Analysis of Sediments from Port Everglades Inlet (PEI) for Microbiome Characterization, Phase II

Florida Department of Environmental Protection
Coral Reef Conservation Program
CRCP Project 13

Analysis of Sediments from Port Everglades Inlet (PEI) for Microbiome Characterization, Phase II

Final Report

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EXECUTIVE SUMMARY

Our laboratory was funded for two years to conduct a study of microbial communities ("microbiomes") to characterize Port Everglades Inlet (PEI) and reef microbiomes with the standard 16S rRNA gene marker (CRCP Project 13). Phase I was completed in June 2020 (Lopez et al, 2020). This report summarizes Phase II (which we may refer to as 2021 herein) of the same CRCP project that began in 2020, using essentially the same exact collection and laboratory methods, sampling sites and computational analyses. The latter combines DNA sequencing with traditional and novel water chemistry analyses (ion chromatography, high resolution mass spectrometry) to determine the following overarching goals: i) a comprehensive spatial profile of the microbial communities (and potential pathogens) present in PEI sediments (P) and adjacent Florida's Coral Reef sediments (R); and ii) begin cursory analyses that may link sediment parameters with microbiome profiles.

We realize that the port must be periodically dredged in order to accommodate ship traffic, and so part of the Phase II project design was to characterize PEI microbes after regular operations and maintenance dredging (OED) was carried out. This activity necessarily disturbs bottom sediment which can be resuspended and transported to the reef and other natural habitats. Therefore, we have used state of the art microbial genetics methods, such as high throughput sequencing (HTS) of 16S rRNA amplicon variants, in order to profile the alpha and beta diversity of sediment microbiomes found within Port Everglades Inlet (PEI) and the adjacent Florida's Coral Reef. This method provides one of the most reliable and fastest ways to obtain a comprehensive profiling of bacterial diversity, including the majority of unculturable taxa.

We have thus expanded analyses by combining the 2020 and 2021 microbiome datasets in order to determine any temporal based changes over the one-year time period. Having two years of microbiome data now increases the power of our analyses and allows us to focus on specific taxa that may have been significantly affected by an operations and maintenance dredging (OED) in 2021.

The CRCP Project 13 Phase II results now provide two time points, and the ability to compare four cohorts of environmental data: 2020 port (P) and reef (R), and 2021 port and reef communities. A large 2021 dataset of over 14 million sequence reads was generated by HTS. This data translates to at least 1097 identified bacterial families in the port and 960 families in the reef. The results indicate several similar findings in microbiomes from last year, as similar microbial taxa can be identified across all four datasets, but with varying relative abundances, dependent on time and location. Again, the most dominant phyla at both site types (P and R) was Proteobacteria, although reef sediments continued to show greater abundances of cyanobacterial taxa. The strongest differences were between 2021 port and reef communities, with the former providing the greatest significant differences in any pairwise comparison of alpha diversity. Port microbiomes also changed between 2020 and 2021 but with weak significance. The taxa contributing to changes in 2021 port communities were mostly anaerobic Archaea and in

the Phylum Chloroflexi. The increase of Chloroflexi could be associated with sites P13, P16, P17 in 2021.

Moreover, R communities shifted to appear less structured than 2021 P communities compared to 2020 in beta diversity analyses. In general, the R communities between 2020 and 2021 had an increase of Thaumarchaeota (Archaea), Rhodospirillaceae, and Saprospiraceae (Bacteroidetes). An increase of Chloroflexi could be associated with sites P13, P16, P17 in 2021. Concomitant increases in the Archaeal phyla Euryarchaeota and Crenarchaeota also appeared at these same port sites. Site P14 was the only port site with elevated Chloroflexi in 2020. When chemical profiles were analyzed in the context of the microbiome data, we find less influence of Cd, while total Phosphorus (TP) affected port communities similar to 2020. Potential pathogenic bacterial taxa were once again identified in port and reef communities, but as in 2020 and most cases, they appear as part of the natural community.

Taking both Phase I and II together, the CRCP Project 13 provides an interesting comparison and contrast of microbiomes in a human "built" environment (P) adjacent to nearby "natural" reef environments (R). The two year dataset indicates that microbiomes at most sites are dynamic and can be affected by a temporal gradient. There is evidence of possible acute disturbances via the O&M dredging within the port which could have affected its microbial communities. However, the once a year annual sampling provided by this study cannot account for other possible factors which could have also affected both P and R microbiomes. To determine these factors further, we recommend more frequent sampling, and the application of other informatics tools which can track the transfer of discrete taxa from various locales.

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LIST OF ACRONYMS

P - Port
R - Reef
ASV -Amplicon sequence variants
FIU - Florida International University
NSU - Nova Southeastern University
PEDP - Port Everglades Deepening Project
PEI - Port Everglades Inlet

1 INTRODUCTION

1.1 Port Everglades Inlet

Port Everglades Inlet (PEI) lies adjacent to sensitive coral reefs, mangroves and popular beaches and is also one of the most active cargo ports in the United States, including a main seaport in southern Florida for the delivery of petroleum products (Banks et al, 2008). PEI is located on the East coast of Florida situated in three municipalities: Fort Lauderdale, Dania Beach and Hollywood (www.portofeverglades.net). This highly engineered port is 641 meters in length by 295 meters wide with a depth of 13 meters (Stauble, 1993). The port waters are subject to a high volume of commercial and recreational boat traffic and likely influenced by local and regional human-derived anthropogenic inputs through a myriad of urban land uses well beyond its boundaries into its associated watershed.

