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BRIEF REPORT

Microbial Communities Display Key Functional Differences between Reference and Restored Salt Marshes

Kathryn L. Campbell[1,2] · **Anna R. Armitage**[1] · **Jessica M. Labonté**[1]

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Abstract

Salt marshes, despite their ecological importance (i.e., carbon sequestration) and rapid decline due to climate change and sea-level rise. Salt marsh ecosystems provide essential services such as removal of pollutants, carbon sequestration, and protection of coastal lands from storm surges. These services are strongly influenced by plant productivity, which is closely linked to microbial processes such as biogeochemical cycling of carbon, nitrogen, and sulfur. To retain carbon sequestration and other ecological functions, substantial efforts are currently directed towards coastal marsh restoration. Restoration efforts often lack comprehensive assessments of ecosystem functioning. Here, in an effort to assess ecosystem functions, we compared the microbial and viral community composition, as well as the genetic potential between reference and 10-year-old restored marshes in Galveston Bay, TX, USA. Duplicate bulk surface sediment in stands of *Spartina alterniflora* were sampled for metagenomic analysis. Metagenome assembled genomes analysis showed that while the microbial community composition was largely similar among sites, the overall metabolic potential was dissimilar. Restored sites displayed a higher abundance of carbon and nitrogen cycling functions compared to reference sites, which mainly consisted of sulfur cycling. Although the restored sites developed sediment microbial communities that approached reference microbial composition, the differences in the metabolic functions suggest that even after 10 years, the restored sites were still in a transitional stage of development. The differences between the reference and restored sites were even more differentiated in the viral community's predicted host composition. Additionally, viruses potentially play a variety of roles within the sediment community, including population control and biogeochemical cycles participation through auxiliary metabolic genes. These results highlight the prolonged timeline of functional development in restored salt marshes and highlight the need to develop approaches to boost the development of soil microbial communities in newly created habitats.

Keywords Salt marshes · Restoration · Sediment · Microbial communities · Viral communities

Climate change, specifically sea-level rise and increased extreme weather events, is a global crisis that will directly affect coastal marine ecosystems and has already contributed to the loss or degradation of half of the salt marshes worldwide [1–3]. Salt marshes provide a variety of critical ecosystem services, including nursery habitat for multiple important fishery species, erosion protection, nutrient cycling, pollution reduction, and carbon sequestration [4–7]. Habitat restoration is a critical tool to mitigate salt marsh loss and maintain the ecological and economic integrity of the coastal zone. Current assessments of restoration success are often limited to vegetation evaluations [8] or to microbial community composition assessed using 16 S rRNA gene surveys [5, 7, 9, 10], but rarely include characterization of ecosystem functions.

Salt marshes host an abundance of microbes (upwards of 10^9 cells/g soil) that play important roles in biogeochemical cycling, stimulating highly productive, carbon-rich ecosystems [4, 5]. They cycle essential nutrients like nitrogen (denitrification, nitrification, anammox, and nitrogen fixation), carbon (aerobic and anaerobic detrital degradation), and sulfur (sulfate reduction and sulfide oxidation), which

✉ Kathryn L. Campbell
campbell.2910@osu.edu

[1] Department of Marine Biology, Texas A&M University at Galveston, Galveston, TX 77554, USA

[2] Department of Evolution, Ecology, and Organismal Biology, The Ohio State University, Columbus, Ohio, USA

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can promote organic matter degradation and carbon storage [5, 9, 10]. Microbial community composition can affect the overall functioning of these ecosystems [11] as well as the composition of the associated viral community. Viruses, in turn, can affect community structure through lysis and impact ecological functions through the expression of auxiliary metabolic genes (AMGs). In sediment, viruses account for 10–60% of microbial mortality [12] and are coupled with sulfate reduction and dissolved inorganic carbon (DIC) availability [13]. Very little is known about viruses in salt marshes [14], but in freshwater wetlands, the majority of viruses are bacteriophages (or phages, viruses infecting bacteria) [15] and have been shown to be involved in global geochemical cycles [16, 17].

Due to the vital role microbial communities play, their composition and functions could be used in assessing ecosystem health and functioning within restored salt marshes. However, the scope at which microbes have been explored is limited to either 16 S rRNA gene or other phylogenetic marker surveys [5, 7, 18], or the cycling of a single nutrient [11, 13]. Assessing the functional potential of the microbial community using, for example, metagenomics could reveal dominant microbial taxa that serve as a keystone species or recruiters of other organisms that would be vital for resilience of the soil community [19]. Additionally, it has been suggested that shifts in microbial communities occur prior to shifts in vegetation, highlighting microbes as potential indicator species for resilience and ecosystem shifts in response to climate change [5]. Microbial communities are therefore essential for determining the health and ecosystem functioning of restored marshes.

