degrading marsh due to erosion at the marsh edge, loss of vegetative cover, and reduction of belowground biomass [24, 26]. We sampled five replicate plots on each marsh island.

Vegetation and Sediment Characteristics

We found significant differences in belowground biomass among sites, but detected no clear patterns in the other vegetation variables with respect to marsh age (Table 1). Belowground biomass was greater in the older restored sites and the natural marsh site relative to the two youngest restored marsh sites (YB and EW; Table 1). Stem heights and density, leaf area, and aboveground biomass were similar among sites.

We observed greater sediment organic content and organic carbon content in the natural marsh (BB) in both the surface and subsurface samples, relative to restored sites. Surface sediment organic content and organic carbon were similar among the restored marsh sites, ranging from

Fig. 1 Map of sampling locations in Jamaica Bay, NY. Yellow Bar (YB), Elders West (EW), Elders East (EE), Big Egg (BE), Black Bank (BB)

0.38–1.13% to 0.14–0.53%, respectively (Table 1). The subsurface samples showed a similar pattern. Extractable NH_4^+ ranged from 3.82 to 9.49 mg l^{-1} in the surface sediment, with YB having the lowest and BB having the greatest concentrations. YB also had the lowest NH_4^+ concentration in the subsurface sediment. Other extractable nutrients were similar across sites (Table 1).

Alpha Diversity Estimates Differ by Both Location and Sampling Depth

Sequence reads were classified into a total of 3887 surface amplicon sequence variants (ASVs) and 2569 subsurface ASVs for community analysis. Surface ASVs were classified into 29 phyla and 159 families, while subsurface samples were classified to 29 phyla and 123 families. Surface and subsurface alpha diversity values were estimated using Breakaway [31] for sample richness, and DivNet [32] for Shannon Diversity (Fig. 2). Minimum and maximum richness estimates and Shannon estimates are provided by location and sampling depth in Table S1.

Within each sampling depth, we detected no significant differences in richness among sites. However, there were significant differences between surface and subsurface samples at each site. Richness estimates ranged from 3669 to 4838 ASVs at the surface level, and 2454 to 3004 at the subsurface level (Table S1). Similarly, a salt marsh chronosequence spanning over 100 years showed no age-related differences in richness [18]. The observed differences between sampling depths in the current study could result from both more stringent selective pressures at the subsurface level than at the surface level, as well as less frequent perturbation and taxonomic turnover at subsurface levels. Sediment oxygen conditions are typically a major driver of community composition and could restrict the number of taxa able to persist at the subsurface level.

Shannon diversity, which takes into account evenness among taxa, showed differences among the five marsh sites at both surface and subsurface levels, consistent with our hypothesis. Estimates by location ranged from 6.14 to 6.86 at the surface level and 5.17–6.25 at the subsurface level (Table S1). While we were not able to establish a relationship between diversity and age or restoration status across sites, the two youngest sites had the lowest Shannon diversity. We hypothesize that these younger restored marshes may be farther away from ecological equilibrium due to their sediment composition or nutrient limitations.

It is important to note that alpha diversity estimation in microbiome studies is an area of active research, and values may be under- or overestimated due to the compositional nature of the data [33–36]. However, as long as no biases exist across samples, Shannon diversity remains useful for exploration of broad relative trends. In this study, we note that environmental factors may be driving differences in alpha diversity among sites and between depths. Our results are broadly consistent with a previous study that found differing Shannon diversity and richness between sampling depths in mudflat sediment [37]; however, in that study, two of three sites showed higher

Table 1 Environmental variables (mean ± standard error) measured at four restored salt marshes and a natural degraded salt marsh (Black Bank) in Jamaica Bay (NY) during July 2015

Site	*n*	Yellow bar	Elders west	Elders east	Big egg	Black bank
Vegetation variables						
Leaf Width (cm)	10	0.94 (0.03)	0.96 (0.05)	1.06 (0.04)	0.94 (0.06)	0.95 (0.06)
Specific leaf area (cm^2 g^{-1})	10	104.30^a (2.65)	105.95^a (4.26)	102.20^a (4.98)	134.34^b (7.24)	118.87^{a,b} (6.26)
Leaf carbon (%)	10	41.78^a (0.18)	43.67^b (0.35)	42.49^a (0.27)	43.03^{a,b} (0.31)	42.14^a (0.27)
Leaf nitrogen (%)	10	1.70 (0.10)	1.87 (0.11)	1.64 (0.07)	1.83 (0.14)	1.54 (0.10)
Avg. stem height (cm)	5	52.0 (5.3)	70.4 (6.3)	75.5 (9.9)	51.1 (7.6)	66.6 (4.7)
Max. stem height (cm)	5	91.2^{a,b} (6.0)	118.1^b (10.0)	109.3^{a,b} (10.6)	83.7^a (4.8)	91.9^{a,b} (4.9)
Stem density (m^{-2})	5	153 (22)	155 (30)	211 (110)	205 (60)	266 (39)
Aboveground biomass (g m^{-2})	5	147 (28)	365 (66)	614 (265)	202 (81)	664 (115)
Belowground biomass (g m^{-2})	5	43^a (17)	233^a (156)	777^{ab} (362)	2150^b (616)	781^{ab} (273)
Sediment variables (0–5 cm)						
Chlorophyll a (μg cm^3)	6	5.78 (1.21)	6.69 (1.63)	8.00 (1.05)	4.72 (0.79)	5.67 (0.50)
Organic content (%)	6	0.38^a (0.06)	0.55^a (0.41)	1.13^a (0.25)	0.69^a (0.11)	15.98^b (2.84)
Organic carbon (%)	6	0.14 ^{a} (0.01)	0.23 ^{a} (0.02)	0.53 ^{a} (0.09)	0.27 ^{a} (0.04)	7.66 ^{b} (1.04)
Total nitrogen (%)	6	0.02 (0.01)	0.03 (0.00)	0.05 (0.01)	0.06 (0.01)	0.50 (0.08)
Ext. NH_4^+ (mg N L^{-1})	6	3.82^a (0.54)	7.72^{a,b}(0.51)	9.32^b (1.64)	4.69^{a,b} (0.75)	9.49^b (1.83)
Ext. NO_2^- (mg N L^{-1})	6	0.07^{a,b} (0.01)	0.12^a (0.02)	0.09^{a,b} (0.01)	0.05^b (0.00)	0.07^{a,b} (0.01)
Ext. NO_3^- (mg N L^{-1})	6	0.05 (0.00)	0.08 (0.03)	0.13 (0.08)	0.03 (0.00)	0.03 (0.02)
Ext. SRP^- (mg P L^1)	6	0.06 (0.01)	0.12 (0.02)	0.16 (0.07)	0.10 (0.05)	0.28 (0.12)
Sediment variables (5–10 cm)						
Organic content (%)	6	0.85^a (0.67)	0.37^a (0.10)	0.70^a (0.10)	1.52^a (0.88)	9.62^b (1.67)
Organic carbon (%)	6	0.08^a (0.02)	0.15^a (0.04)	0.23^a (0.03)	0.17^a (0.04)	5.45^b (1.04)
Total Nitrogen (%)	6	0.00^a (0.00)	0.01^a (0.00)	0.02^a (0.01)	0.06^a (0.01)	0.35^b (0.06)
Ext. NH_4^+ (mg N L^{-1})	6	1.6 (1.0)	2.36 (1.28)	1.82 (0.59)	3.02 (0.77)	2.43 (0.21)
Ext. NO_2^- (mg N L^{-1})	6	0.09^a (0.01)	0.08^a (0.00)	0.13^b (0.01)	0.07^a (0.01)	0.07^a (0.01)
Ext. NO_3^- (mg N L^{-1})	6	0.08 (0.01)	0.04 (0.02)	0.18 (0.09)	0.04 (0.00)	0.04 (0.01)
Ext. SRP (mg P L^{-1})	6	0.02 (0.00)	0.06 (0.06)	0.02 (0.01)	0.04 (0.01)	0.01 (0.00)

Letters indicate significant differences between study sites

richness at subsurface depths, whereas two of three sites showed lower Shannon diversity at the subsurface [37].

