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Sediment microbiomes associated with critical habitat of the Juvenile American Horseshoe Crab; *Limulus polyphemus*

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ABSTRACT

Plumb Beach, Brooklyn, New York in USA is an important horseshoe crab breeding and nursery ground that has experienced substantial anthropogenic influence, including pollution, erosion and subsequent restoration. Since little is known about the relationship between sediment microbial communities and juvenile horseshoe crab survival, next generation sequencing was used to characterize and compare the sediment microbiome of three distinct areas of Plumb Beach:- a tidal creek with abundant juveniles, East Beach with moderate number of juveniles, and West Beach- a highly disturbed area where juvenile crabs are rarely seen. The microbiome of juvenile crab intestinal content (both dissected gut content and fecal flush content) from the tidal creek site was also examined. The results showed that in our 2017 survey, the overall dominant sediment orders at all beach sites were Vibrionales (30%), Flavobacteriales (22%) and Alteromonadales (21%). Although alpha diversity was similar among the three beach sites, Bray-Curtis distances assessed by Permanova revealed significant differences in Beta diversity, with a unique microbial assemblage found in the tidal creek. Both crab gut and fecal flush samples did not sequence well, showing low species diversity and very high variability. This study is the first to use next generation sequencing to characterize Plumb Beach sediment microbes and the first attempt to examine the gut microbiome of juvenile horseshoe crabs. This information will contribute to understanding the relationships between sediment microbial assemblages and juvenile crab populations within this important urban habitat.

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50	6	2

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INTRODUCTION

Plumb Beach, at the mouth of Jamaica Bay in Brooklyn, New York is an important spawning, nesting and foraging site for the American horseshoe crab (*Limulus polyphemus*) but has experienced a significant decline in habitat quality. It is part of Gateway National Park (founded in 1972), which has experienced 150 years of development including dredging and filling, resulting in increased water depth and shifted tidal flux. Increases in stormwater runoff, water temperature and wastewater pollution have resulted in nitrogen loading (Benotti *et al.*, 2007) and eutrophication of Jamaica Bay (Wigand *et al.*, 2014). In 2012, Super Storm Sandy further decreased water and sediment quality. Such anthropogenic disturbances are likely to impact many species that rely on this beach for habitat, including the American horseshoe crab. *Limulus polyphemus* is a key species in estuarine food webs (Botton, 2009) throughout its range from Maine to Mexico's Yucatan. Its importance as a food source for migratory shorebirds (Karpanty *et al.*, 2006), as a source of bait for eel and whelk fisheries, and use of its blood to detect bacterial contamination in medical supplies (Novitsky, 2009) has been well-documented. Catch restrictions have attempted to mitigate population declines in recent years (ASMFC, 2018). However, the loss of spawning and nursery habitat on sandy beaches due to erosion, sea level rise, increased storm frequency, pollution and shoreline hardening pose a greater threat to the survival of this species (Mattei *et al.*, 2015). *L. polyphemus* is listed as Vulnerable by the IUCN Red List of Threatened Species (Smith *et al.*, 2016). Only three larvae per 100,000 survive their first year of life on sandy nursery beaches under natural conditions (Botton and Loveland, 2003) and it takes 10 to 12 years for individuals to reach maturity (Smith *et al.*, 2009). Therefore, it is critical to understand all factors that may contribute to juvenile survival, including habitat quality and sediment microbial diversity. The population dynamics of juveniles, eggs, and spawning adult horseshoe crabs on Plumb Beach have been surveyed annually since 2009. Spawning adults prefer the calmer eastern portion (East Beach) to dig nests and lay eggs, perhaps because of the less compacted sediment in this area (vs the highly disturbed western flats, (Botton *et al.*, 2017). Juvenile crabs are also found predominantly on East Beach and in an adjacent tidal creek. Despite high interannual variation and patchy distribution of juvenile populations on the East Beach and Tidal Creek, these areas still support far more juveniles than the West Beach, where virtually no juveniles have been found despite several hatching events (Colon *et al.*, in press). Since juvenile horseshoe crabs rely on Plumb Beach sediment for both shelter and foraging, sediment microbial assemblages could be closely linked to juvenile crab survival. It is possible that the microbial community in these distinct areas at Plumb Beach would exhibit significant differences that may impact juvenile crab survival. Currently, there are no studies that have explored a possible connection between sediment microbiome and crab survival. Similarly, information about the gut microbiome of any of the four species of horseshoe crab has yet to be determined, and could be critical to understanding the health and survival of these vulnerable organisms. The aims of this study were to: 1) describe the sediment microbial community at Plumb Beach using next generation sequencing, 2) compare microbial community composition in areas with abundant juvenile crabs vs. those where juveniles are absent or less abundant; 3) describe the microbial community of crab fecal flush samples and gut contents. This study is the first to describe bacterial communities found in an important juvenile horseshoe crab habitat, as well as the first attempt to examine the gut microbiome of juvenile crabs. This juvenile horseshoe crab survey and sample collection for this study took place at Plumb Beach during the day time low tide in 2017.