PEI will soon be one of the ports looking to expand traffic by deepening and widening. This follows other developments across the globe to accommodate Neo-Panamax ships that were added to the fleet after the expansion of the Panama Canal in 2016 (Ashe, 2018). Port Everglades Deepening Project (PEDP) received federal authorization in December of 2016 for the U.S. Army Corps of Engineers to move forward with the deepening and widening of the Ports channels as part of the Water Infrastructure Improvements for the Nation (WIIN) Act (https://www.usace.army). However, urban growth and port development can also have detrimental effects on surrounding natural habitats, including coral reef systems (Walker et al., 2012). Recent studies have shown that sediments and accompanying increased turbidity of seawater can degrade coral reef health around the world (Fabricius 2005; Wolanski et al, 2009).

1.2 Marine Microbes

Microbes occur almost everywhere and thus have important ecological and biogeochemical roles in the world's oceans (Thompson et al 2017). They not only provide sourcing information about water masses, they may serve as indicators of degradation in water quality. Microbes also are integral and thus interact within sensitive ecosystems like coral reefs and more importantly do have the potential to directly affect the health of human and marine life.

To protect vital and sensitive coastal habitats, the specific chemical and microbial threats and their origins should be accurately located, characterized, and eventually identified. Marine microbial communities are often complex, habitat-specific and may quickly respond to minor environmental changes. Thus, we can use them as very sensitive biological indicators (Urakawa et al., 2012). In the last few decades, technological advances in molecular methods, including high throughput sequencing (HTS) of DNA, have been actively applied to find and characterize microbiomes with important ecological functions or potential disease pathogens on local reefs and organismal hosts (Negandhi et al 2010). The Illumina platforms will assist in these goals, as they can

produce greater than 50 Gbases daily, and over 1.6 billion reads in ten days (Caporaso et al. 2012).

Since last year, more information has been released regarding dredging activities in PEI. For example, the Army Corp of Engineers Environmental Impact Statement (EIS) was released for public comment in 2020. The EIS describes the possible effects of deep dredging from the current mean 42 ft. to a depth of 48 ft. in PEI. Also, operations and maintenance dredging (OED) has been conducted over the life of the Port, with the latest activity occurring in March 2021. Therefore, the sampling of CRCP 13 Phase II samples was designed to capture possible disturbances in sediment communities.

This study aims to combine HTS with traditional and novel water chemistry analyses (ion chromatography, high resolution mass spectrometry) to determine the following overarching goals: i) a comprehensive spatial profile of the microbial communities (and potential pathogens) present in PEI sediments (P) and adjacent Florida's Coral Reef (R) sediments; ii) begin cursory analyses that may link sediment parameters with microbiome profiles. It will be beyond the scope of this project to determine causal effects, natural phenomenon (tides, currents, offshore upwelling) that may be responsible for microorganismal movements and possible microbiome re-structuring.

Microbial community profiling provides valuable information on almost any ecosystem. In this specific project, the data can provide a baseline for the types of bacteria which may be resuspended in the water column after human disturbances (e.g. dredging, boat use).

Therefore, our laboratory has generated microbiome data with assistance from the DEP to provide possible insight on differences in microbiomes that appear to be resident within port and reef sediments.

2 METHODS

2.1 Sampling Sites

For CRCP 13 Phase II, a total of 120 sediment samples were collected from 40 sites spread over Port Everglades and the adjacent Florida reef habitat to characterize the microbial community of sediment in the area (Table 1). In order to characterize the chemical and physical composition of the water and sediment supporting that community, a subset of nine sediment samples and three water samples (split into multiple bottles) were analyzed. Water and sediment samples for delivery to FIU for chemical analyses were taken at the locations in the table below. The sample IDs reflect the site shown in the Figure 1 map, and the collection location on Transect ("-00, 15, or 30" m from the buoy). Therefore, each site was essentially taken in triplicate.

Table 1 *– List of all sample sites and coordinates. Each site had three replicates taken for a total of N=120.*

Sample Number R = Reef P = Port	Latitude	Longitude	Location Name	Samples for Extra Analyses
R01	26.11233	-80.09223	2000N-IRL-Control	Sediment Chemistry
R02	26.10795	-80.09242	1500N-IRL	
R03	26.09893	-80.09682	500N-CPS	
R04	26.09893	-80.09320	500N-IRL	
R05	26.10070	-80.08772	700N-MRL	
R06	26.10070	-80.08320	700N-ORL	
R07	26.09713	-80.08780	300N-MRL	
R08	26.09713	-80.08352	300N-ORL	
R09	26.09545	-80.10138	50N-NRC	Sediment Chemistry
R10	26.09545	-80.09462	100N-IRL	
R11	26.09587	-80.08882	150N-MRL	Sediment and Water Chemistry
R12	26.09512	-80.08400	50N-ORL	Sediment Chemistry
R13	26.09193	-80.10272	100S-NRC-RS	
R14	26.09193	-80.09365	100S-IRL	
R15	26.09193	-80.08888	100S-MRL	Sediment Chemistry
R16	26.09240	-80.08418	50S-ORL	
R17	26.08988	-80.09945	300S-NRC-CPS	
R18	26.08988	-80.09382	300S-IRL	
R19	26.08988	-80.09125	300S-MRL	
R20	26.08988	-80.08413	300S-ORL	
R21	26.07895	-80.09447	1500S-IRL	
R22	26.07447	-80.09548	2000S-IRL-Control	Sediment Chemistry
P01	26.11111	-80.11028	Control-N	
P02	26.09833	-80.11806	NTB-1	Sediment Chemistry
P03	26.09444	-80.11750	NTB-2	
P04	26.09389	-80.11222	EC-1	
P05	26.09417	-80.10694	EC-2	
P06	26.09222	-80.11972	MTB-1	
P07	26.09139	-80.11306	MTB-2	
P08	26.09278	-80.10833	EC-3	
P09	26.09028	-80.11583	MTB-3	Sediment and Water Chemistry
P10	26.08750	-80.11861	STB-1	
P11	26.08639	-80.11806	STB-2	
P12	26.08583	-80.11278	SAC-1	