Community Composition Differs Significantly Among Marsh Sites

Normalized weighted UniFrac [38] distances, NMDS ordinations (Fig. 3) and PERMANOVA analysis using Bray-Curtis [39] distances demonstrate that sites were grouped significantly by location at the surface level (Table 2), despite being proximate geographically (< 4.6 km between the two most distant sites). This result is consistent with the findings of Dini-Andreote et al. [18] which looked at a 100 year naturally occurring chronosequence. In contrast, significant groupings were not observed at the subsurface level.

Hierarchical Clustering

We used hierarchical clustering methods to estimate beta diversity, or differences in microbial community composition among sites. Unweighted pair group with arithmetic mean (UPGMA) clustering was performed to visualize normalized weighted UniFrac sample distances (Fig. 4). At the surface level (Fig. 4A), BB clearly separates from all other locations, while younger restored EW and YB marshes are grouped together, and older restored EE and BE marshes are grouped together despite being the sites most geographically distant from each other (Fig. 1). While we cannot draw firm conclusions from this clustering due to the complex nature of the study system and limited sample size, the clusters share some distinctive characteristics. EE and BE are the two oldest restored marshes, and both have demonstrated loss of plant cover

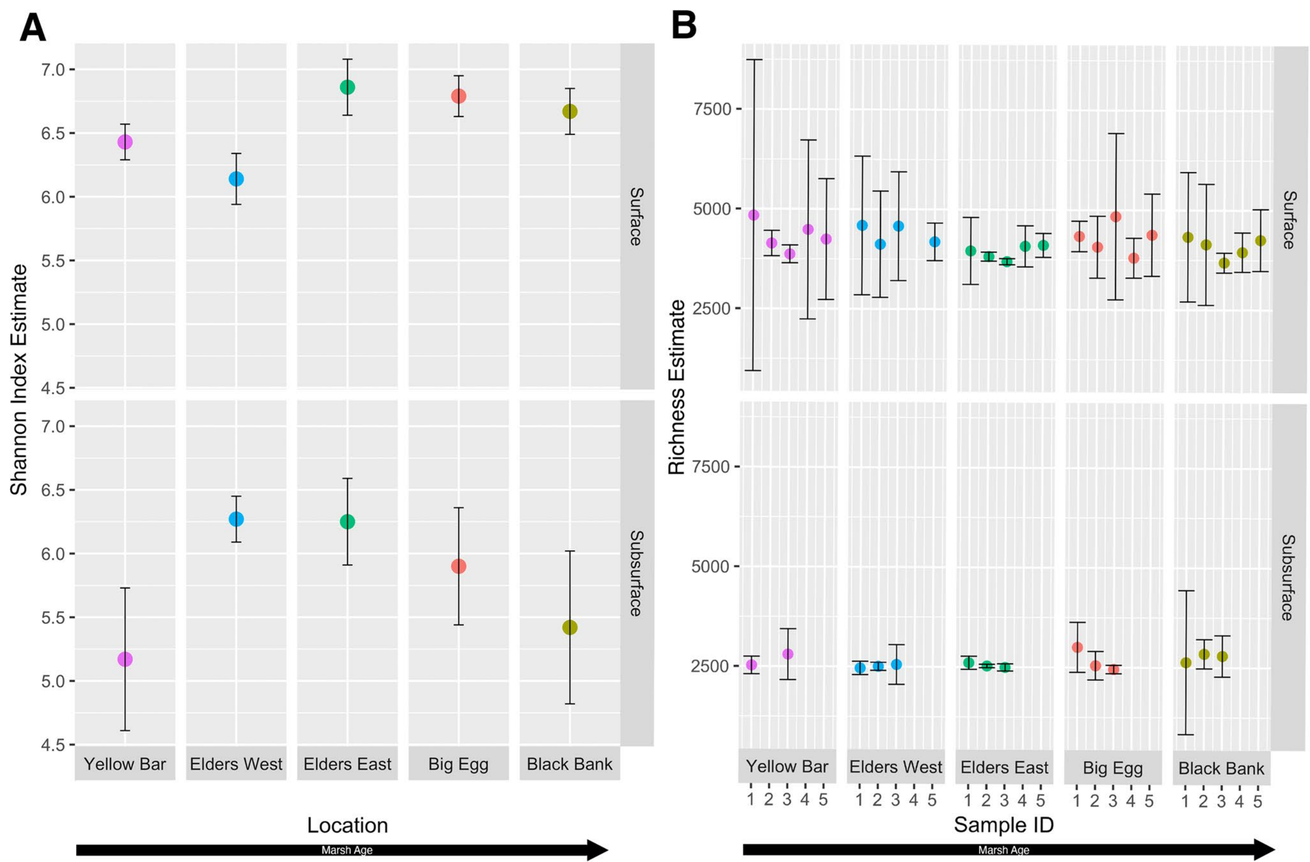

Fig. 2 Alpha diversity estimates displayed by marsh (**A, B**) and sampling depth (surface and subsurface). **A** Shannon diversity estimates with confidence intervals (using parametric bootstrapping) made with DivNet, site-wide estimations made at the ASV level. **B** Richness estimates with confidence intervals using Breakaway. Two outliers EWS4 and YBD2 were excluded from the richness plot