MATERIALS AND METHODS

Description of the study area and context

Plumb Beach, at the mouth of Jamaica Bay in Brooklyn, New York (Fig. 1) is part of Gateway National Park (founded in 1972), that has experienced over 150 years of development including dredging and filling which has increased water depth and shifted tidal flux. Increased stormwater runoff, water temperature and pollution from four combined wastewater treatment plants have resulted in nitrogen loading (Benotti *et al.*, 2007) and eutrophication of Jamaica Bay (Wigand *et al.*, 2014). In 2011, Super Storm Sandy further impacted water and sediment quality.

Permit information

Collection of the 30 Plumb Beach sediment



Fig. 1: Geographic location of the study area in Plumb Beach study area in Brooklyn, New York, USA

samples, 5 juvenile horseshoe crabs (for gut dissection), and 6 juvenile horseshoe crab fecal flush samples used in this study was authorized by the National Park Service under NPS permit GATE-2014-SCI-0036 issued to Mark L. Botton.

Juvenile crab abundance survey

Three areas of Plumb Beach (Fig. 1) were surveyed for the presence of juvenile horseshoe crabs during the day time low tide on July 06, 2017 using a 10-minute timed visual surveys. Results were converted into catch per man hour of effort. Juveniles were collected, measured with dial calipers at the widest portion of their prosoma, then released back into the habitat. All juvenile horseshoe crabs used for gut and fecal analyses were collected from Tidal Creek.

Sampling locations

Tidal Creek (TC): (40°34'58.73" N, 73°54'55.2" W): A dune slack or hollow behind the foredunes that is surrounded by salt marsh and connected to a tidal creek inlet. Sediment in the tidal creek foraging ground is medium in size and moderately well sorted and coarse skewed. In 2017, the average catch for juvenile crabs was 40.6 crabs per hour of effort with prosoma widths ranging from 20mm to 93mm. These were predominantly from the slow moving and shallow portions of the tidal creek (designated at Tidal Creek A); virtually no juveniles were found in the deeper faster flowing areas (Tidal Creek B). East Beach: (EB) (40°34'55.0"N, 73°54'41,0" W): A calm, wide intertidal flat with a recovering population of juvenile horseshoe crabs (27.5 crabs per hour of effort; prosoma widths 11.7 to 43mm) most of which were found in a single shallow pool near the shore (East Beach A). Sediments were also collected in a nearby tide pool that contained no juvenile crabs (East Beach B). Sediment in the eastern tidal flats is fine, moderately well sorted and very fine skewed. West Beach (BW): (40°34'58.9"N, 73°55'37'3"W): This beach area experiences high erosion and was renourished with sand in 2012. The broad tidal flat is dominated by *Ulva* and mud snails (*Ilyanassa obsoleta*). Two distinct areas of West Beach were sampled: West Beach A: a still, deep narrow channel behind a breakwater with no crabs. West Beach B: an intertidal flat where one juvenile crab (0.2 crabs per man hour; prosoma width of 12.4 mm) was found in 2017. Sediment here is fine, moderately well sorted and very fine skewed making it more compacted and less oxygenated.

Water and Sediment properties

Water and sediment parameters were recorded in the field concurrent with sediment collection at each location. A YSI Professional Plus Pro Comm II was used to measure temperature, pH, orthophosphorus, ammonia and other water and sediment parameters (Table 1) following the manufacturer's instructions.

Table 1: Water and sediment properties taken on sediment collection day (July 6, 2017) and sediment structure (collected July 12, 2019). mg/L- milligrams/liter; mEq/L-milliequivalents/liter Ppt-parts per thousand;; mg/kg- milligrams/kilogram

Collection site	Tidal Creek A	Tidal Creek B	East Beach A	East Beach B	West Beach A	West Beach B
Water properties						
pH	7.73	8.28	7.97	8.27	8.33	8.53
Temperature (°C)	24.1	24.2	24.0	24.0	22.9	24.6
Dissolved Oxygen (mg/L)	11.6	12.5	8.8	10.1	2.7	10.5
Alkalinity (meq/L)	2.44	2.35	1.78	1.67	2.39	2.15
Salinity (ppt)	28.3	28.5	28.9	29.1	28.5	28.9
Orthophosphorus (mg/L)	0.111	0.105	0.175	0.129	0.100	0.073
Ammonia N (mg/L)	<0.010	<0.010	0.098	0.03	0.024	<0.010
Nitrite N (mg/L)	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
Nitrate N (mg/L)	0.134	0.02	<0.020	<0.020	0.022	<0.020
Sediment properties						
Orthophosphorus (mg/kg)	0.334	<0.177	0.446	0.399	0.882	0.941
Ammonia N (mg/kg)	5.18	5.73	4.65	3.99	6.54	4.60
Nitrite N (mg/kg)	<0.037	<0.035	<0.035	<0.035	<0.037	0.05
Nitrate N (mg/kg)	<0.248	<0.236	<0.235	<0.235	<0.245	<0.248
Total Solids (%)	80.4	84.0	84.7	85.0	78.8	79.2
Sediment Structure						
Grain size (µm)	234.01	236.13	419.25	230.85	348.58	256.63
Descriptor	Fine	Fine	Medium	Fine	Medium	Medium
Sorting	Moderately Well	Moderately Well	Poorly	Moderately	Moderately Well	Moderately Well
Skewness	Very Fine	Very Fine	Very Coarse	Very Fine	Coarse	Fine

Sediment samples for determining structure were collected on July 12, 2019 and analyzed by Mark Botton using GRADISTAT Version 8.0 (Copyright 2010 Simon Blott).