In this study, we used metagenomics of bulk surface sediments of salt marshes that were the subject of a large restoration project (completed in 2010) in West Galveston Bay, Texas, USA, to evaluate ecosystem functioning. We aimed to characterize the dominant microbial communities of reference and restored salt marshes. We also characterized the viral communities and identified the potential role of viruses in coastal wetlands. Assessing the microbial communities of both reference and restored salt marshes will aid in understanding the dynamics of coastal marshes, which is imperative to evaluating the outcomes of restoration efforts and the factors that affect the resiliency of these important ecosystems threatened by climate change.

Materials and Methods

Sampling

Galveston is an over-developed industrial shipping and cruise port, that is subject to annual hurricane seasons, oil drilling operations, as well as urban and agricultural runoff from the San Jacinto and Trinity Rivers. Parts of Texas' Gulf Coast have already experienced ecosystem shifts and loss of salt marshes due to mangrove expansion [20, 21], which is projected to worsen in the near future. Understanding how well ecosystem functioning has been restored in a vulnerable coastal marsh on Galveston Island was the motivation behind our sampling site choice. Jumbile Cove, located on the west end of Galveston Island, TX, was part of a massive restoration effort to combat the rapid loss of intertidal wetlands to relative sea level rise due to a lack of sediment accretion and wave-induced erosion in the Galveston Bay system [22] (Fig. 1). The restoration effort was completed in October 2010 and consisted of depositing dredged fine

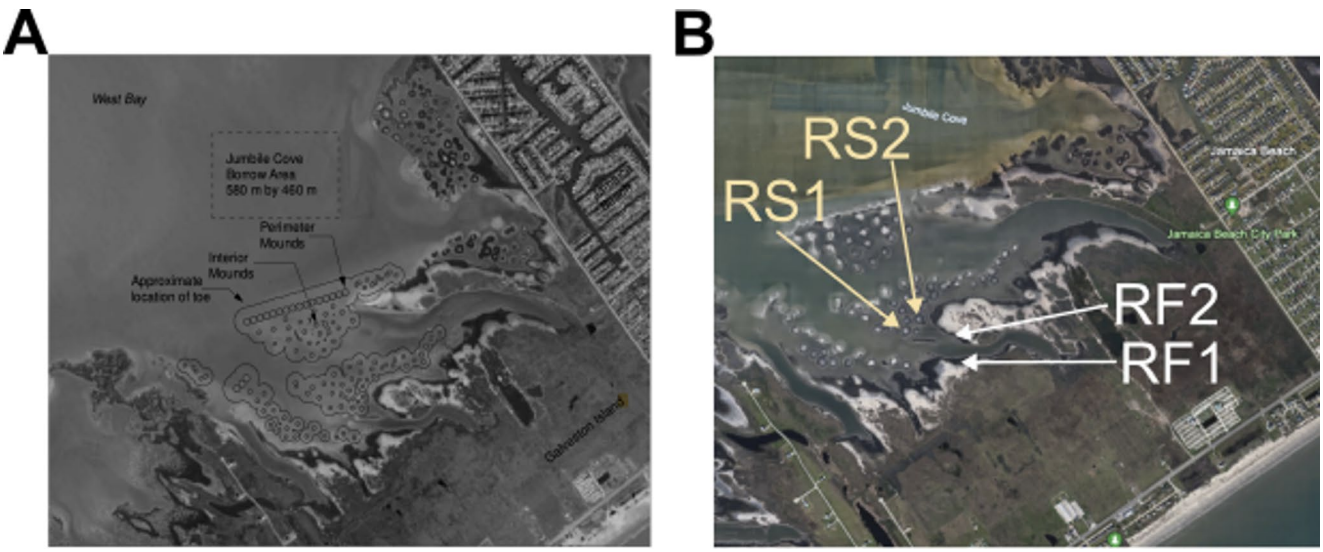

Fig. 1 Aerial views of Jumbile Cove, Galveston, TX (**A**) The layout for restored marsh sediment planned for Jumbile Cove [Image from Augustin et al. (2011)]. (**B**) GoogleEarth image of Jumbile Cove indicating areas used for reference (RF1&2) and restored sites (RS1&2)

sand from the native bay bottom to create both perimeter and interior mounds planted with smooth cordgrass, *Spartina alterniflora* sourced from a nursery located in Baytown, TX [22, 23] (Fig. 1A). The current status of Gulf of Mexico salt marshes are projected to be threatened by sea-level rise within the next two decades [2]. Jumbile Cove fits within this 20 year time frame. Reference sampling sites consisted of marsh along Galveston's natural shoreline just south of the interior mounds; restored sampling sites targeted the interior mounds (Fig. 1B).