P13	26.08250	-80.11333	SAC-2	
P14	26.07806	-80.11417	SAC-3	
P15	26.07444	-80.11778	STN-1	
P16	26.07306	-80.11667	STN-2	Sediment and Water Chemistry
P17	26.06583	-80.11472	SAC-4	
P18	26.04833	-80.11639	Control-S	

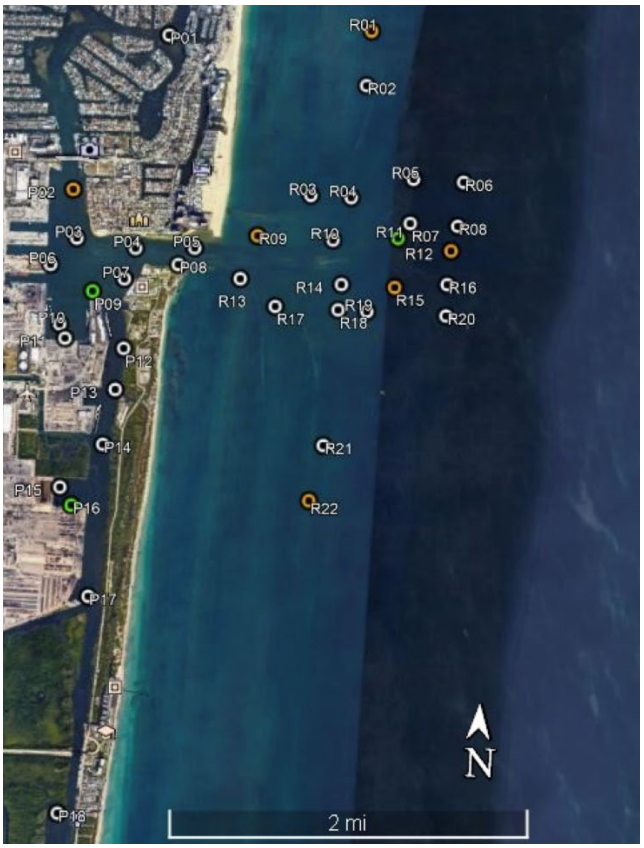


Figure 1 *- Map of collection sites provided by DEP. Orange and green labels indicate where a) chemical and microbial sediment and b) water and chemical sediment samples were collected, respectively*

Table 2 *- Site locations for samples which were further analyzed for nutrients and chemicals by FIU*

Sample Number R = Reef P = Port	Latitude	Longitude	Location Name (from former study)	Samples for Extra Analyses
R11	26.09587	-80.08882	150N-MRL	Sediment and Water
P09	26.09028	-80.11583	MTB-3	Sediment and Water
P16	26.07306	-80.11667	STN-2	Sediment and Water
P02	26.09833	-80.11806	NTB-1	Sediment
R01	26.11233	-80.09223	2000N-IRL-Control	Sediment
R09	26.09545	-80.10138	50N-NRC	Sediment
R12	26.09512	-80.08400	50N-ORL	Sediment
R15	26.09193	-80.08888	100S-MRL	Sediment
R22	26.07447	-80.09548	2000S-IRL-Control	Sediment

2.2 Site Stations

At sites R11, P09, and P16, water samples were taken from mid-depth for the water analytes in the Table 2. At P02, P09, P16, R01, R09, R11, R12, R15, and R22, sediment samples were collected in the middle of the transect for the sediment analytes also shown in Table 2.

Table 3 *- Nutrients and chemicals analyzed by FIU*

Analyte	COC Code	Type	Analyte Code for Bottle/Tube
Nitrate-nitrite	N+N	Filtered nutrients water sample	NNH
Ammonium	NH4	Filtered nutrients water sample	
Soluble Reactive Phosphorus	SRP	Filtered nutrients water sample	PNO

Nitrite	NO2	Filtered nutrients water sample	
Total Organic Carbon	TOC	Carbon water sample	TDOC
Dissolved Organic Carbon	DOC	Carbon water sample	
Trace Metals	-	Sediment sample	TMN
Soil Total Phosphorus	TP	Sediment sample	
Soil Total Carbon/Total Nitrogen	TC/TN	Sediment sample	

2.3 Microbiome Analysis

2.3.1 DNA Extraction

All 120 sediment samples provided were processed for DNA analyses. Preparation of DNA samples from sediment and water acquired and/or the mesocosms for high-throughput 16S rRNA gene sequencing were conducted as described in detail in previous studies from the Lopez NSU molecular genomics and microbiology laboratory (Campbell et al, 2015; O'Connell et al, 2018; Easson and Lopez, 2019). Briefly, after pelleting 1-2 grams of sediment, and filtration of the water and genomic DNA purification of the filters, DNA was extracted using the Qiagen PowerLyzer Powersoil DNA isolation kit following standard protocols. DNA quality was evaluated for molecular weight integrity by electrophoresis on a 1.0% gel.