Sediment collection

When crabs were detected, sediment was collected nearby. Five subsamples were taken from each of the three beach regions (Tidal Creek, East Beach and West Beach) and two subsections of each region (A and B; described above). Samples were collected with plastic spoons and stored in soil sampling bags. Samples were kept in a cooler on ice for transport back to the lab and stored at -20°C until processed.

Gut contents (GC) collection

Five juvenile horseshoe crabs ranging in size (prosoma width) from 67 to 91.5 mm were transported to Queensborough Community College in a cooler, where they were stored at -80°C. Frozen crabs were thawed and dissected for total gut content. A 0.25 g subsample of gut content was reserved for DNA extraction; the rest was used for stereoscopic gut content analysis to determine juvenile crab diet (Hoffgaard, 2017).

Fecal flush (FF) collection

Fecal material was collected on August 8th 2017 from six live crabs located in the tidal creek. Trained staff from the Wildlife Conservation Society's New York Aquarium used 3ml of a 0.9% sodium chloride solution in a 3mm Monoject syringe with a 4 mm urethral catheter coated with Surgilube (per Aquarium vet staff protocol) to gently administer saline into the crab's cloaca, and then draw out the fecal content into a 15 ml conical tube. Crabs were immediately released back to the area where they were collected.

DNA extraction

Total community DNA was extracted from sediments, gut contents and fecal flush samples using the PowerSoil DNA extraction kit (Qiagen) according to the manufacturer's instructions. A 0.25g sample of sediment was used for each of the extractions. All DNA samples were stored frozen (-20°C) prior to shipping for DNA sequencing.

DNA sequencing

The bacterial communities were sequenced using Ilumina MiSeq targeting the V1-V3 region of the 16S rRNA gene (Molecular Research Labs, Shallowater, TX). Using the primers 27F/519R (AGRGTTTGATCMT-

GGCTCAG/GTNTTACNGCGGCKGCTG) each sample underwent polymerase chain reaction (PCR) using HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute; with a final elongation step at 72°C for 5 minutes. Following PCR, barcoded amplicon products from all samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were pair-end sequenced utilizing the Illumina MiSeq chemistry for a read length of up to 300 basepairs, following manufacturer's protocols.

DNA Sequence Analysis

Sequence files were imported into QIIME2 (v2018.2; Bolyen *et al.,* 2019). For each file, samples were demultiplexed, depleted of barcodes and primers, and trimmed of poor-quality bases with an average read length of 290 basepairs remaining. Chimeras were filtered out using dada2 (Callahan *et al.*, 2017) and amplicon sequence variants (ASV) determined with the statistical error correction model (set at 50000 reads). Resulting data tables and representative sequences were merged in with QIIME2. Sequences were aligned using MAFFT (Yamada *et al*., 2016); fasttree (Price *et al.*,2010) was used to create a rooted phylogeny. Taxonomy was assigned using a pre-trained Naïve Bayes classifier from Greengenes (13_8) 99% operational taxonomic units' database. The generated taxonomy table was then exported as a plain text file to manually remove non-target taxa sequences (unassigned, archaea, chloroplasts, and mitochondria) as well as amending the taxonomy assignment for the bacteria Family *Pseudoalteromonadaceae* from within the Order Vibrionales to the correct Order Alteromonadales. The resulting taxonomy table was imported back into QIIME2 for downstream bacterial community analysis. Alpha diversity metrics (Chao1, Shannon-Wiener index and Faith's phylogenetic diversity) were calculated. ANOVA was used to determine if alpha diversity significantly varied across collection sites. Beta diversity was calculated using Bray-Curtis distances between groups followed by a principal coordinate analysis (PCoA), with significance assessed using Permanova (Permutational analysis of variance). Constrained Analysis of Principal Coordinates (CAP), (Anderson and Willis 2003; Xia *et al.*, 2018) was completed for comparing sediment bacterial communities with sediment chemistry. ASV that were found in at least half of the sediment samples were identified using National Center for Biotechnology Information (NCBI) BLASTn. Since there was high variation in sequences per sample, a sequence depth of 2000 sequences for sediment was used to normalize comparisons. Significant differences between bacterial communities at sample sites were assessed using pairwise Kruskal-Wallis test. High variability within the horseshoe crab digestive tract samples limited analyses to general trends based on samples normalized to 500 sequences.