We sampled two sites for each type of marsh sampled in a 30 m transect that was 2–3 m from the water's edge. Quadrats (0.25 m^2) were placed every 5 m and used to assess percent cover (total and for each plant species) and to take two benthotorch readings to determine the benthic microalgae biomass (five samples of each). The average values of each of the sites with their associated standard error are in Table 1. Bulk surface sediment samples, at a depth of ~2.5 cm, were taken every 10 m (three samples total) per site for microbial analysis. The collected samples were placed into sterile 50 ml centrifuge tubes and Whirlpak bags and stored at 4 °C until back in the lab for further DNA extractions.

DNA Extraction and Sequencing

To determine the microbial communities associated with each site, two samples from each site were selected for metagenomic analyses (total of eight samples; four reference, four restored). Total DNA was extracted using Qiagen PowerMax Soil kit from 10.8 to 12.9 g of sediment. The DNA extracts were further purified using Cytiva Sera-mag SpeedBeads. The DNA (1.1–2.3 μ g per sample) was sent for sequencing to the Texas A&M AgriLife Bioinformatics and Genomics facility for library preparation and Illumina sequencing using an S4 flowcell with ~32 M reads per sample.

Bioinformatic Analyses

Quality control of the sequences was performed using BBtools by first trimming the adapter sequences (bbduk), then removing contamination (bbduk), and masking of low-complexity regions (bbmask) [24]. Sequences from all samples were *de novo* assembled using MEGAHIT v1.2.8 [25] with '--presets meta-large'. Prodigal v2.6.3 [26] was used with setting '-p meta' for predicting open reading frames and then annotated for functions with DIAMOND v2.0.15 [27] using a blastP search [28] with settings '--max-target-seqs 10 --evalue 0.00001 –sensitive'. The reads that passed quality control of each sample were then mapped back to the co-assembly with bwa-mem2 v2.2.1 [29] and MetaBAT2 v2.14 [30] was used with default settings for binning sequences into metagenome assembled genomes (MAGs). CheckM [31] was used to determine MAG quality in terms of completeness ($\geq$ 50%) and contamination/redundancy (<10%). Bins taxonomy was determined using anvi'o's 'anvi-estimate-scg-taxonomy' function [32]. METABOLIC v4.0 [33] was used to determine the metabolic capabilities of MAGs, with default parameters. phyloFlash was used on the raw reads with default parameters to identify small subunit ribosomal RNA sequences and determine the composition of the metagenomic datasets [34].

Viruses were called using checkV v1.0.0 [35] and VIBRANT v1.2.1 [36] with default parameters and VirSorter2 v 2.2.4 [37] with the setting '--high-confidence-only' all using the assembled contigs. The viral calls were concatenated and deduplicated from the three software. The resulting deduplicated viral sequences were clustered using cd-hit-est v4.8.1 using '-c 0.95 -aS.85' to get viral operational taxonomic units (vOTUs). The vOTUs were further annotated for AMGs using DRAM-v [38] and assigned taxonomy using GeNOMad v1.5.2 [39]. Host predictions were performed through iPHoP v1.3.0 [40] using default parameters. Analysis and graphical representations of data were created using packages in RStudio [41] and ggplot2.

Statistical Analyses

A PERMANOVA was performed for both the MAGs and viral communities using Bray-Curtis distance matrix and adonis2 of the vegan package [42] in R [41]. To assess the differentially abundant MAGs between sites the community was assessed using a linear discriminant analysis effect size

Table 1 The average percent plant cover and benthic microalgae biomass (µg/cm^2) for reference and restored sites

Site	Site type	Total plant cover	S. alterniflora	B. maritima	Salicornia sp.	Cyano	Green Algae	Diatoms	Total conc.
RF1	reference	61 (6.2)	60 (6.5)	1 (1)	0 (0)	ND	ND	ND	ND
RF2	reference	72 (8.5)	71 (8.3)	0.2 (0.2)	0.8 (0.5)	0.132 (0)	0.051 (0)	0.845 (0.1)	1.028 (0.1)
RS1	restored	56 (8)	33 (7.2)	1.4 (0.6)	21.6 (11.6)	0.19 (0.1)	0.01 (0)	1.063 (0.3)	1.263 (0.3)
RS2	restored	66 (11.7)	54 (11)	0 (0)	12 (8.7)	0.314 (0.1)	0.02 (0)	1.617 (0.4)	1.951 (0.4)

Values are averages of the five samples taken with standard error in parentheses; S. alterniflora: Spartina alterniflora, B. maritima: Batis maritima, Cyano: Cyanobacteria, Total conc.: total concentration of benthic microalgae biomass, ND: Not determined

(LEfSe) [43]. The vOTU Bray-Curtis dissimilarity matrix used to generate a nonmetric multidimensional scaling (NMDS) plot using ggplot2 [44] in R.