2.3.2 Microbiome DNA Sequencing

Microbial 16S amplicon libraries were generated using the universal V4 region primer sets 515F and 806R with the Golay barcodes and Illumina adapters attached to the reverse primer. These primers have been selected, because they can amplify and provide the most comprehensive diversity of both bacteria and archaea. The V4 regions of the 16S rRNA molecule were sequenced on an Illumina MiSeq instrument with standard protocols in Lopez's NSU laboratory. The standard Earth Microbiome Project protocols (www.earthmicrobiome.org) were applied using the same methods described (Earth Microbiome Project 2016 and Thompson et al, 2017). Amplicon PCR was performed as per the EMP sequencing protocol for the Illumina MiSeq platform. PCR products were cleaned using AMPure beads and underwent a quality control check on an Agilent Bioanalyzer Tapestation 2200. Quality control is followed by normalization to 4 pM and then library pooling. The final product was loaded into an Illumina MiSeq system for 16S metagenomics DNA sequencing following a modified Illumina workflow protocol.

2.3.3 Data Analysis and Statistics

FASTQ DNA sequence files were run through Quantitative Insights into Microbial Ecology (QIIME2) and then R Studio to determine overall microbial community structure, including alpha and beta diversity. Sequences will first be quality filtered to remove chimeras and scores under 25 (1 error in 10,000 base pairs based off the PHRED system).

We did not analyze data that is < Q30 Phred. Sequences were sorted into operational taxonomic units (OTUs = operational taxonomic units or ASVs = Amplicon Sequence Variants) or amplified sequence variants (ASVs) with a 97% similarity or greater. This experiment followed the standards which Knight et al. (2012) set for metadata and sampling sizes. QIIME2 used for demultiplexing, quality filtering, OTU picking, taxonomic assignment, phylogenetic reconstruction, and diversity analysis and visualizations. The 113 FASTQ files from the 2021 dataset were run through QIIME2 (Quantitative Insights into Microbial Ecology) for quality control to reach a Phred score of no <Q30. Then the 113 samples were processed using RStudio's vegan packages to statistically describe the difference in the microbial community between the Port and Reef sites. Some of these vegan packages included checking alpha diversity, as well as running Simper, ANOSIM, and NMDS (Oksanen et al 2017). After the 2021 dataset was processed through RStudio, the FASTQ files from both datasets were combined. In order to combine the data, the 2021 dataset had to be trimmed in the same way the 2020 dataset was using the same methods. It is worth noting that between the datasets, QIIME2 was updated, and version 2020.11 was used for trimming the 2021 dataset. The raw FASTQ files from both datasets were then uploaded to CosmosID to create phylogenetic trees and organize the abundance of species within the samples according to either Phylum, Order, or Family. CosmosID was used to calculate the alpha and beta diversity between the samples and to create different visualizations of the combined dataset. To statistically describe the differences in microbial communities between the years the combined dataset was processed through Primer-E v7 to run Simper, ANOSIM, and NMDS. Other data analyses and visualizations were performed by uploading all processed FASTQ data onto the CosmosID bioinformatics pipeline by CosmosID (see https://www.cosmosid.com/bioinformatics). CosmosID pipeline methods for metagenomic analyses are on their webpage but also reproduced here:

> "For taxonomic profiling based of amplicon data, the CosmosID 16S data analysis pipeline starts with preprocessing of the raw reads from either paired-end or single-end Fastq files through read-trimming to remove adapters as well as reads and bases of low quality. If the reads are in a paired-end format, the forward and reverse overlapping pairs are joined together; the unjoined R1 and R2 reads are then added to the end of the file. The file is then converted to Fasta format and used as input for OTU picking. OTUs are identified against the CosmosID curated 16S database using a closed-reference OTU picker and 97% sequence similarity through the QIIME framework. The final results are then presented in tabular format with the taxonomic names, OTU IDs, frequency, and relative abundance. Results can be downloaded or compared to other 16S samples for visualizations through the CosmosID Comparative Analysis tool."

2.3.4 Metadata

The possible effects of environmental drivers (metadata, chemical data) on microbial community composition is well recognized in microbiome analyses (Knight et al, 2012).

Therefore, field and chemical were incorporated and applied to 16S rRNA sequence data using Canonical Correspondence analysis (CCA) (Oksanen et al., 2017). Raw data is available by DEP. Processed data will be submitted for public access to the National Center of Biotechnology Information (NCBI Sequence Read Archive (SRA). NCBI SRA accession # is PRJNA742832, release date: 2022-04-01.

3 RESULTS/DISCUSSION

3.1 Chemical Analysis

Chemical and nutrient analyses were again performed on the same subset of sites as 2020. Therefore, nine sediment samples were collected, analyzed and yielded chemical profiles shown in Table 4. Histograms of Hg, Cu and Cd are placed in the appendix. These data will be incorporated into the Canonical Correspondence Analysis (CCA) analyses discussed in section 3.2.3.2

Table 4 *- Results of chemical analyses across three selected port and six selected reef sites. Source data was provided by FIU, in the file "TM210316_SW031621_AC91784-AC91792_Sediment report_Final_210420 combined 2021 2020.xlsx"*