RESULTS AND DISCUSSION

Water and sediment chemical properties

All measured sediment and water chemical analyses can be found in Table 1. Water quality data revealed some variability between locations. Salinity

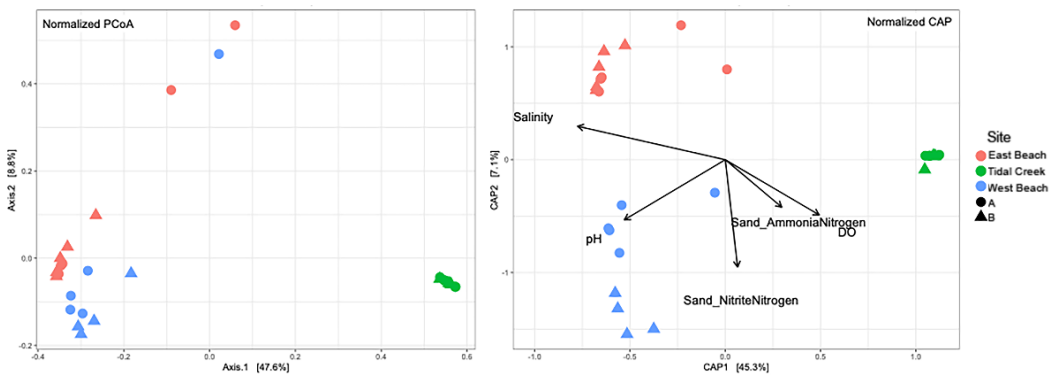

Fig. 2: Principal Coordinates Analysis using the Bray-Curtis distance matrix of normalized sediment bacteria communities (a-left) and the Constrained Analysis Plot (CAP) evaluating the influence of environmental parameters (b-right).

was slightly lower (28.3 and 28.5 ppt) at Tidal Creek versus elsewhere (28.5 to 29.1 ppt). Nitrate levels were also considerably higher here (0.134 mg/L) compared to elsewhere (0.020 to 0.022 mg/L). pH was lower in Tidal Creek A where the juvenile horseshoe crabs were abundant (7.73) compared to the other locations (7.97 to 8.53). Dissolved oxygen was somewhat higher in the tidal creek overall (11.6 and 12.5 mg/L) compared to elsewhere (2.7 to 10.1 mg/L). Sediment orthophosphorus appeared slightly lower in the tidal creek (<0.177 and 0.334 mg/kg) compared to elsewhere (0.399 to 0.941 mg/kg). Although water nitrite levels in water were consistent throughout sites, higher sediment values were observed for sediment nitrite in West Beach B (0.05 mg/kg) than in other sediments (<0.037 mg/kg).

Sequence analysis

Initial demultiplexed samples had a total of 3,958,102 sequences with a range of 4,853 to 676,623 sequences per sample. Following QIIME2 and dada2 sequence processing and ASV determination, the merged data table had 41 samples with 258,545 sequences that resulted in a total of 2,750 ASV or unique bacteria present. When non-target sequences were removed, the filtered data table had 246,054 sequences and 2,665 ASV - this is the data set used for all further analyses.

Sediment profiles

Sediment samples had the greatest number of sequences for each sample; however, there was high variability. Following the normalization to 2000 sequences, two samples (East Beach 5A and West Beach 1A) were excluded from the analyses. Sediment bacterial communities were similar across the beach sites, but had enough differences to distinguish individual sites (Fig. 2). Replicates from the same beach regions clustered together but were distinct from each other (PCoA, Fig. 2a). Environmental conditions of salinity, pH, dissolved oxygen, and sediment nitrogen (nitrite and ammonia) appear to correlate with these differences (CAP, Fig. 2b). Salinity has been identified as an important factor influencing intertidal bacterial assemblages (Piccini and Garcia-Alonso 2015). However, since only one measurement was obtained for each parameter, further investigation would be needed to confirm correlations between water/sediment chemistry and microbial community composition. Few differences were detected when the areas were compared with and without juvenile crabs within each site. For example, Tidal Creek A (abundant crabs) and B (few crabs) shared similar taxonomic profiles, indicating

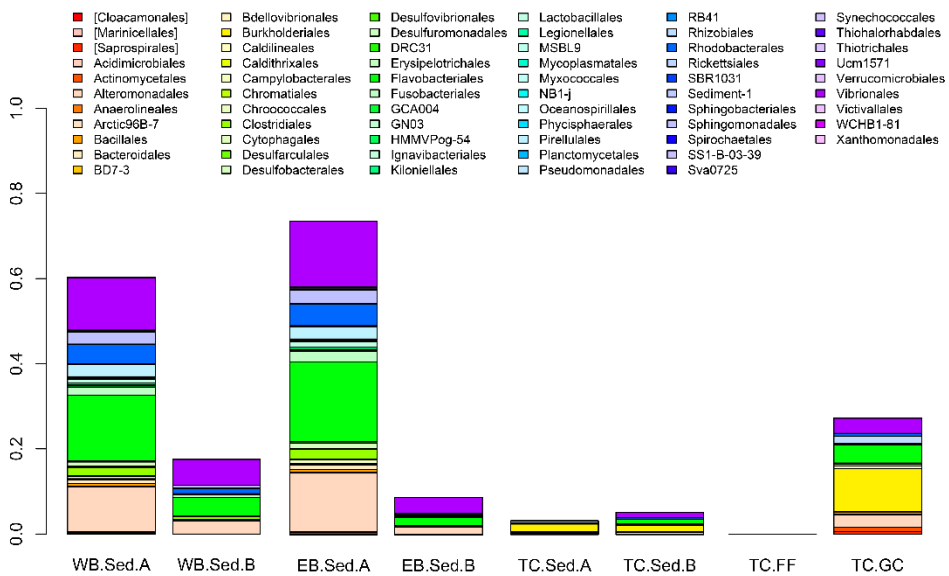

Fig. 3: Sample site bacterial community organized by bacterial Orders and represented as the relative abundance excluding unidentified taxa. Note: Tidal Creek fecal flush (TC FF) was composed of almost all unidentified taxa. TC-Tidal Creek; EB- East Beach; WB- West Beach; GC- Gut Content

that the presence or absence of juveniles was not correlated with any particular sediment microbial taxa.