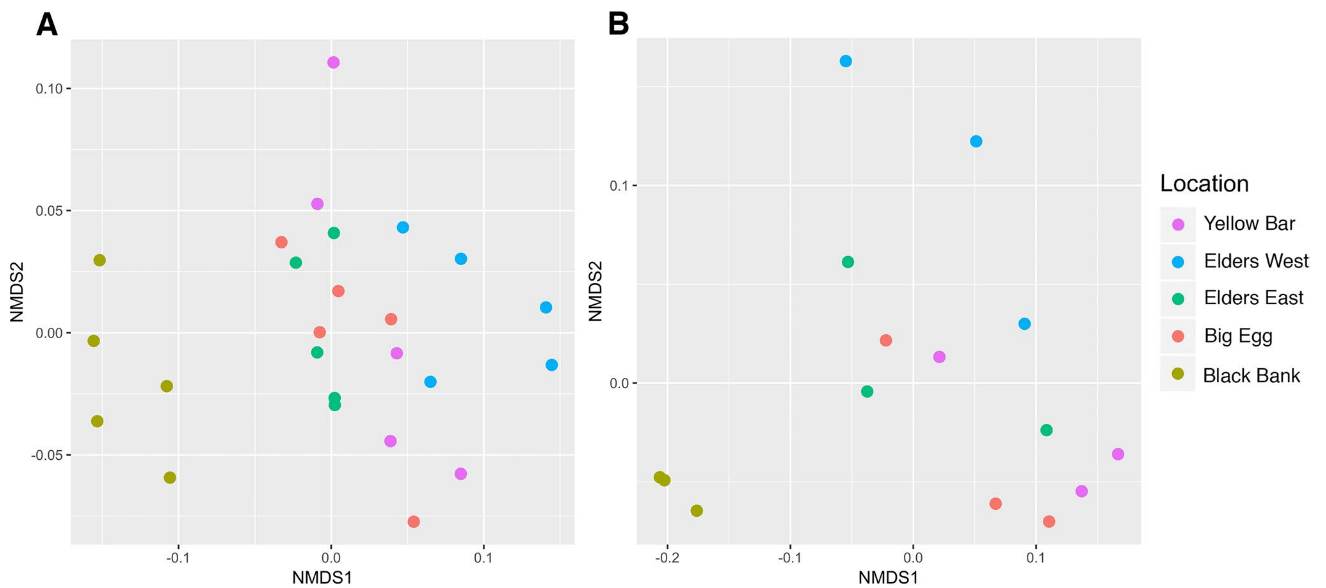

Fig. 3 Non-metric multidimensional scaling (NMDS) of weighted UniFrac distances. Prior to UniFrac calculations ASV counts were variance stabilized using DESeq2. Surface samples (**A**) significantly grouped by location in PERMANOVA analysis (p = 0.001*** R^2 = 0.301), while subsurface samples (**B**) grouped non-significantly by location (p = 0.151, R^2 = 0.258).

Table 2 PERMANOVA results of surface and subsurface sediment microbial communities in relation to the environmental variables

		p value	R^2
Surface sediment[a]			
	Belowground biomass	0.597	0.016
	Organic content %	0.361	0.021
	Organic C %	0.517	0.018
	Total N %	0.621	0.016
	Ext. NH_4^+	0.433	0.020
	Ext. NO_2^-	0.438	0.019
	Ext. NO_3^-	0.519	0.018
	Ext. SRP	0.597	0.016
	Location	0.002***	0.192
Subsurface sediment[b]		*p* value	R^2
	Belowground biomass	0.803	0.043
	Organic content %	0.902	0.035
	Organic C %	0.967	0.028
	Total N %	0.983	0.025
	Ext. NH_4^+	0.503	0.067
	Ext. NO_2^-	0.852	0.038
	Ext. NO_3^-	0.938	0.032
	Ext. SRP	0.822	0.041
	Location	0.793	0.207

[a] $n = 5$

[b] $n = 3$

post-restoration between 2008 and 2012 [26]. EW and YB are the two youngest restored marshes, and experience a shorter period of daily tidal inundation relative to the older restored sites [40]. They have also gained more plant cover from 2008 to 2013 [27]. Consistent with our predictions, the microbial community at the unrestored site, BB, was clearly dissimilar from all other sites. These differences could be related to marsh elevation as other studies [24, 41, 42] have demonstrated that increased inundation may alter sediment and vegetation characteristics [24, 41]. Changes in patterns of inundation, vegetation, and sediment traits would likely affect microbial communities as well. Subsurface samples (Fig. 4B) show another distinct separation of BB with less distinct clustering of the other sites. Like many restored coastal marsh sites, the restored marshes included in our study were built on dredged material and thus have a higher percentage of sand and accumulate less organic matter and total N in subsurface layers [24, 43, 44]. Nutrient availability and sand content have previously been identified as significant drivers of salt marsh microbial community structure across a chronosequence [18, 19]. It is possible that subsurface sediments experience less site-to-site variability in environmental conditions than surface sediments, leading to slower divergence in community composition at subsurface depths. As a consequence, bacterial communities

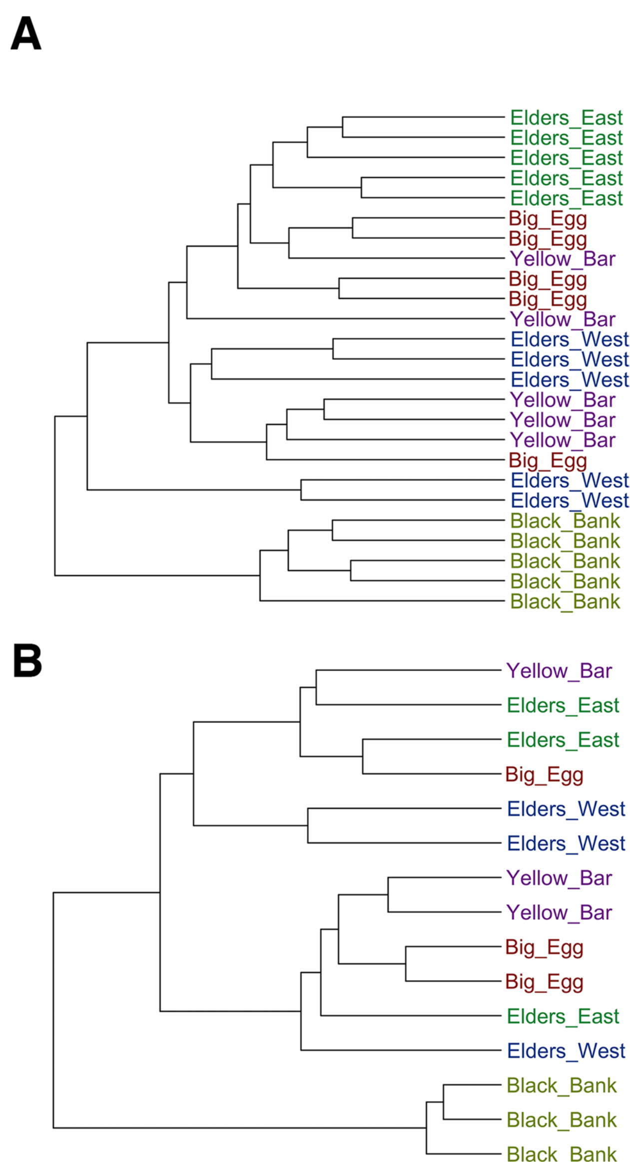

Fig. 4 UPGMA dendrograms of weighted UniFrac distances of surface (**A**) and subsurface (**B**) sediment communities. Prior to UPGMA clustering, ASV tables were variance stabilized using DESeq2. Yellow Bar (YB), Elders West (EW), Elders East (EE), Big Egg (BE), Black Bank (BB)

below the surface may change more slowly than at the surface following restoration. Several studies have shown that microbial community structure, functional potential, and nitrogen cycling processes change across a natural marsh chronosequence [18–20, 45]; however to our knowledge post-restoration succession within the microbial community has not been examined previously at the subsurface level. More research is needed to determine if these results are due to a difference in the rate of community succession