Results and Discussion

Microbial communities are vital components of salt marshes. The various ecosystem functions, such as carbon sequestration, erosion and flood protection and nutrient cycling are all fueled by the metabolic processes of microbes. Despite being so integral to healthy salt marsh ecosystems, microbial communities are rarely assessed beyond the 16 S rRNA gene level as a way to assess ecosystem functioning within restoration sites. Metagenomics can assess the functional potential in addition to revealing the microbial community composition. The restoration sites that were used for this study have only been surveyed for performance in terms of the engineering constraints used to create them (e.g. subsidence of restored mounds) [22, 23]. This is the first attempt at assessing microbial (bacterial, archaeal, viral) composition and function between the reference and restored sites to assess if the restoration efforts were successful in restoring ecosystem functions. A successful effort being a restored marsh sediment that taxonomically and functionally resembles reference marsh sediment. Assessing the bacterial, archaeal and viral communities creates a unique comparison between the restored and reference sites that includes not only taxonomy but metabolic contributions and virus–host interactions that affect potential metabolic functions.

MAG Taxonomic Composition and Metabolic Potential Accentuate Key Differences Between Sites

A total of 21,476,162 contigs between reference and restored sites were assembled. The community composition largely consists of classes from phyla Proteobacteria, Desulfobacteria, and Bacteroidia (Fig. 2A), with higher diversity observed for the reference sites, although not statistically significant (p-value > *0.05*) (Fig. 2B). The functional potential of the whole metagenomes was similar between each site (Fig. 2C).

From the contigs 135 metagenome assembled genomes (MAGs) of medium- to good-quality ($\geq$ 50% complete, $\leq$ 10% contaminated) were assembled, representing the dominant microbial members at both sites (7.5–14.6% of mapped reads). For the purpose of relating metabolic potential to species within the community, the remainder of the analysis will focus on the MAGs. Taxonomic composition (Fig. 3A) and functional potential (Fig. 3B) were highly similar at both sites. Carbon utilization pathways, nutrient cycling (nitrogen, sulfur) and oxygen metabolisms were present at both sites. The high degree of similarity between our two sampled sites indicates that, at time of sampling, the restored and reference sites had similar functions.

Despite these similarities, a PERMANOVA analysis showed that the overall taxonomic composition was statistically different (p-*value = 0.022*). To further determine which MAGs were more abundant at each site, we used a linear discriminant analysis effect size analysis (LEfSe). There were 26 and 42 MAGs statistically significantly more abundant in the reference and restored sites, respectively (Fig. 4A). Reference sites having more carbon cycling associated genes could indicate a more stable and established community that is associated with organic matter degradation and carbon sequestration. The restored sites were enriched in MAGs with functional investment in sulfur cycling, oxidative phosphorylation and oxygen metabolism (Fig. 4B), while the reference sites were enriched in MAGs invested primarily in carbon related metabolism, complex carbon degradation, fermentation and C1 metabolism (Fig. 4B). Sulfur cycling accounts for most of the respiration processes in salt marshes, typically in the form of sulfate reduction, which links organic matter degradation to the terminal organic carbon oxidation to CO_2 [45, 46]. Restored sites having higher abundance of sulfur cycling metabolisms suggests that the community is predominantly investing in the major characteristic biogeochemical cycle that drives the other cycles within the marsh. The higher abundance of oxidative phosphorylation and oxygen metabolisms could indicate larger demands for energy/respiration as well as adaptation to low-oxygen conditions. These results indicate that the marsh may not be as functional as reference marshes as similar results have been shown in marsh loss areas, where exposed organic matter lead to photo-oxidation and aerobic microbial degradation, increasing emissions of carbon dioxide and limiting carbon sequestration [47, 48]. Together these genes could be an indication that restored sites, even after 10 years, are still in a transitional state where there the community is adapting to the variable conditions of tides, where high tide would limit oxygen availability. Wetland restoration efforts often consist of dredge slurry amendments, frequently behind erosion control structures (e.g., breakwaters or sills), followed by planting of flood-tolerant species such as the grass *Spartina alterniflora* [49]]. Restored sites develop through successional processes, and therefore display a range of biotic and abiotic interactions.

Viral Assemblages and Host Prediction Further Highlight Differences Between Sites

Viruses have been modeled to have stimulatory effects on whole ecosystems, including increased organic matter recycling, reduced transfer to higher trophic levels, and