Client Sample ID			P02-TMN	P09-TMN	P16-TMN	R01-TMN	R09-TMN	R11-TMN	R12-TMN	R15-TMN	R22-TMN
Receive Date	03/16/21		03/16/21	03/16/21	03/16/21	03/16/21	03/16/21	03/16/21	03/16/21	03/16/21	03/16/21
Collection date			NA	NA	NA	NA	NA	NA	NA	NA	NA
Matrix	Tissue/Sediment		Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment
QC Batch	TM0212		TM0212	TM0212	TM0212	TM0212	TM0212	TM0212	TM0212	TM0212	TM0212
SDG	TM210316		TM210316	TM210316	TM210316	TM210316	TM210316	TM210316	TM210316	TM210316	TM210316
FIU ID (LIMS #)											
Lab Sample ID			AC91784	AC91785	AC91786	AC91787	AC91788	AC91789	AC91790	AC91791	AC91792
Dig. Method	EPA 3050B		EPA 3050B	EPA 3050B	EPA 3050B	EPA 3050B	EPA 3050B	EPA 3050B	EPA 3050B	EPA 3050B	EPA 3050B
Method	EPA 6020A		EPA 6020A	EPA 6020A	EPA 6020A	EPA 6020A	EPA 6020A	EPA 6020A	EPA 6020A	EPA 6020A	EPA 6020A
ICPMS Analysis Date	03/31/21		03/31/21	03/31/21	03/31/21	03/31/21	03/31/21	03/31/21	03/31/21	03/31/21	03/31/21
Sample Description	Tissue/Sediment		Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment	Tissue/Sediment
Sample Weigth (g)			0.2016	0.2023	0.2017	0.2092	0.2023	0.2181	0.2045	0.2136	0.2063
		3*MDL									
Reporting Units	MDL Terr	Terr	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)	Conc. (mg/Kg)
Element											
Be / 9	0.03	0.10	0.05	0.04	0.05	0.03	0.03	0.02	0.03	0.02	0.02
Al / 27	168.15	504.44	1302.82	857.62	1307.63	282.04	237.55	165.35	137.40	204.08	152.59
V / 51	1.08	3.23	4.66	2.85	3.77	3.82	2.97	1.82	1.40	2.17	3.39
Cr / 52	1.4	4.1	5.65	4.59	4.99	4.72	3.00	3.34	1.90	3.12	4.73
Mn / 55	0.14	0.41	15.79	14.96	16.04	17.52	15.26	11.59	9.03	13.62	17.15
Fe / 57	975.43	2926.28	2548.84	1847.71	3005.06	1247.69	1054.16	704.11	416.33	1042.44	1021.13
Co / 59	0.01	0.02	0.15	0.10	0.15	0.05	0.07	0.04	0.04	0.04	0.04
Ni / 60	0.09	0.3	1.50	1.03	1.45	0.66	0.44	0.43	0.33	0.47	0.51
Cu / 63	0.23	0.69	24.43	14.01	26.19	1.35	0.76	0.39	0.47	0.45	0.57
Zn / 66	0.63	1.90	20.80	12.14	30.25	2.59	2.10	1.06	0.95	1.54	1.63
As / 75	0.25	0.75	0.72	0.69	0.89	1.44	0.97	0.82	0.51	1.09	1.68
Se / 82	0.09	0.26	0.42	0.36	0.43	0.29	0.21	0.19	0.20	0.19	0.25
Mo / 95	0.11	0.32	0.13	0.10	0.19	0.06	0.03	0.04	0.02	0.03	0.07
Ag / 107	0.01	0.03									
Cd / 111	0.00	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.03
Sn / 118											
Sb / 121			0.06	0.03	0.04	0.08	0.04	0.05	0.02	0.05	0.08
Ba / 137	0.30	0.91	6.98	7.42	6.93	7.11	5.15	6.04	4.20	7.31	6.52
Hg / 202	0.07	0.21	0.09	0.03	0.05	0.00					
Pb / 208	0.19	0.58	10.29	5.95	8.69	1.95	1.46	1.23	1.06	1.64	1.48

Microbiome Analysis

3.1.1 MiSeq DNA Sequencing Output

All details of DNA extractions and quality are provided in a separate Excel file (titled "CRCP Phase II DNA Log (1).xlsx"). After all 120 samples were processed, a total of 113 16S rRNA samples from the 2021 sample set were sequenced and completed on the MiSeq DNA sequencer for high quality data (80% Q30 Phred quality scores).
Seven samples either failed to amplify or did not meet QC standards. Four samples were not run due to poor PCRs, (R06.00, R02.30, P08.15, P08.30) while three samples yielded too few sequences to score (R03.00, R07.30, P13.30)

Thus, after QC and sequence processing, the final dataset included 113 samples with the widely accepted high quality level of Q30. The average number of 16S rRNA reads per sample was 124,663/sample which are 40,000 higher than 2020 results and also 70,000+ reads higher than the minimum (50,000) considered to be sufficient to assess the diversity of sediment samples. Out of the 113, three samples remained problematic and could not be included in our final analyses because the number of reads was below 35,000 (R15.15 had 15,587 reads; P07.0 had 31,594 reads) (Table 5).

Overall, this resulted in a total of 62 reef and 51 port samples in the final 2021 microbiome analyses. Because all sites were collected in triplicate, the missing samples will likely not affect the final conclusions.

Both 2020 and 2021 CosmosID data are accessible on their website and through our NSUworks dropbox indefinitely (the URLs are shown in the Appendix). User driven CosmosID interactive graphs also display actual relative abundances as percentages.

Table 5 *- 2021 sample IDs and Number of 16S rRNA sequences (reads) for each 2021 port and reef sample*

Sample	# of MiSeq Reads
R01.00	81,155
R01.15	85,722
R01.30	83,627
R02.00	87,274
R02.15	82,831
R03.15	90,168
R03.30	103,571
R04.00	65,912
R04.15	82,689
R04.30	73,018
R05.00	131,002
R05.15	81,090
R05.30	121,775
R06.15	121,350