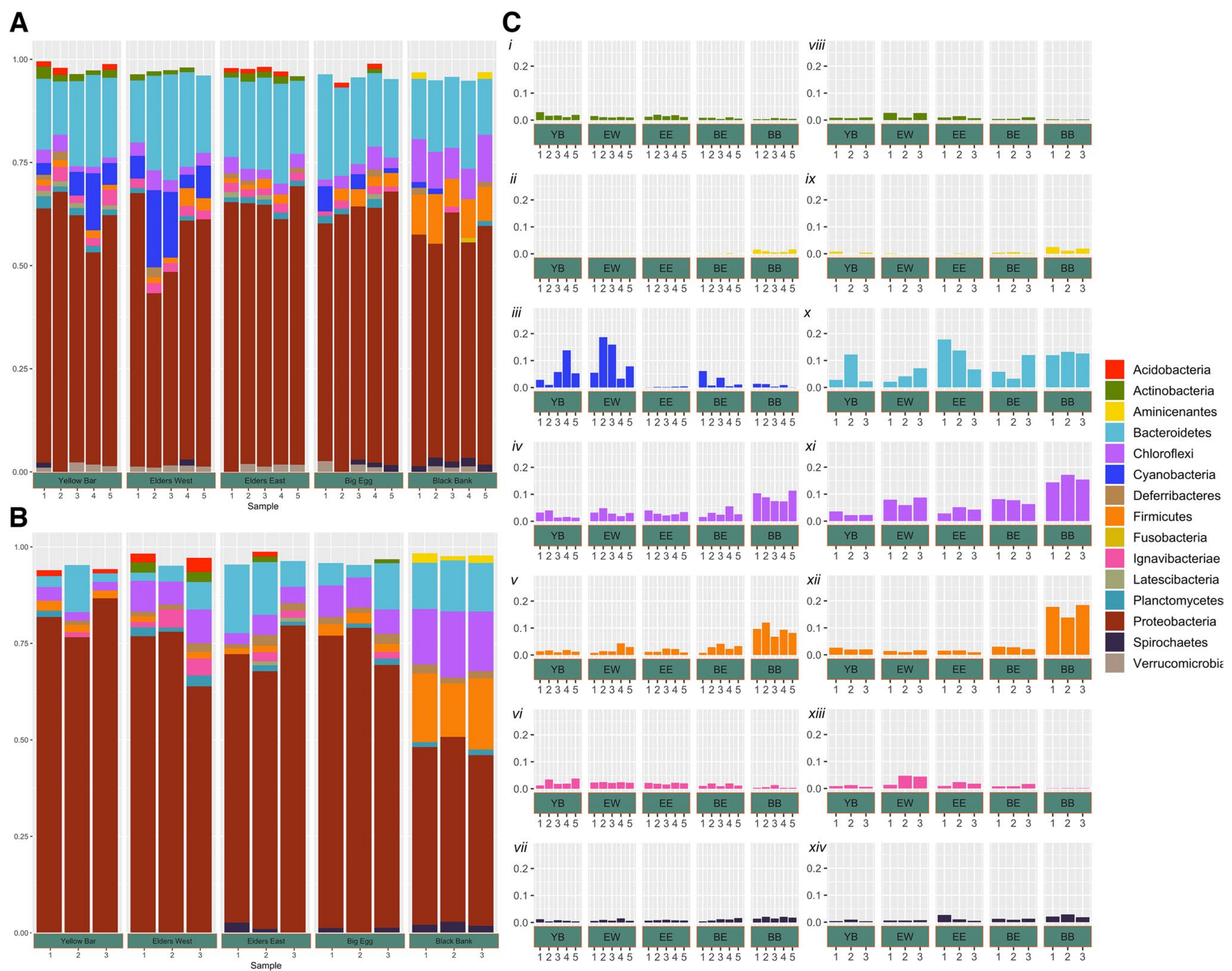

Fig. 5 Taxonomic composition in relative abundance at the phylum level of sediment bacterial communities from the surface (**A**) and subsurface (**B**) and grouped by sampling marsh. Only phyla representing >= 1% of the total community are displayed (**A**) and (**B**). Individual phyla from the surface (Ci-Cvii) and subsurface (Cvii-Cxiv) that display trends across the chronosequence or are differentially abundant between restored and unrestored

between surface and subsurface, or if subsurface microbial communities are similar among unrestored marsh sediment as well.

Taxonomic Composition by Relative Abundance

Figure 5A, B shows the taxonomic composition at the phylum level of all ASVs found at greater than 1% relative abundance. The two most dominant phyla, *Proteobacteria* and *Bacteroidetes,* observed at all sites were also most dominant in other studies performed in estuarine systems [18, 46–48]. Seven different phyla were found to be differentially abundant between restored and unrestored locations at the surface level using DESeq2 [49] or displayed clear trends with age (Fig. 5C). This result was consistent with our hypothesis that microbial communities would differ among sites along the chronosequence.

The relative abundance of the phylum *Chloroflexi* was found to be highest at BB (mean ± sd 9.1 ± 1.8%) compared with all other sites (2.8 ± 1.1%) in surface samples, and in subsurface samples at BB (15.6 ± 1.4%) at any other site (5.4 ± 2.4%). Families within the phylum Chloroflexi were also more diverse at BB, with Anaerolineaceae, Dehalococcoidetes, and clades which could not be classified to below the phylum level equally well represented. At the restored site, *Anaerolineaceae* was the dominant family, a finding that is consistent with a previous study in mudflat sediment [37, 50, 51]. The phylum *Chloroflexi* has been shown to contain a wide array of carbohydrate hydrolytic genes [52], which correlates with the high amount of accumulated organic

matter and decomposing belowground biomass at BB [24], and aligns with CAP results showing organic content and C to be positively associated with the BB site. This observation accords with our third hypothesis that communities in older marshes would harbor more taxa with the metabolic capacity to break down organic matter. Previous studies have also shown that *Chloroflexi* was significantly more abundant in older marshes across a natural chronosequence [18], and was associated with N- and organic-rich sediments [18, 47].

Firmicutes was also more common at BB than any other site at both the surface (9.2 ± 1.9% vs 2.0 ± 1.1%) and subsurface (16.7 ± 2.5% vs. 1.8 ± 0.7%). In contrast, a previous study showed *Firmicutes* to be associated with younger sites in a natural chronosequence [18]. Unlike *Chloroflexi*, *Firmicutes* was dominated by unclassified *Firmicutes* in our results, and the proportions of classified families within the *Firmicutes* phylum varied from site to site. It is possible that metabolic capacities within *Firmicutes* may be similar enough that different families may fill the same metabolic role at different sites. More research is needed to determine what role each *Firmicutes* ASV is filling in this system.

At the surface *Cyanobacteria* were more common at the two youngest sites, EW (10.3 ± 6.7%) and YB (5.7 ± 4.9%), relative to EE, BE or BB (0.2 ± 0.1%; 2.4 ± 2.4%; 0.8 ± 0.6%, respectively). This result is consistent with studies previously conducted in marsh chronosequences [18, 20] and is likely due to the ability of many *Cyanobacteria* to fix atmospheric nitrogen as early colonizers of marsh communities. The youngest sites are likely to be nitrogen-limited [53, 54], which is supported by our nutrient analysis results (Table 1).