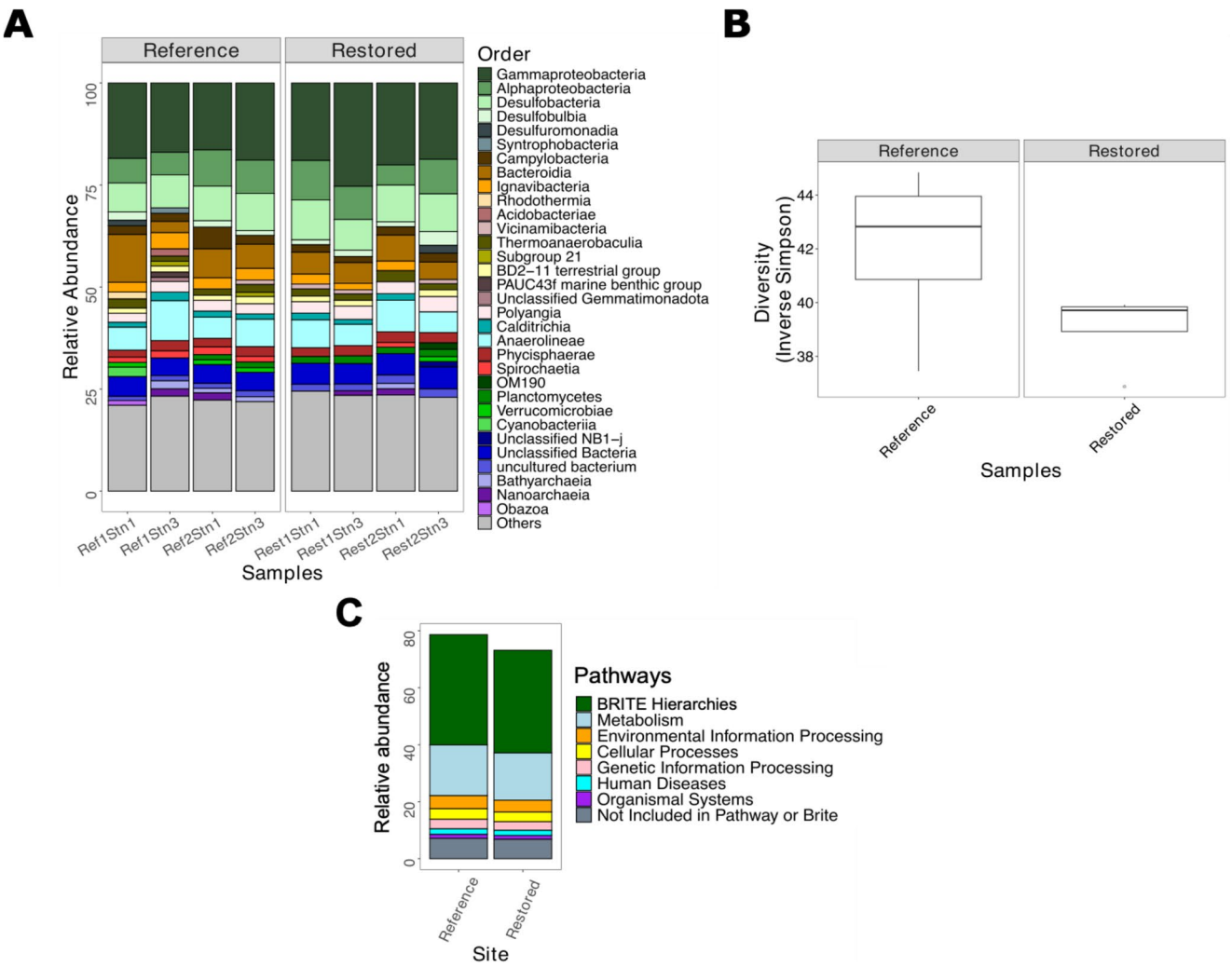

Fig. 2 (**A**) Relative abundance of the metagenome (**B**) Inverse Simpson index of diversity for the metagenome between reference and restored sites (**C**) Relative abundance of the proteins found in various KEGG pathways in the metagenome

increased net primary productivity [50]. Few assessments of the viral assemblages found in salt marshes are available, despite the importance of viruses in terrestrial, marine, and freshwater environments. In freshwater marshes, viral abundance has been positively correlated with critical carbon and respiration processes within marsh sediments [13] as well as shown to account for a high rate of microbial mortality [12, 13]. The viral community identified in the Galveston marshes consisted of 587 viral operational taxonomic units (vOTUs) $\geq$ 5 kb that were different at 95% ANI. The majority of the viral assemblages at both sites were unclassified or belonged to unclassified orders of Caudoviricetes (Fig. 5A), suggesting a unique composition of viruses, which is consistent with other marsh sediment [51]. While the taxonomy of the viral assemblages, as observed with the microbial community, were highly similar, the sites were determined by PERMANOVA to be significantly different (p-value= 0.026, S Figure 1) and diverged through the predicted hosts (Fig. 5B). Different virus–host interactions can indicate certain aspects of the host's physiology, growth rate, and/or abundance, as well as affect biogeochemical cycling with the use of AMGs and contributions to the dissolved organic matter (DOM) pool through lysis [52].