The distribution of taxa at the order level for all sediment, gut content and fecal flush samples are shown in Fig. 3. All sediment samples had a high fraction of unidentified taxa, ranging from 26 to 96%. When unidentified taxa were removed, sediment samples had between 8 and 64 orders represented.

The species richness for all sediment sites were not significantly different based on the observed ASV (p=0.1967) and for the Shannon-Wiener index (p=0.6458; Fig. 4). The current study revealed lower diversity in Plumb Beach sediments than in other studies of benthic and pelagic ocean systems (Nogales *et al.,* 2011). It is possible that this difference is due to the lower sequencing efficiency in the study. Previous studies have identified factors that influence alpha diversity in beach sediments. For example, a comprehensive study of 11 marine and freshwater beaches (Staley and Sadowsky, 2016) showed that distance from shoreline was the most important factor, with diversity decreasing away from the shoreline. While the present study did show a unique microbial community composition in the Tidal Creek sediments that are furthest from the shoreline (Fig. 2), alpha diversity was similar in these sediments as compared to those taken closer to shore (Fig. 4).

When phylogenetic relatedness of ASV of each site was considered, differences across sites were present (Faith's PD, p=0.0193). This trend was further supported by the Bray-Curtis distances between sample sites: all were significantly different (Permanova, p=0.001) with Tidal Creek being most distinct from East and West Beach. After normalizing to the same number of sequences for each sediment sample, 18 identified bacterial orders remained (Fig. 5). The most common bacterial orders for all sediment was Vibrionales (30% of all remaining sequences), Flavobacteriales (22%) and Alteromonadales (21%), none were significantly different in abundance at each site (ANOVA p = 0.546, p=0.532, p=0.506, respectively). Alteromonadales and Vibrionales were relatively abundant at West and East Beach sites and in much lower abundance in the Tidal Creek. Notable differences in sediment profiles include the presence of three orders: (Saprospirales, Actinomycetales, and Rhizobiales) found only in Tidal Creek A sediment, and four orders (Alteromonadales, Camplylobacterales, Cytophagales and Rhodobacterales) found only in

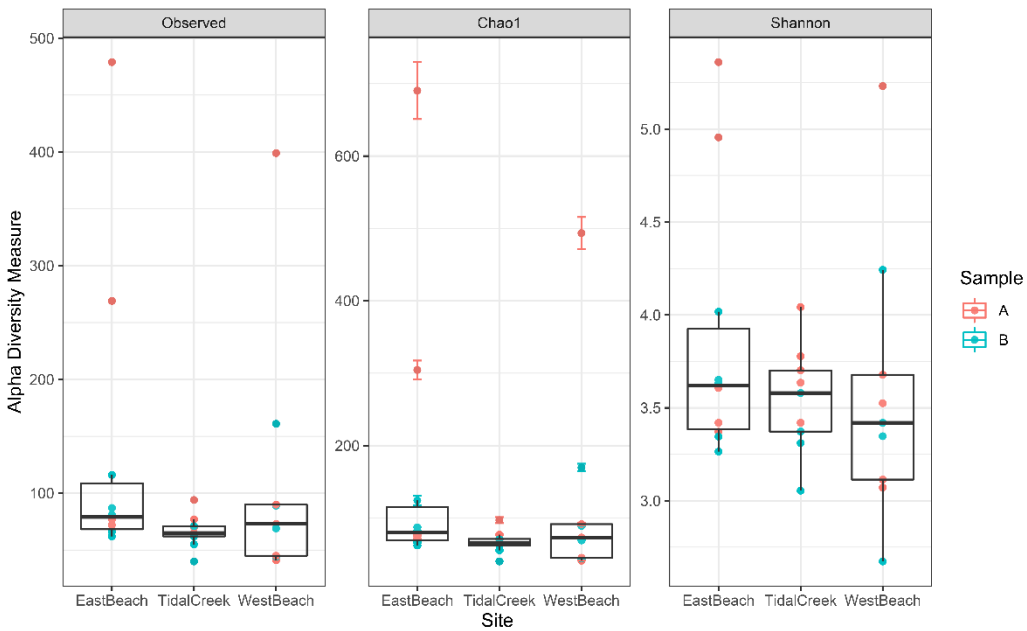

Fig. 4: Sediment alpha diversity metrics following normalization for the observed ASV (left) and the estimated richness with Chao1 (middle) as well as the Shannon-Wiener Diversity Index (right)