At the subsurface level the phylum *Aminicenantes* contributed more to the community at BB (1.8 ± 0.7%) than any other site (0.2 ± 0.3%), while *Acidobacteria* were more prevalent at the two youngest sites with 2.3 ± 1.3% at EW and 1.1 ± 0.3% at YB compared to BB (0.4 ± 0.07%), BE (0.7 ± 0.2%), and EE (0.8 ± 0.4%). Finally, the phylum *Bacteroidetes* made a larger overall contribution to community structure at the two oldest restored sites EE (12.7 ± 5.6%) and BE (7.0 ± 4.5%), and BB (12.6± 0.6%) compared to the two youngest restored sites EW (4.4 ± 2.5%) and YB (5.7 ± 5.6%). This difference could be due to accumulation of organic matter over time and the previously shown characteristic of *Bacteroidetes* phylum to have a broad array of carbohydrate-degrading genes [52]. A recent study, however, examined diversity at the surface level of a natural marsh chronosequence, and found *Bacteroidetes* to be associated with younger sites (0 and 5 years of age) [18], possibly indicating that *Bacteroidetes* fulfills a different role in subsurface samples than at the surface.

Constrained Analysis of Principal Coordinates (CAP) and Weighted Regressions

We used constrained ordinations to explore how environmental variables (Table 1) were associated with changes in community composition at both the surface and subsurface depths (Fig. 6). The unrestored and degrading site (BB)

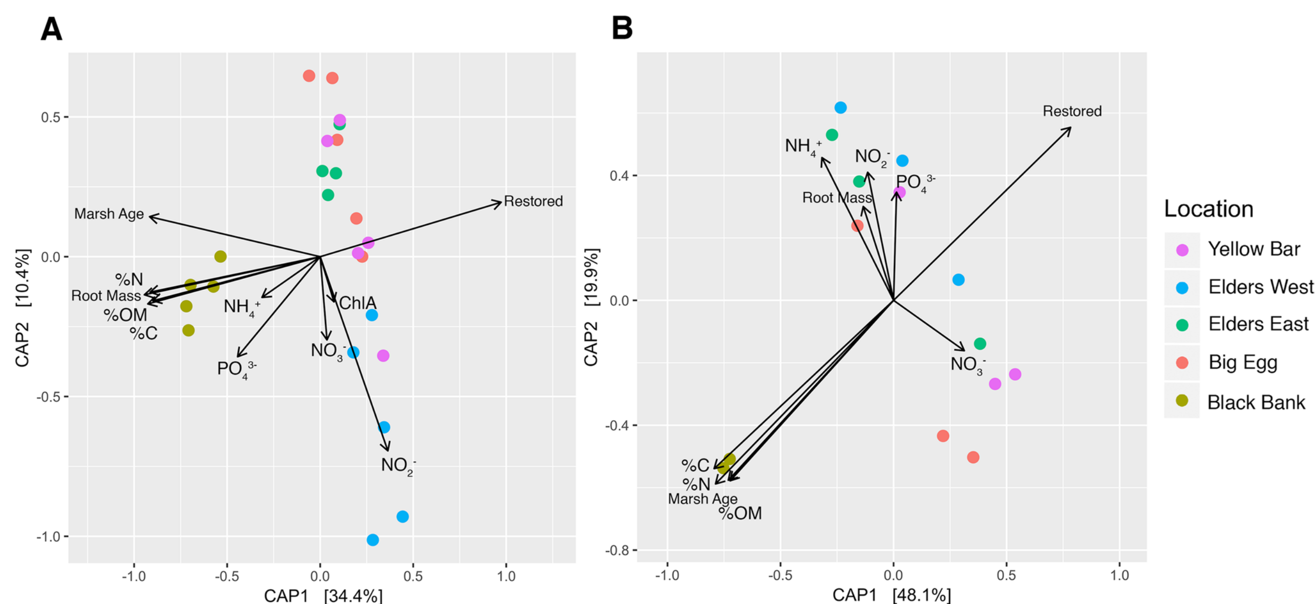

Fig. 6 Canonical analysis of principal coordinates (CAP) ordinations of weighted UniFrac distances. Bacterial communities of surface (**A**) and subsurface (**B**) sediment are colored by sampling location. Fitted vectors of environmental variables represent the direction of the gradient and the length represents the strength of the variable

was distinct from the other locations, with percent N, C, organic content, belowground biomass, and marsh age positively correlated with this separation. Differences in organic matter accumulation and belowground biomass have been observed between degrading and restored marshes previously in Jamaica Bay and elsewhere [24, 43]. These differences suggest that unrestored marshes harbor distinct microbiomes when compared to those from newly constructed marshes that may be related to belowground root growth, decomposition, or greater accumulation of recalcitrant forms of carbon in older marshes. Newly constructed marshes are likely to differ from natural marshes in the amount of accumulation of belowground plant biomass and organic carbon. As a consequence, new plant growth and labile carbon may provide more important sources of energy for microbes in newly constructed marshes. However, an important caveat is that we were unable to determine with our dataset how this difference may be affected by the degraded status of Black Bank. We expect degraded marshes to show less new root growth and a higher fraction of standing root mass that consists of recalcitrant (i.e., difficult to decompose) material, relative to healthy ones. Future studies should incorporate comparisons of stable and degrading natural marshes wherever possible in order to determine how marsh degradation affects these factors. While the factors separating BB from the restored marshes were relatively clear, surprisingly, neither belowground biomass nor percent organic content, N, or C, had a statistically significant effect on groupings from PERMANOVA analysis. Therefore, factors driving statistically distinct communities among the restored marsh islands may not have been captured in our environmental data.

Subsurface communities from EE and EW were separated from BE and YB, and this difference in community composition was associated with extractable NO_2^-, NH_4^+, soluble reactive phosphate (PO_4^{3-}) and sediment organic content. In addition, regressions were made on NMDS and CAP axes weighted by the inverse of the standard error of the predictor variable (Fig. 7), to assess their effect on bacterial community composition. Black Bank separates out based on sediment organic content, but the trend in