A notable difference in the restored sites is the prediction of Nitrososphaerales, an ammonia-oxidizing archaea [53], as a host, which is also observed in the MAGs, along with other diazotrophic taxa within the Gammaproteobacteria. Nitrososphaerales can have rapid localized growth due to enhanced nutrient uptake [54], suggesting different community dynamics with regards to nitrogen between the restored and reference sites. Additionally, the presence of Nitrososphaerales could indicate a response to plant interactions. Marshes with stands of *Spartina alterniflora*, the dominant plant in Texas salt marshes, as well as other vegetated marsh sediments have found high rates of nitrogen fixation as well as a depletion of ammonia in the top 10 cm of sediment

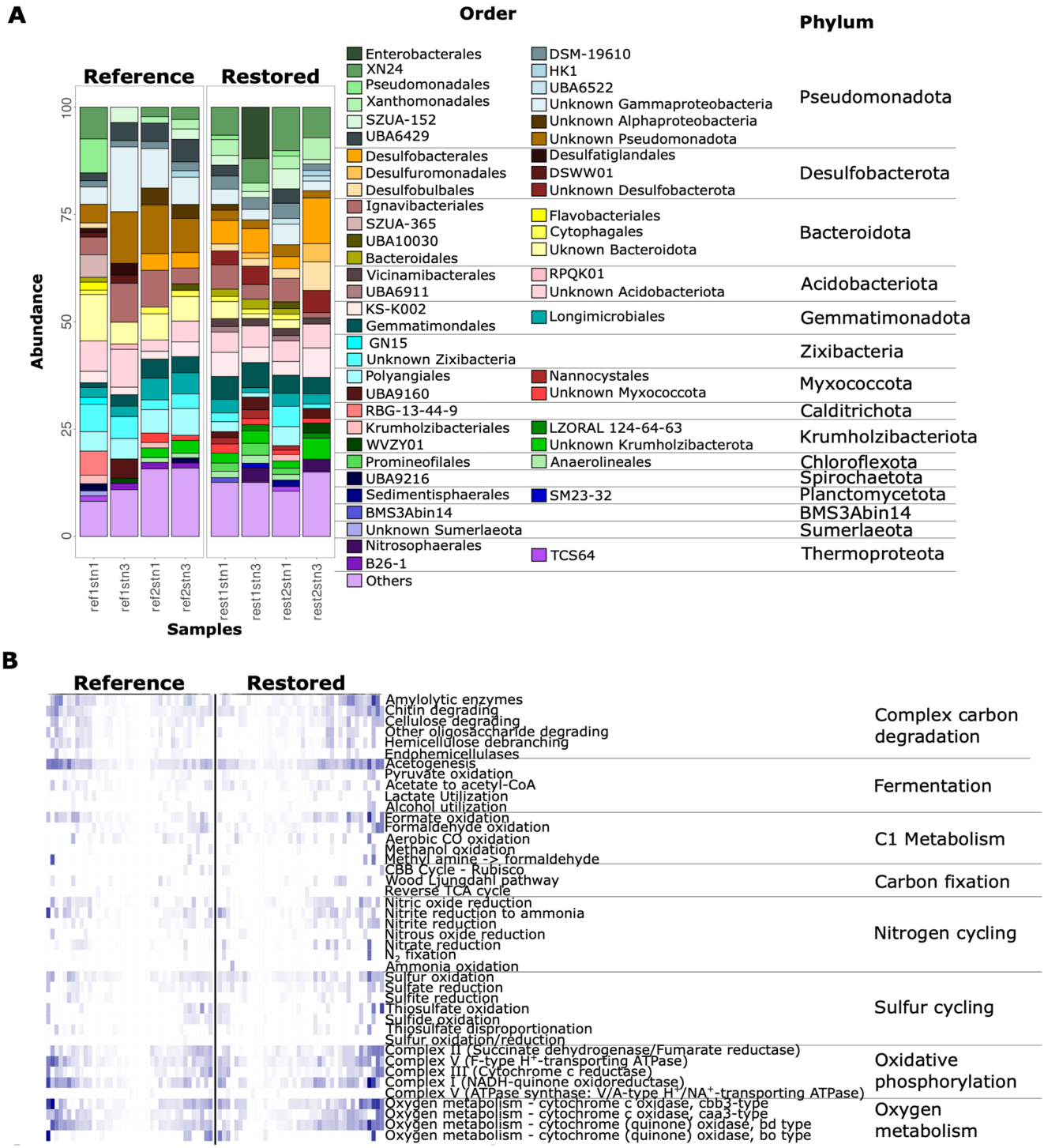

Fig. 3 (**A**) Taxonomic and abundance comparison of the medium- to good-quality MAGs between the reference and restored sites (**B**) Functional potential comparison of the 135 shared MAGs (present on the x-axis) between the reference and restored sites

[55–57]. Furthermore, ammonia oxidizing archaea such as Nitrososphaerales and other archaea are known to help plants adapt to high salinity or temperature, as well as support plant productivity and nutrient management [58]. The higher demand for ammonia-oxidizing archaea could be in response to plant demand during establishment [59, 60], i.e., transplantation from a nursery into previously submerged sediment. Here, we only looked at bulk surface sediment, but it is common for coastal wetland plants to have symbiotic relationships with microbes that can fix nitrogen, which