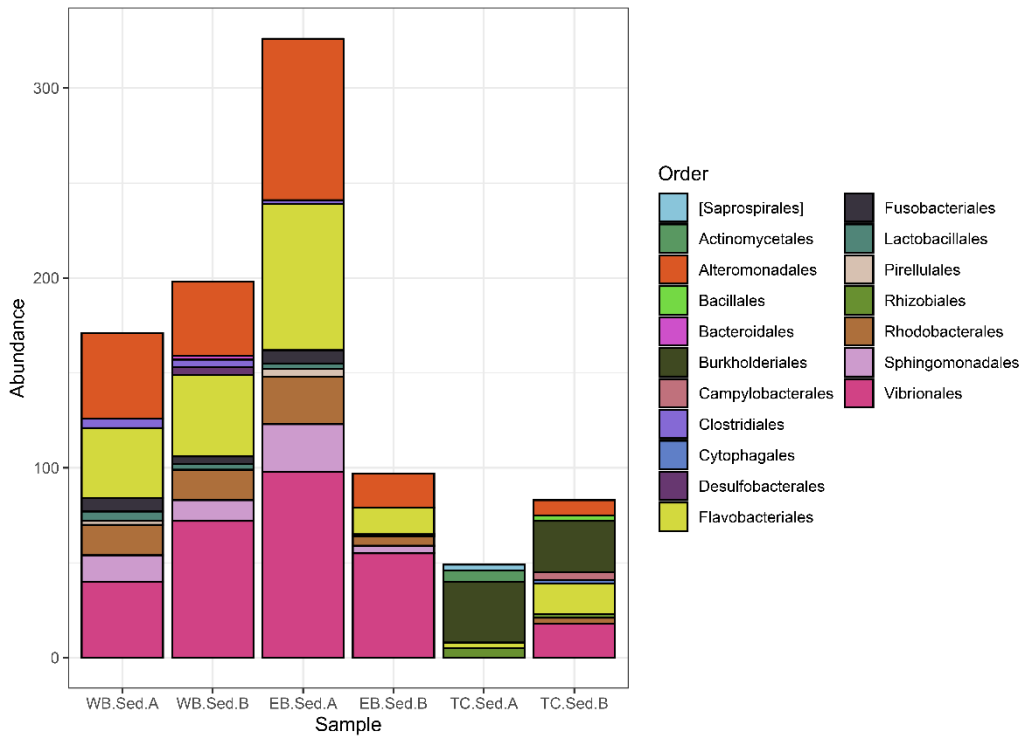

Fig. 5: Sediment profiles at the order level after normalization to the same number of sequences/sample. WB- West Beach; EB- East Beach; TC-Tidal Creek.

Tidal Creek B. These differences, however, were based on taxa with relatively low abundances. Burkholderiales were found in both Tidal Creek A and B but not in the West or East Beach sediment. The Tidal Creek sediment microbiome was particularly distinctive, being the only beach site where Burkholderiales and Saprospirales were found. Only two orders were present in all sediments: Flavobacteriales were prevalent at all sediment sites, whereas Bacillales was also present but in low abundance. A few sequences matched to those from environments with anthropogenic influence. For example, MSBl-9 were isolated from anoxic marine sediments (Pachiadaki *et al.,* 2014); BD7-3 from arsenic-contaminated tropical sediments (Suhadolnik *et al.,* 2017), and DRC-31 from wastewater treatment areas (Reboleiro-Rivas *et al.,* 2016). It is possible that the numerous anthropogenic disturbances at Plumb Beach may contribute to the presence of these taxa. In this study, many of the species that appear to differ between sites were low in abundance, however they could still play an important role in the sediment microbial community. A study by Gobet *et al.,* (2012) suggested that the proportion of rare species may be correlated with environmental conditions, including levels of primary production. Their results also showed that only 3-5% of bacterial types were present on all six sampling dates, and 60-70% occurred only once per year. The microbial community composition in Plumb Beach sediments described in this study could therefore contribute to future studies of how these communities may vary temporally or after disturbances (see Newton *et al.,* 2013).

Despite the low diversity and unique communities found at each site, a few taxa seem to have a cosmopolitan distribution at Plumb Beach. Thirteen ASV were found in at least 50% of all the sediment samples: these were identified to 10 bacterial species (Table 2). Overall dominant taxa were within the orders Vibrionales, Flavobacteriales and Alteromonadales. At the species level, *Vibrio alginolyticus, V. parahaemolyticus,* and *V. campbellii* were the most prevalent. Vibrios have been identified as major taxa in several studies of marine waters

Table 2: Amplicon Sequence Variants (ASV) present in 50% of all sediment samples

NCBI Accession	Species ID	Sequence frequency
MH547110, NR_113781	*Vibrio alginolyticus*	1631.96
NR_113604	*Vibrio parahaemolyticus*	303.26
NR_119050	*Vibrio campbellii*	132.68
NR_125458	*Pseudoalteromonas shioyasakiensis*	156.58
NR_113299	*Pseudoalteromonas phenolica*	208.3
NR_152003	*Pseudoalteromonas gelatinilytica*	46.4
NR_114053	*Alteromonas macleodii*	130.58
NR_044349	*Actibacter sediminis*	348.56
NR_116329	*Muriicola jejuensis*	81.08
NR_113841	*Tenacibaculum mesophilum*	107.02