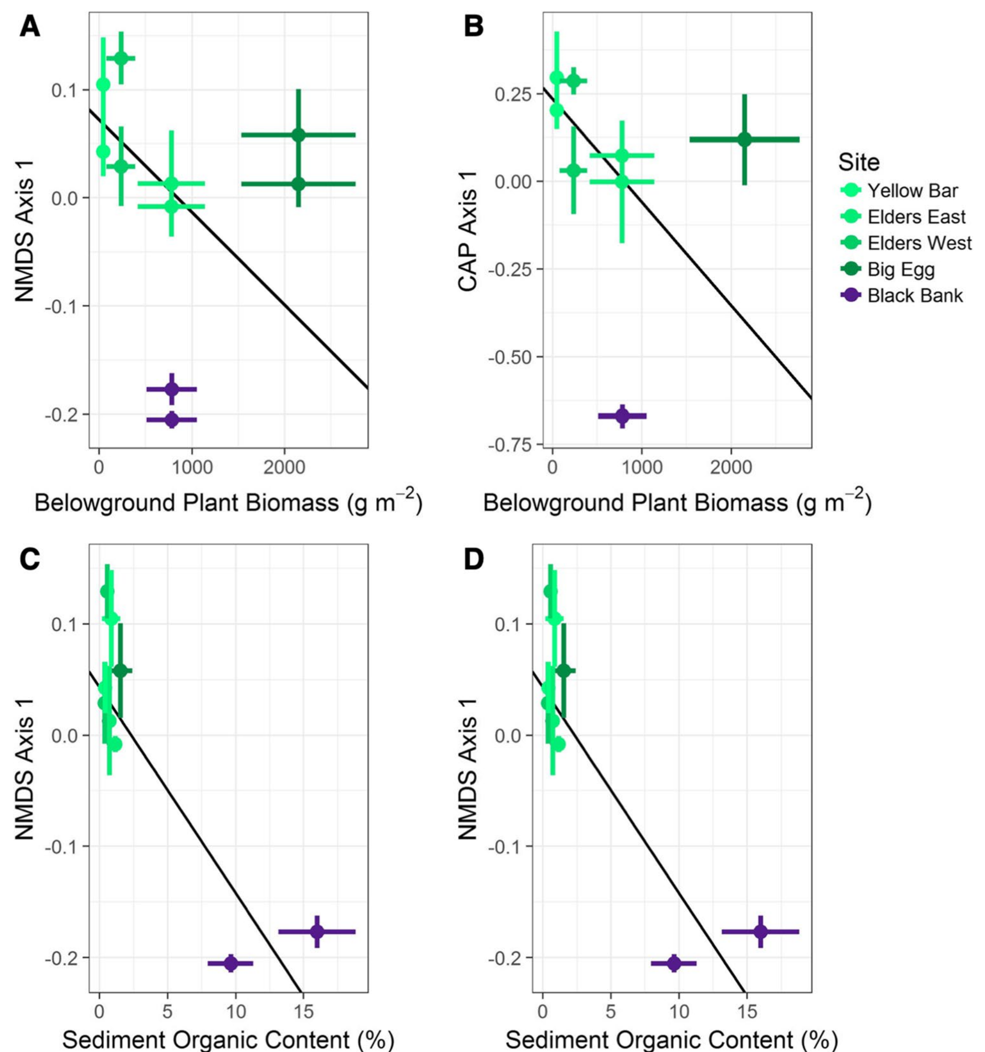

Fig. 7 Weighted regressions using environmental factors as predictor variables weighted by SE

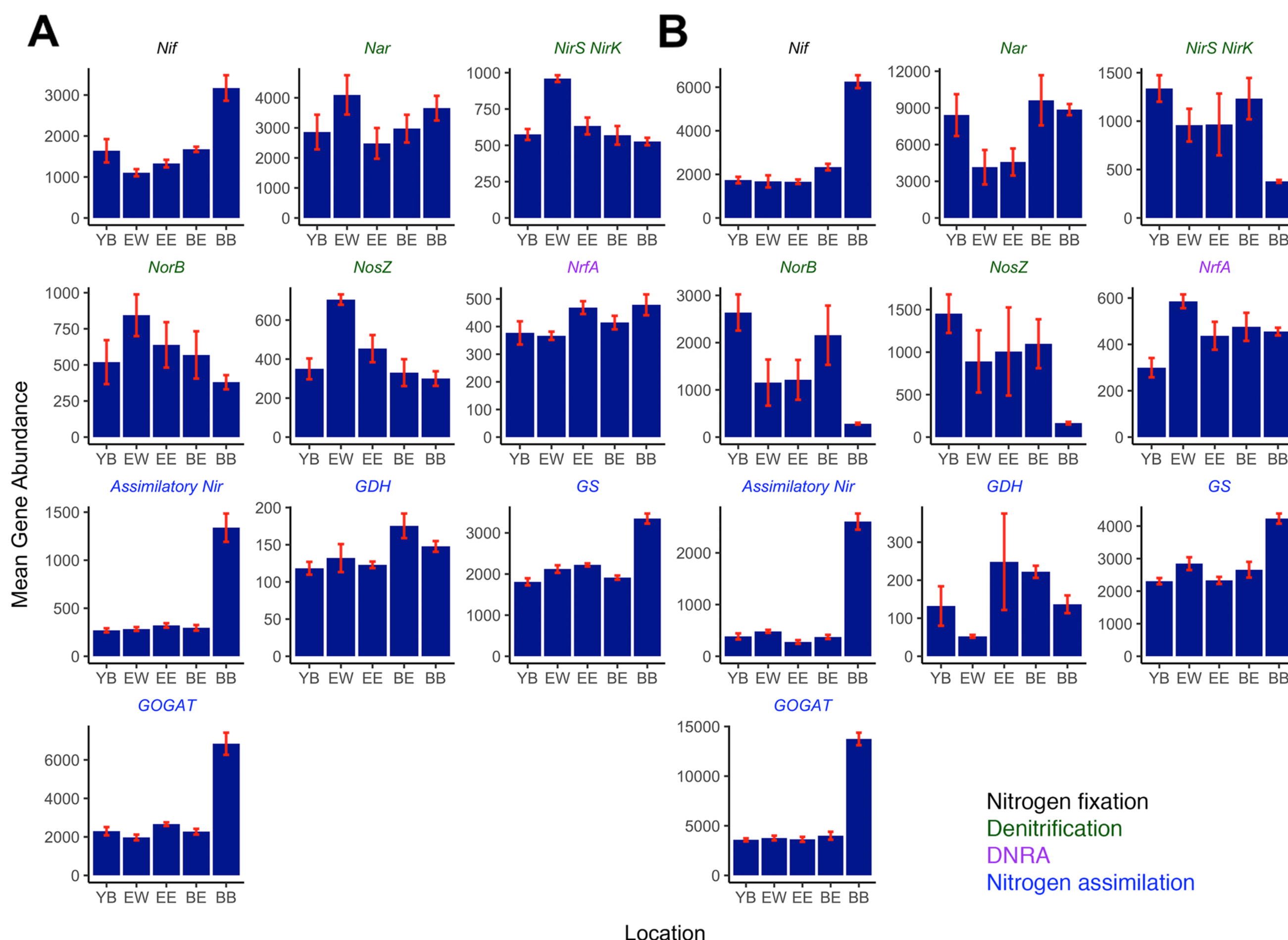

Fig. 8 Predicted nitrogen cycle gene abundances by location (Yellow Bar=YB, Elders West=EW, Elders East=EE, Big Egg=BE, Black Bank=BB) and sediment depth. Nitrogen fixation (Nif), denitrification (NirS, NirK, NorB, NosZ), DNRA (NrfA), and nitrogen assimilation (*Nas*, GDH, GS-GOGAT) were predicted for both surface (**A**) and subsurface (**B**) sediment communities using PAPRICA. Prior to gene prediction sequences were randomly subsampled to even depth across all samples at both sampling depths

community structure in the restored marshes was more correlated to belowground biomass. The separation of Elders West from other restored sites was associated with differences in extractable NO_2^-, NO_3^-, and chlorophyll a (ChlA).

Predicted Nitrogen Cycling Gene Content

ASV sequences were run through the PAPRICA [55] metabolic inference software to predict the abundance of genes involved in N cycling (Fig. 8). Spearman correlation coefficients show which environmental variables exhibited positive and negative correlations with predicted N gene content (Table 3).