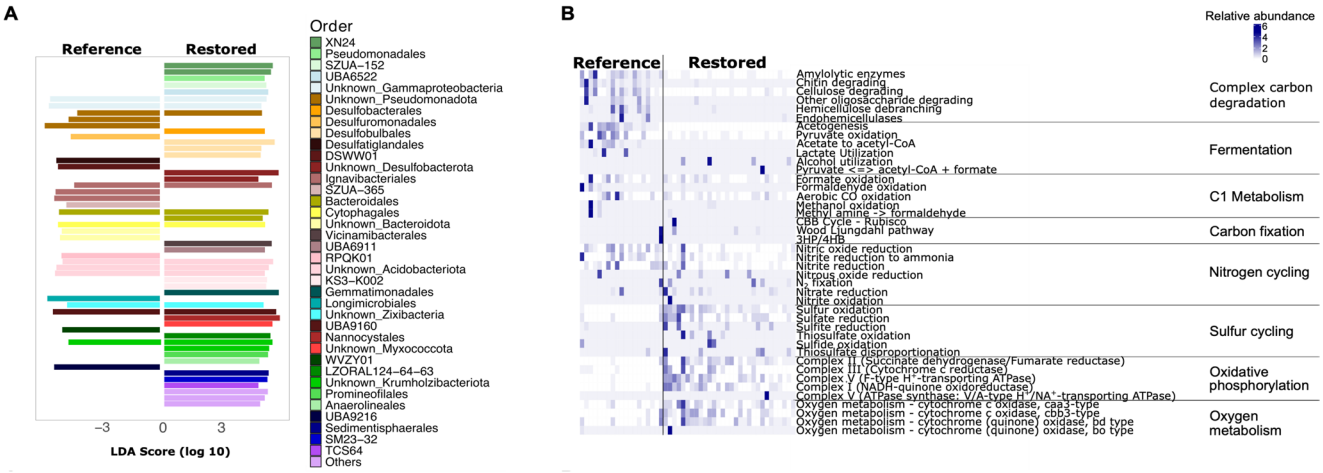

Fig. 4 (**A**) The 26 and 42 MAGs differentially more abundant in the reference and restored sites, respectively (**B**) Functional potential between the differentially abundant MAGs (x-axis) between the reference (26 MAGs) and restored sites (42 MAGs)

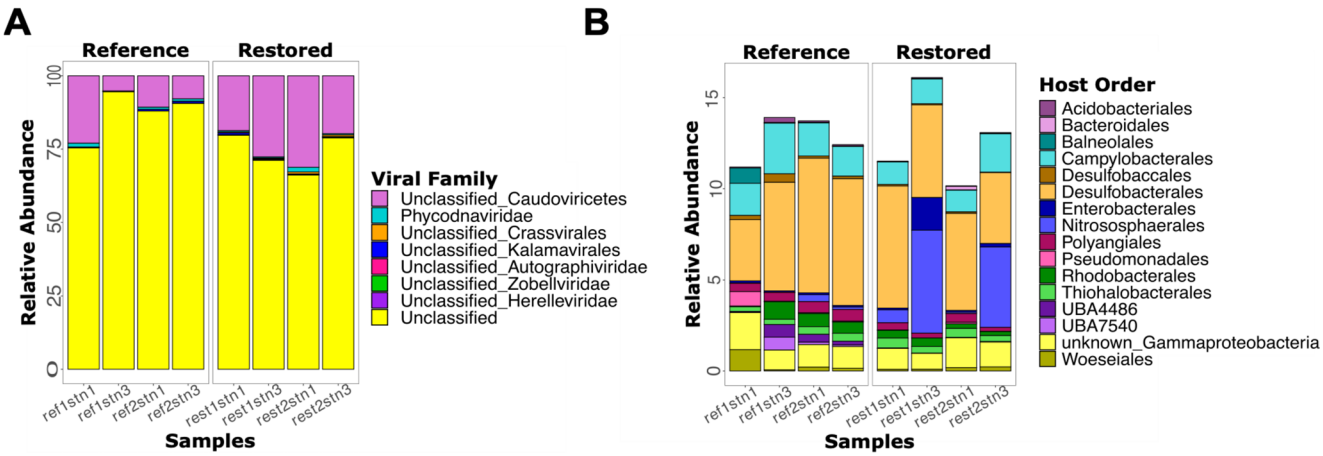

Fig. 5 (**A**) Relative abundance of the viral assemblages identified in each of the sampling sites for the whole viral assemblage (**B**) Relative abundance of the hosts predicted for the various vOTUs. ref: Reference sites; rest: restored sites; stn: sampling station

is usually a limiting nutrient in coastal wetlands [61] or use the carbon provided by the roots for decomposition, specifically for sulfate reduction [62, 63].