R06.30	142,681
R07.00	131,947
R07.15	67,200
R08.00	99,057
R08.15	76,815
R08.30	86,341
R09.00	78,290
R09.15	83,030
R09.30	64,428
R10.00	90,466
R10.15	56,536
R10.30	75,904
R11.00	98,635
R11.15	68,434
R11.30	110,112
R12.00	63,745
R12.15	73,422
R12.30	106,510
R13.00	107,331
R13.15	99,834
R13.30	101,661
R14.00	101,166
R14.15	102,714
R14.30	83,771
R15.00	148,804
R15.15	15,587
R15.30	124,965
R16.00	74,928
R16.15	88,591
R16.30	116,891
R17.00	122,739
R17.15	92,756
R17.30	88,733
R18.00	70,488
R18.15	119,212
R18.30	135,836
R19.00	53,585
R19.15	79,812
R19.30	71,580
R20.00	77,116
R20.15	125,653

R20.30	128,382
R21.00	85,906
R21.15	118,126
R21.30	130,385
R22.00	136,255
R22.15	115,038
R22.30	146,674
P01.0	149,017
P01.15	113,930
P01.30	138,808
P02.00	193,324
P02.15	157,524
P02.30	156,797
P03.0	210,425
P03.15	93,428
P03.30	185,206
P04.00	133,747
P04.15	125,556
P04.30	97,354
P05.0	163,624
P05.15	106,239
P05.30	180,446
P06.00	181,295
P06.15	124,471
P06.30	105,951
P07.0	30,594
P07.15	60,561
P07.30	94,932
P08.00	119,552
P09.00	143,719
P09.15	203,652
P09.30	165,775
P10.00	197,909
P10.15	174,419
P10.30	145,567
P11.00	142,131
P11.15	208,506
P11.30	192,905
P12.00	229,599
P12.15	204,089
P12.30	194,495

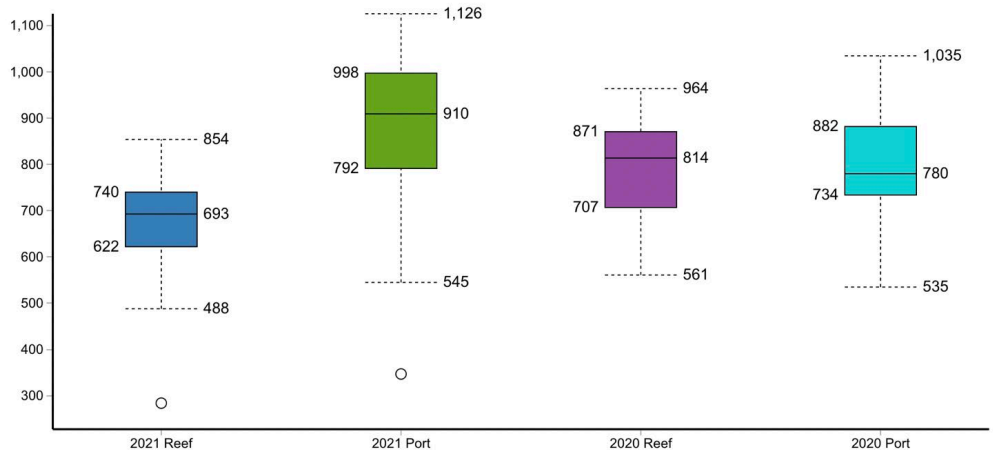
P13.00	192,539
P13.15	155,594
P14.00	229,308
P14.15	219,848
P14.30	213,096
P15.00	217,162
P15.15	149,734
P15.30	144,116
P16.00	183,071
P16.15	155,733
P16.30	189,510
P17.00	166,491
P17.15	156,619
P17.30	205,810
P18.00	222,666
P18.15	179,254
P18.30	51,611
Total Reads	**14,086,965**
Avg Reads/sample	**124,663**

3.1.2 Bacterial Microbiome Composition in Port and Reef Sites

For microbiome studies, the 16S rRNA gene has proven to be a suitable marker for measuring alpha diversity, the number (richness) and distribution (evenness) of ASV's expected within a single population, as well as beta diversity, which is the similarity (or difference) in organismal composition between different samples. The data of amplicons derived from the 16S rRNA V4 region can provide bacterial taxonomic identifications, but reliability fades below the Family or Genus level, so bacterial species identification is not considered as unequivocal (Wang et al, 2007). A total of 58,189 bacterial taxa were identified in the total 2021 dataset. This includes 1,097 families in the port and 960 families in the reef. Detailed taxonomy is provided in large Excel tables which are available on the dropbox repository. These family level taxa are fully listed in the supplemental spreadsheet excel file named "ca-pei-2021-2020_2021_06_11_21_28 family.tsv".

As mentioned in the Phase I report, sediments are known to have high species richness compared to other types of habitats (Delgado-Baquerizo et al, 2018). The mean alpha diversity (species richness) for the total 2021 dataset was found to be 1,412 species (Min: 245; SD: 518.8601). In the total 2021 port and reef dataset, 2,620 genus level taxa were identified through CosmosID taxonomy platform. The genus level taxa are fully listed in the supplemental spreadsheet excel file named "ca-pei-2021-2020_2021_06_11_21_31 genus.tsv".

Figure 2 shows a box plot comparison of the alpha diversity for composite data for all 113 2021 and 117 2020 P and R samples, using the Chao1 richness estimator, with significance tests based on Wilcoxon Rank Sums. This metric approximates the relationship between the number of sequences drawn from each sample and the number of taxa expected to be present based on detected abundances. The groups show similar amounts of alpha diversity, but the Wilcoxon scores show significant differences between 2021 Port and Reef samples.