In contrast to our expectations based on the observed higher abundance of *Cyanobacteria* at the younger sites, nitrogen fixation predicted gene content (nitrogenase, *Nif*) increased slightly across the chronosequence. Gene content in surface sediment at the unrestored site (BB) was substantially higher, with twice as many predicted *Nif* gene copies than any other site, and three times higher counts in subsurface samples. Due to a higher abundance of *Cyanobacterial* autotrophic N fixers at EW and YB, and preliminary evidence that has shown that denitrification was the dominant N_2 flux at BB, we might expect higher *Nif* abundance at younger sites. The method presented here reports predicted gene content, rather than a direct reflection of genes being actively expressed. Many of the genomes containing *Nif* genes may be facultative N fixers who rarely fix N. It is likely that these gene copy results were likely due to a mismatch between predicted *Nif* content and actual rates of N fixation [56].

We expected to see an increase in denitrification gene predictions at the natural unrestored marsh (BB) since a previous study showed significant increases in denitrification gene content in a marsh creek that had experienced extensive

Table 3 Spearman correlation coefficients of the relationships between environmental variables and the predicted nitrogen cycling gene abundances from the surface (0–5 cm) and subsurface (5–10 cm) sediment samples

Surface										
	Nif	*Nar*	*NirS_NirK*	*Nas*	*NorB*	*NosZ*	*NrfA*	*gdh*	*GS*	*GOGAT*
Belowground biomass	**0.481**	− 0.038	− 0.323	**0.594**	− 0.163	− 0.235	0.353	**0.398**	**0.657**	**0.508**
Chl *a*	− 0.121	− 0.202	− 0.077	0.294	− 0.082	− 0.059	− 0.088	− 0.023	− 0.021	− 0.016
Org. con. %	**0.595**	0.043	− 0.520	**0.713**	− 0.272	**− 0.436**	0.539	0.050	0.600	**0.763**
Org. C %	**0.440**	− 0.077	− 0.373	**0.678**	− 0.200	− 0.258	**0.468**	0.366	**0.681**	**0.580**
Total N %	**0.629**	− 0.092	**− 0.528**	**0.571**	− 0.375	**− 0.493**	**0.473**	**0.492**	0.394	**0.544**
Ext. NH_4	− 0.151	− 0.097	− 0.004	0.310	− 0.061	0.110	0.061	0.045	**0.524**	0.123
Ext. NO_2^-	− 0.536	0.070	0.390	− 0.097	0.165	**0.402**	**− 0.461**	− 0.375	0.255	− 0.283
Ext. NO_3^-	− 0.277	− 0.204	0.056	0.130	− 0.216	0.017	− 0.053	− 0.288	0.181	− 0.157
Ext. SRP	− 0.052	0.177	0.196	0.017	0.056	0.202	− 0.230	− 0.112	0.342	− 0.015
Subsurface										
	Nif	*Nar*	*NirS_NirK*	*Nas*	*NorB*	*NosZ*	*NrfA*	*gdh*	*GS*	*GOGAT*
Belowground biomass	0.084	− 0.466	**− 0.572**	− 0.191	**− 0.653**	− 0.510	0.348	0.299	0.132	0.020
Org. con. %	0.226	− 0.002	− 0.455	0.174	− 0.464	− 0.525	0.015	0.213	0.297	0.240
Org. C %	0.320	− 0.271	**− 0.642**	0.311	**− 0.736**	**− 0.650**	0.322	0.320	0.428	0.384
Total N %	**0.631**	0.253	− 0.382	0.291	− 0.378	− 0.442	0.173	**0.518**	0.411	**0.627**
Ext. NH_4^+	0.020	− 0.262	− 0.332	0.059	− 0.345	− 0.411	0.178	**0.582**	− 0.086	− 0.059
Ext. NO_2^-	− 0.235	− 0.376	− 0.103	− 0.187	− 0.108	0.011	− 0.226	− 0.051	− 0.165	− 0.147
Ext. NO_3^-	− 0.257	0.305	**0.451**	− 0.407	0.393	0.442	**− 0.486**	− 0.314	− 0.169	− 0.196
Ext. SRP	0.442	− 0.073	0.029	0.055	0.037	− 0.046	0.169	0.363	− 0.248	− 0.116

Org. = organic, *con.* = content

Statistically significant relationships are indicated by bold font

nutrient loading [57, 58]. Jamaica Bay has been experiencing increased N loading for > 110 years [31]. Contrary to our expectation, the gene predictions demonstrated that BB microbes had less denitrification gene content than any restored marshes. Gene predictions related to the process of denitrification (*Nar, NirS/NirK, NorB, NosZ*) were lowest for most genes at BB, in both surface and subsurface samples. Preliminary results from these locations indicate significantly higher rates of microbial denitrification at BB relative to restored sites [56]. As with our nitrogen fixation results, we expect that many denitrifying bacteria are facultative. In that case, local environmental conditions that favor expression of denitrification genes may prove to be better predictors of actual N-removal rates than characteristics of the microbial community itself.

Conclusions

Complex System, Complex Communities

Salt marsh sediments are complex systems with diverse microbial communities that perform a wide array of biogeochemical processes critical to the world's nutrient cycles. While much is known about the processes that microbial communities are able to perform as a whole in these systems, more research is needed to elucidate the specific taxonomic and functional composition of these communities, and the environmental drivers that shape them. Here our results showed that five marsh islands within the same estuary exhibited distinct differences in Shannon diversity, as well as beta diversity, taxonomic composition, and predicted functional capacity among sampling sites. These differences were also observed when comparisons were made between surface and subsurface samples within the same sampling location, attempting to tease out differences in community composition under different redox conditions. Some differences in community structure among sites and depths can be correlated to the environmental data, or known functional characteristics of taxa. Other results, however, do not have a clear explanation, and may be influenced by daily inundation times, aspects of restoration such as sediment origin, or variables not measured such as hydrology or erosion.

Our results indicated distinct differences in bacterial communities, and their metabolic capacities, among five marsh islands despite their close geographic proximity within the same estuary. The most dissimilar community at both surface and subsurface sediment depths was Black Bank, the oldest and the only unrestored marsh. The two youngest sites showed an increased relative abundance of taxa known to

be early colonizers of salt marsh sediment and known autotrophic N fixers. Restored sites contained more predicted genes related to the ability to perform denitrification. Analysis of predicted gene content suggested that metabolic capacity for N assimilation was greater at BB than restored sites. We speculate that this unique community composition at BB was driven by environmental factors at this site including high C, N, and organic content. We cannot determine with the present dataset, however, whether marsh age, degradation, or its unrestored status are the proximate causes for these differences. It is also important to note that the method used to predict gene abundance (Paprica), has known limitations, as its inference is based on representation of genomes from the sampled environment in the phylogenetic reference tree, but performs favorably when compared with similar methods (e.g., [59]). Many N processes are facultative, especially denitrification, so measuring gene expression should be considered as an alternative for estimates of gene abundance based on taxonomy. In addition, we are unable to estimate the potential contribution of endospores to our results based on the methods used. Future work within this and similar systems should seek to experimentally link the biogeochemical processes ongoing within the marsh to specific bacterial taxa through RNA-seq, proteomics, metabolomics, and should also include stable natural marshes in addition to degrading natural marshes.