Sulfur cycling taxa were prevalent at both sites, with predicted hosts in Desulfobacterales and Campylobacterales [64] present in high abundance; however, only Desulfobacterales were observed in the MAGs. Unclassified Gammaproteobacteria, Rhodobacterales (class Alphaproteobacteria) and Polyangiales were present in the MAGs, and may be contributing to carbon turnover within the sediments. Thiohalobacterales, known as a chemoautotrophic clade, was prevalent in the MAGs and could be contributing to carbon fixation within the sediments [65]. The predicted hosts suggest that taxa capable of carbon degradation and/or carbon fixation were important in both sites, as are taxa capable of sulfur cycling. The taxa predicted as hosts accounted for a small proportion of the microbial community. Their virus–host interactions may be following a boom and bust pattern, where they are abundant locally and lytic infections potentially alleviate nearby competition for nutrients within the marsh sediment [15, 66, 67]. Additionally, the differences in predicted host could be an indication of environmental selection as a result of habitat characteristics that are known to shape wetland viral communities [51, 68]. Overall, the viral assemblages further highlighted the community differences between the sites while providing another potential indicator that restored sites are in a transitional state.

Viral AMGs Reveal Viruses Play a Variety of Roles within Marsh Sediment

Viruses depend on the physiological state of their hosts and on host machinery to replicate and produce progeny. As a

means of acquiring host resources and machinery, viruses can encode genes that exploit existing machinery in terms of upregulating a particular pathway or providing a novel pathway. In this way, viruses are able to impact biogeochemical cycles within the marine environment. To better understand the ecological impact of viruses within salt marshes, we analyzed virally encoded AMGs. Out of 587 vOTUs, 23 (3.9%) contained AMGs associated with a variety of putative functions. The pathways targeted by AMGs consisted of nucleotide metabolism (viral DNA synthesis); carbohydrate metabolism (plant polymer degradation [69] and the citrate cycle); metabolism of cofactors and vitamins (production of polyamines that contribute to the labile dissolved organic nitrogen pool in coastal waters [70]) and signaling and cellular processes (degradation of sulfated polysaccharides [71], lipopolysaccharide or exopolysaccharide biosynthesis and fatty acid degradation, and antibiotic production [72]). While we detected few AMGs (11.3% of annotated viral proteins), our data suggest that viruses may play a variety of roles, including population control of the microbial community and affecting metabolic functions that include carbon and energy production, sulfur and nitrogen related processes. The identified AMGs are putative and the majority have not been experimentally confirmed; therefore they may perform differently than host homologues [52]. Overall, viral AMGs potentially reflect the complex dynamics within the microbial community that could have community-wide implications [52].

Metagenomics to Assess Marsh Restoration

This study provides a novel approach to assessing salt marsh restoration success, compared to only vegetation assessments or microbial surveys, by focusing on the microbial community structure, function, and dynamics. As microbial communities are at the base of the food web for salt marshes as well as indicators of plant community shifts [5], they serve as an important metric for ecosystem functioning. Employing a metagenomic approach revealed many different dimensions to the functioning of salt marshes and provided deeper understanding of the microbial community dynamics of coastal marshes. Additionally, with metagenomics, we were able to tease apart the functional differences between highly similar sites. However, we were limited in our ability to know if or when the communities were using the identified genes. Metatranscriptomics would be needed to help fill this gap, by providing the gene expression of the community as a whole. Furthermore, to better quantify the impact of the viral community, assessing the microbial mortality due to viral lysis and/or more targeted virus-host interactions would be necessary.

Conclusion

Our analysis indicated the community composition was largely similar between the sites but differed in overall metabolic potential, which was supported by differences in the viral community and predicted host composition. Overall, the restoration project achieved sediment microbial composition and functions that closely resembled that of the reference marshes. However, the differences in the metabolic functions may indicate that restored sites are still in a transitional stage of development [73], which may affect the structure and activity of the deeper subsurface communities with implications to plant root health and ecosystem resilience to future disturbances. With coastal wetlands becoming increasingly susceptible to loss from anthropogenic disturbance and sea-level rise, it is vital to understand the outcomes of restoration efforts for important wetland ecosystems like salt marshes, as this informs better restoration strategies and helps identify conservation priorities to mitigate localized habitat loss.

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Author Contributions KLC, JML, and ARA designed the study. Sample collection was performed by KLC and ARA. KLC performed all sample processing, i.e., DNA extractions, DNA purification, and bioinformatics and statistical analyses. KLC wrote the manuscript with input and revisions from all co-authors.

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Data Availability The DNA sequences have been deposited in the Sequence Read Archive (SRA) database of NCBI under BioProject ID: PRJNA1263884.

Declarations

Competing interests The authors declare no competing interests.

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