(Johnson *et al.,* 2012). Members of this group are ubiquitous in marine environments as free-living organisms as well as on the surface of zooplankton and within tissues of marine organisms such as sea breem (Balebona *et al.,* 1998), blue crab (Davis and Sizemore 1982); sea horse (Martins *et al.,* 2010) and shrimp (Wang *et al.,* 2015). Vibrio have wide salinity and temperature ranges, and it is predicted that their contribution to microbial communities may increase with global climate change (Baker-Austin *et al.,* 2017; Vezzulli *et al.,* 2015) and eutrophication (Liu *et al.,* 2018). Two genera within the order Altermonadales were also well-represented in Plumb Beach sediment samples. Four species were particularly prevalent: *Pseudoalteromonas phenolica, P. gelatinolytica, P. shioyasakiensis* and *Alteromonas macleodiii*. Members of the genus *Pseudoalteromonas* are of interest as anti-biofouling agents and production of antimicrobial compounds (Papa *et al.,* 2013) as well as their role in biofilm formation. *Pseudoalteromonas gelatinilytica* was very recently isolated and characterized from surface seawater (Yan *et al.,* 2016), and *Alteromonas macleodii* is widely distributed in deep seawater environments (deep and surface; Weyman *et al.,* 2011). Four other genera (*Alteromonas, Actibacter, Muricola*, and *Tenacibaculum*) were also represented. The type strain of *Tenacibaculum mesophilum* was isolated from green algae and sponge on the coast of Japan (Suzuki *et al.*, 2001). *Actibacter sediminis* type strain was isolated from tidal flats in South Korea (Kim *et al.,* 2008) and has also been found in pelagic sediment in the Andaman Sea (Sundarakrishnan *et al.,* 2012). *Alteromonas macleodii*¸ whose type strain was isolated near Jeju Island (Korea), was also found to be abundant in intertidal sites, including those exposed to anthropogenic influence, off the Atlantic coast of South America (Piccini and Garcia-Alonso 2014).

Three of the common microbiome species identified at Plumb Beach were in the order Flavobacteriales: *Muriicola jejuensis*, *Tenacibaculum mesophilum,* and *Actibacter sediminis.* Flavobacteriales have been detected in similar environments including coastal areas in Greece (Meziti *et al*., 2015). Other studies have identified taxa with broad distributions among sites. Across California beaches there were 1000 unique taxa found in the bacterial communities at 10 or more beaches of 49 that were surveyed (Boehm *et al.,* 2014). Taxa clustered by grain size, organic carbon content, wave pattern, and anthropogenic influence. In this study, sediment characteristics could also explain observed differences in taxa, as Tidal Creek had both a distinct microbial assemblage and a coarser-skewed sediment than the other beach sites. Observed differences in microbial diversity in this study are therefore consistent with observations elsewhere. Knowledge about the ecological patterns of microbial biodiversity across habitats in coastal communities is limited, but sediment microbiomes appear to vary according to specific environmental conditions and disturbances (Chase *et al.,* 2017) including carbon levels, water saturation and sample depth (Zhou *et al*., 2002). Stiborova *et al*., (2020) found that 85% of the variance in sediment bacterial community structure could be ascribed to a handful of environmental pollutants. In an Amazon floodplain system, spatial habitat heterogeneity and flood pulse were the main factors shaping differences in free-living microbial diversity (Camara dos Reis *et al*., 2019). In contrast to seawater, sediment has approximately 500-fold greater diversity of bacteria, archaea, and microbial eukaryotes, despite high

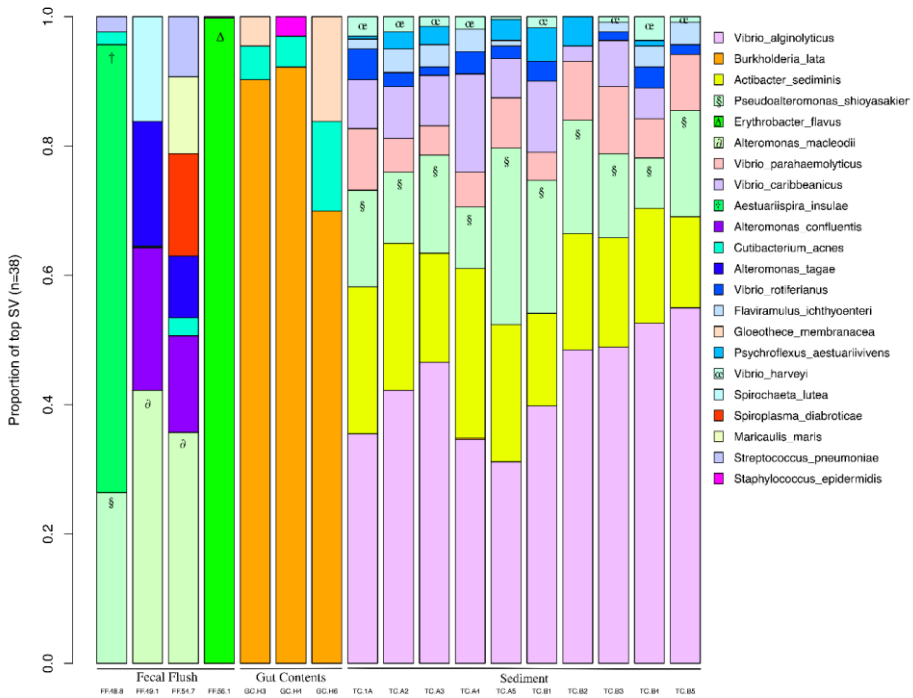

Fig. 6: Fecal flush and gut content bacterial species, shown with Tidal Creek A sediments (location where crabs were collected). FF- Fecal Flush; GC- Gut Content; TC-Tidal Creek.

consistency in microbial community composition between sites (Ul-Hasan *et al*., 2019).