Cohorts	Statistic	P-value
2021 Reef ↔ 2021 Port	-6.5086	0.0000
2021 Reef ↔ 2020 Reef	-5.5632	0.0000
2021 Reef ↔ 2020 Port	-5.2891	0.0000
2021 Port ↔ 2020 Reef	3.5374	0.0004
2021 Port ↔ 2020 Port	3.3047	0.0010

Figure 2 - *Alpha diversity box plots based with the Chao1 species richness indicator for composite 2020 and 2021 Port and Reef 16S rRNA datasets. Wilcoxon Rank Sum Tests of the data show significance between 2021 Port and Reef samples. Shannon indices gave similar results and significance.*

As emphasized in the 2020 Phase I report, microbiomes of most natural habitats are dominated by relatively few (5-20) bacterial taxa, while the majority of bacterial taxa that can number in the 100s or 1000s, appear rarely (Easson and Lopez 2019). Examples of total bacterial taxa and the alpha diversity present in microbiomes can be viewed via sunburst pie (also known as Kronas) charts. This depiction shows the relative abundance

of bacterial taxa at the ordinal level (Figures 3 - 8). This includes three representative samples from Port sites and two from Reef sites (Ondov et al, 2011). These examples were chosen based on their relative distance from each other, their depth on the reef, or distance from the port. Other sunburst plots at different taxonomic levels can be viewed or downloaded from the CosmosID archived dataset. These sites also differ from examples shown in the Phase I report of 2020. Firstly, three port sites P09, P07 and P04 are shown in Figures 3 -5. Recall that site P04 is one P site in the channel itself, and its bacterial community composition reflects both reef and port community compositions.

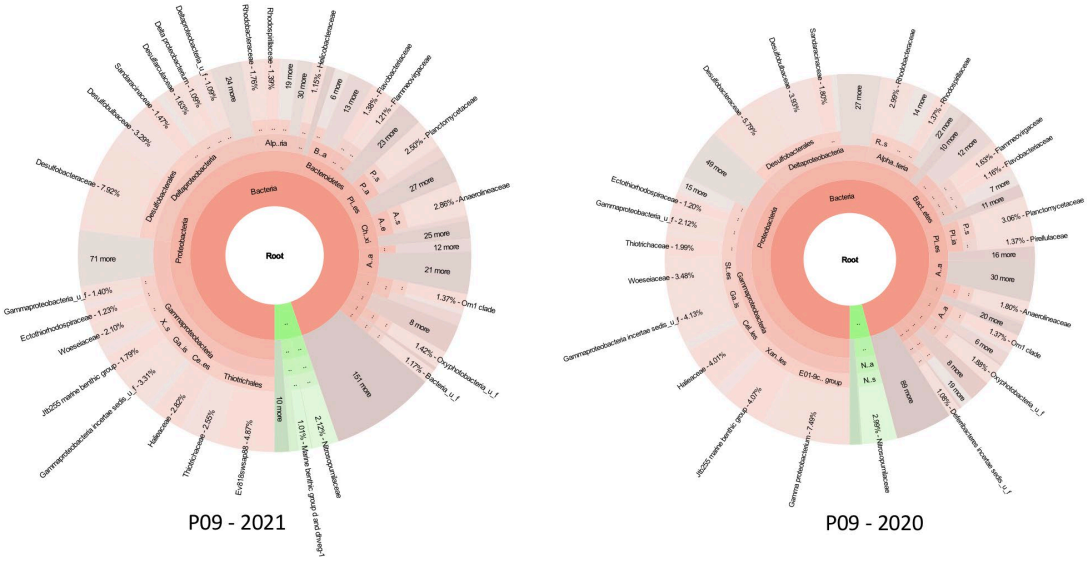


Figure 3 - *Representative sunburst plots showing bacterial taxa at the Order level for Port site, P09 for both 2021 and 2020.*

Figure 4 - *Representative sunburst plots showing bacterial taxa at the Order level for Port site, P07 for both 2021 and 2020.*

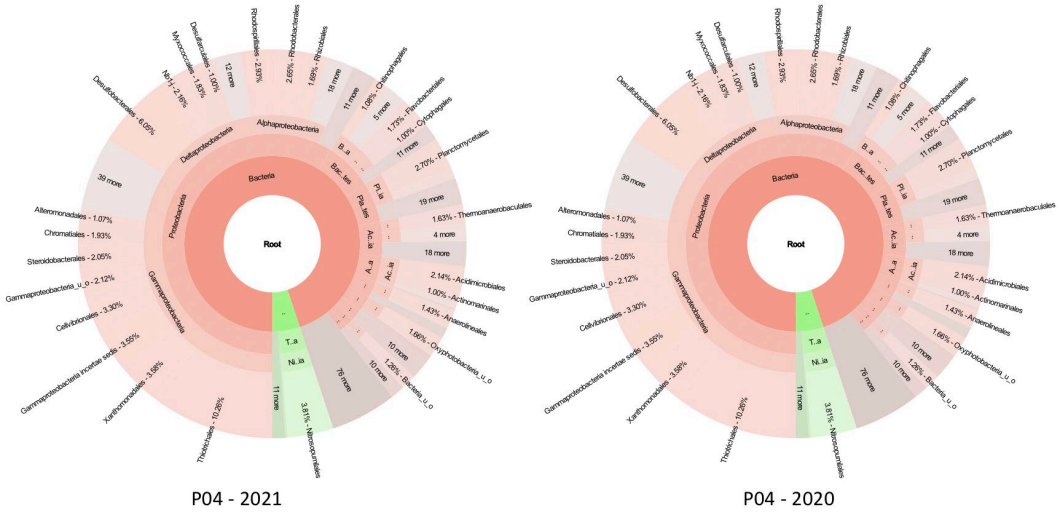


Figure 5 - *Representative sunburst plots showing bacterial taxa at the Order level for Port site, P04 for both 2021 and 2020.*