Materials and Methods

Study Site Sediment and Vegetation Characteristics

Samples were collected from 5 *Spartina alterniflora* salt marshes located in the center of Jamaica Bay (New York City, NY, USA). Four of these marshes were restored marshes while the fifth was a natural degrading marsh [25]: Yellow Bar (YB restored 2012), Elders East (EE restored 2006), Elders West (EW restored 2010), Big Egg (BE restored 2003), and Black Bank (BB natural degrading; Fig. 1). We did not include a reference (non-degraded or degrading) marsh due to the lack of such a site in reasonably close proximity to our study area. Sampling was performed as a single sampling event on 7–8 July 2015. At each site a transect was established parallel to the marsh edge (~ 1 m from marsh edge). A 0.25 m^2 quadrat was haphazardly tossed along the transect to collect five replicate samples from each site. Samples from each quadrat were taken for aboveground biomass by harvesting all plant material inside the quadrat. Measurements of stem density, heights, and leaf characteristics were performed in the laboratory. The belowground biomass was sampled by taking a core (2.76 × 15 cm) from the center of the quadrat to a depth of ~ 10 cm. Samples for microbial DNA were collected immediately in the field as described below, and the core was then brought back to the lab to measure sediment characteristics. A second core was collected from each plot to quantify belowground plant biomass. The belowground biomass core was wet-sieved through a 1-mm mesh. Both above and belowground plant material was dried at 60°C and sub-samples were homogenized with a mortar and pestle. The carbon and nitrogen content of the plant material was measured using a Perkin Elmer Series 2400 CHN element analyzer (Perkin Elmer Inc., Shelton, CT) using acetanilide as a standard.

Sediment subsamples were collected from the microbial DNA core to characterize the sediment environment at surface (0–5 cm) and subsurface (5–10 cm) depths. Subsamples from the sediment surface were taken to measure sediment chlorophyll-a using the acetone extraction method and measured spectrophotometrically [60]. Subsamples were also taken from both the surface (0–5 cm) and subsurface (5–10 cm) and homogenized prior to determination of organic content, organic carbon, total nitrogen, and extractable nutrient concentrations. Sediment was dried at 60 °C and weighed to determine water content. The sediment organic content was determined based on the loss on ignition at 500 °C for 12 h following best practices for estuarine and wetland sediments [61, 62]. Dried sediment samples were treated with 25% hydrochloric acid and re-dried [63] to determine percent organic carbon and total nitrogen. Lastly, we used a fresh ~ 5 g sediment sample to determine extractable nutrient concentrations. Samples were extracted with 10-mL 2 N KCl and analyzed for ammonium, nitrite, nitrate, and soluble reactive phosphate concentrations using a Seal AQ2$^+$ discrete autoanalyzer (Seal Analytical Inc., Mequon, WI, USA) following the methods of [64–66], respectively. A one-way ANOVA was used to compare environmental variables across sites. A Tukey post hoc analysis was used to determine differences among sites. Analyses were performed with SigmaPlot 11 (Systat Software Inc., UK).

DNA Collection and Extraction

Sediment was collected using a sterile scoop into a sterile 50 mL Falcon tube from the surface (0–5 cm) and subsurface (5–10 cm). The surface depth was chosen to capture bacterial communities involved in active biogeochemical transformations, and the subsurface depth was chosen to capture bacterial communities with access to fewer electron acceptors (i.e., oxygen, nitrate, and sulfate), based on previous profiles of sediment oxygen and sulfide concentrations at these sites [40]. Sediment samples were placed on ice and transported back to the lab and were frozen (– 20 °C) until extraction. Sediment was thawed on ice upon removal from freezer then transferred to a weigh boat using a sterile scoopula. Sediment was homogenized and approximately 0.4 g of wet sediment was used in the PowerSoil DNA isolation

kit (MoBio USA) following manufacturers protocol. Extracted sediment DNA was quantified using a Nanodrop 2000 system to check for DNA concentration and purity.

16S Ribosomal RNA Gene Amplification

Five surface samples and three subsurface samples from each site were chosen for amplification based on DNA concentration and purity as determined by Nanodrop 2000. Our final number of replicates reflects a difference in relative DNA concentrations between surface and subsurface samples. Surface samples had DNA concentrations sufficient to provide five replicates per site, while subsurface samples provided only three samples per site. Amplification of the V4 variable region of the 16S small ribosomal RNA gene was completed using 515F/806R [67] primer set with a barcode on the forward primer, using a 30-cycle PCR and HotStarTaq Plus Master Mix Kit (Qiagen, USA) with the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min. A final elongation step at 72 °C for 5 min was performed. Amplified DNA sequences were pooled in equal proportions, and then purified using Ampure XP Beads, purified PCR product was then prepared for sequencing using the Illumina TruSeq DNA library preparation protocol. Amplification, library preparation and sequencing using the Illumina MiSeq platform were performed by Molecular Research LP (Shallowater, TX, USA).

Sequence Preparation. Raw sequence processing produced 594,531 paired end surface level sequences and 316,424 paired end subsurface level sequences. Surface samples had a mean sequence count of 23,671.2 (standard deviation ± 4419.0), while subsurface samples had a mean sequence of 21,904.9 (standard deviation ± 5364.7).

Bioinformatic Pipeline

Raw demultiplexed DNA sequences were first re-orientated and had primers removed using Cutadapt [68]. Forward and reverse sequences free of primers and barcodes were trimmed for quality, denoised, merged, and grouped into ASVs using the DADA2 pipeline [69]. ASVs were assigned taxonomy using the Ribosomal Database Project [70]. ASVs that were classified as an unassigned Kingdom or determined to be of Archaeal, mitochondrial, or chloroplastic origin were discarded from the dataset, resulting in a total of 3887 surface ASVs and 2569 subsurface ASVs. A phylogenetic tree of ASVs was constructed using Decipher [71] and Phangorn [72] in R [73]. An ASV table, taxonomy table, metadata table, and phylogenetic tree were imported into Phyloseq [74] for further community analysis. After rarefaction to account for uneven sequencing depth and correcting for 16S gene copy number, bar graphs of predicted gene abundances were constructed to show differences among sampling sites and sampling depths. Richness estimates were calculated using Breakaway [31] and Shannon estimates were calculated using DivNet [32]. Beta diversity was calculated using both weighted and unweighted UniFrac [38], and taxonomic composition relative abundances were computed using Phyloseq. Non-metric multidimensional scaling (NMDS) calculations, PERMANOVA analysis of beta diversity distance matrices, CAP, weighted regressions and spearman correlations were computed using the R package Vegan 2.3 [75]. CAP implemented in Vegan uses nonparametric permutation which does not assume multivariate normality. Metabolic inferences were made using PAPRICA [44] by first randomly sampling to an even depth of 9499 sequences per sample. In addition to rarefying, sequence counts were normalized using DESeq2 [49] and run through PAPRICA with and without random subsampling and similar trends were observed.

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Authors' Contributions NM, CZ, MA, and SEA designed the study and carried out field work. NM performed all genomic laboratory analyses. CZ and MA collected all environmental data. NM and SEA performed bioinformatic analyses. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Data availability 16S data associated with this study are available on the NCBI Short Read Archive under BioProject: PRJNA551237.

Declarations

Ethics Approval and Consent to Participate No animals, human subjects or human biological material were used in this study.

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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