Horseshoe crab microbiome

Since the number of sequences obtained for both types of crab microbiome samples were low, only four out of six fecal flush and three out of five gut content samples could be used in the analysis. All digestive tract samples yielded limited taxonomic identification using GreenGenes database (Fig. 6).

All Tidal Creek sediment samples were similar to one another and differed considerably from the horseshoe crab gut and fecal flush samples. Only two abundant taxa overlapped between crab microbiome and Tidal Creek sediment samples: *Pseudoalteromonas shioyasakiensis* was detected in all Tidal Creek A sediment samples and one fecal flush sample; *Burkholderia lata* was prominent in three gut samples and detected in low abundance in one sediment sample. Gut samples exhibited the lowest diversity, and were predominantly *Burkholderia lata* (70-92% of taxa). One fecal flush sample was comprised almost entirely of *Erythrobacter flavus,* while another was predominantly *Aestuariispira insulae* (69%). In the remaining two fecal flush samples the dominant taxa were within the genus *Alteromonas* (*A.macleodii, A.confluentis and A. tagae).* Two likely contaminants of the crab microbiome samples were *Cutibacterium acnes* (detected in all three gut samples as well as two of the fecal samples) and *Streptococcus pneumoniae* (2-10%) in two fecal flush samples. The low output and high variability of the horseshoe crab samples is not unexpected. The use of minimally invasive, small samples from separate individual animals are considered inherently difficult to amplify due to low quality and quantity of DNA (Carroll *et al.,* 2017; Andrews *et al.*, 2018). Differences in processing, extraction and amplification methods may dramatically alter taxa observed (Mallott *et al.,* 2019) making it difficult to draw definitive conclusions and comparisons from even well amplified samples.

CONCLUSION

The present study has provided a first look at the sediment microbiome at Plumb Beach- an important nursery habitat for juvenile horseshoe

crabs. Continued disturbances to Plumb Beach have the potential to influence more than the sediment microbiome. Horseshoe crabs and other organisms depend on this habitat that is regularly influenced by broad anthropogenic disturbances: in turn these organisms also contribute to the ecosystem dynamics. The results show that each of the three main areas of Plumb Beach (Tidal Creek, East Beach and West Beach) have bacterial communities with taxa in common, but also have their own unique signatures. The bacterial orders Vibrionales, Flavobacteriales and Alteromonadales were well-represented at all beach sites, whereas Burkholderiales and Saprospirales were found only Tidal Creek. Gut content and fecal flush samples showed very low species diversity and high individual variability, making it difficult to make any generalizations about the microbiome of juvenile horseshoe crabs or to link the gut microbiome of crabs to their environment. Future studies are needed to determine if the results are typical for juvenile crab digestive tract microbiomes, and to determine the relationship between crab digestive tract microbiomes and their sediment. Plumb Beach has been well studied for over a decade by ecologists, geologists, ornithologists, water chemists, taxonomists, engineers, and now by microbiologists. Continued observations of all variables impacting the populations of crabs on this beach will allow researchers to fill in the gaps in understanding of this model system, which serves as a microcosm for environmental change, and a case study for ecological restoration.

AUTHOR CONTRIBUTIONS

J. Petersen and C.P. Colon designed the experiment and collected sediment samples. C.P. Colon performed the juvenile crab survey. J. Petersen performed the DNA extractions. J. Joyner analyzed the next generation sequence data and prepared manuscript figures. All authors particpated in literature review and preparation of the manuscript.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

ABBREVIATIONS

%	percent
°C	Degrees Celsius
16S rRNA	16s ribosomal ribonucleic acid
ASV	Amplicon sequence variant
ANOVA	Analysis of variance
BLASTn	Basic local alignment search tool nucleotide
CAP	Constrained analysis of principal coordinates
DNA	Deoxyribonucleic acid
E	East
EB	East Beach
FF	Fecal Flush
g	Gram
GC	Gut Content
IUCN	International Union for Conservation of Nature
MAFFT	Multiple Alignment using Fast Fournier Transform
µm	Micrometer
mEq/L	Milliequivalents per Liter
mg/kg	Milligrams per kilogram
mg/L	Milligrams per liter
ml	Milliliter

mm	Millimeter
N	North
NCBI	National Cener for Biotechnology Information
NPS	National Parks Service
p	Probability value
PCoA	Principle Coordinate Analysis
PCR	Polymerase Chain Reaction
Permanova	Permutational analysis of variance
pH	Potential of hydrogen
ppt	Parts per thousand
QIIME	Quantitative Insights into Microbial Ecology
TC	Tidal Creek
W	West
WB	West Beach
V1-V3	Variable regions 1-3

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