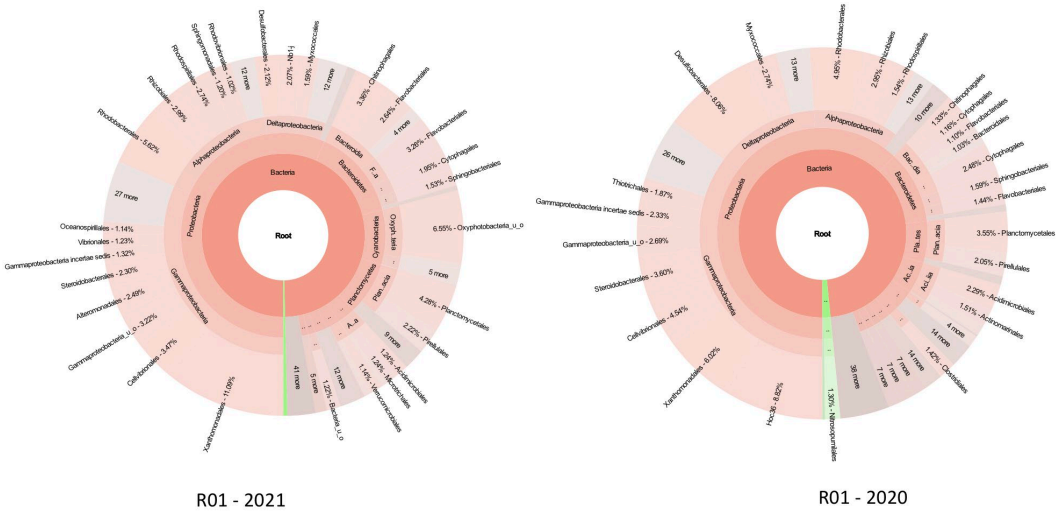


Figure 6 - *Representative sunburst plots showing bacterial taxa at the Order level for Reef site, R01 for both 2021 and 2020.*

As in 2020, most of the sunburst charts show that the most common phylum by far across most of the sites is Proteobacteria (55% port, 61% reef). Bacteroidetes is a common phylum in fecal matter and appeared as the second most common phylum also among both reef and port samples at 6%. As expected, Cyanobacteria had a higher abundance at reef sites than port sites (2.76%).

The predominance of the taxa discussed above are exhibited in the heat maps shown in Figures 8 and 9. These heatmaps show the most abundant taxa and also in which cohort they may occur. For example, the Desulfobacterales appears as the most abundant order and the second most abundant family across all four cohorts, but the Desulfobacteraceae family appears much more pronounced (red) in both port datasets, as well as few 2020 reef sites (R2, R5, R8 and R11 had abundances over 8%).

The Proteobacterial order Thiotrichales (2.56% port, 0.56% reef) and Chloroflexi order Anaerolineales (2.99% port, 0.41% reef) appear with increased abundances in port communities. Past studies indicate these to be anaerobic microbes, with Anaerolineaceae favoring warm conditions. Moreover, these taxa do appear as important members of wastewater communities (Kannan et al, 2020), which underscore the human effects on port communities.

Desulfobacteraceae, Desulfobulbaceae, Anaerolineaceae, Nitrosopumilaceae, Desulfarculaceae, Rhodospirillaceae, and an unknown family of Thermoplasmatales and Crenarchaeota increased in relative abundance in 2021 (across all areas), and Rhodobacteraceae and an unknown family of Cyanobacteria decreased in 2021. Archaea, Desulfarculaceae, and a family within the Gif9 order of Chloroflexi and order Aminicenantes increased in abundance in the intracoastal sites in 2021, while another Desulfobactereceae and unknown Cyanobacterial family decreased. An unknown

Cyanobacteria decreased and Sporichthyaceae (Actinobacteria), Nostocaeae (Cyanobacteria), Comamonadaceae (Proteobacteria) and Terrimicrobiaceae (Verrucomicrobia) were absent in the south reef samples in 2021 compared to 2020 but 2021 south reef samples increased in the Archaea Nitrosopumilaceae. In general, the reef samples between 2020 and 2021 had an increase of Thaumarchaeota (Archaea), Rhodospirillaceae, Saprospiraceae (Bacteroidetes). The Desulfobacteraceae is an anaerobic Proteobacteria family which specializes as sulfate reducers, and sulfur-reducing bacteria (SRBs) are likewise abundant in sludge communities.

Composite stacked bar charts from all 2020 and 2021 Reef and Port samples allow another visualization of relative microbial abundance across grouped Reef and Port sites (Figures 10, 11). For example, the stacked bar chart of Figure 10 supports the Chao metric alpha diversity analyses and statistics (see below) that the aggregate 2021 Port communities show significant differences from last year. (The CosmosID visualization platform allows interactive scrolling to view percentages of all taxa).

Even when the most extreme points on the sampling range were compared, the most northern reef site from port entry (R01) compared to the most distant southern site from port entry (R22), community composition showed no significant microbiome differences.

With regard to the 2020 vs 2021 Port communities, there appears to be a (5.7/3.7 % =) 1.5 fold increase in Chloroflexi (in 2021), and a larger (2.7/0.7 %=_ 3.8) increase Euryarchaeota in the 2021 Port communities over 2020. Another Archaean, Crenarchaeota also increased to 1.25% from 0.19% in 2020. Figure 11 displays these taxa at the Order level and shows the identification of Thermoplasmatales. This bacterial group has been shown to appear in metal-rich and low-pH sediments and environments (Teske et al 2021). Moreover, Oligosphaerales again appears higher in the port.

We did not dwell on Archaea components bacteria taxa last year, but their presence appears to be significantly increased at P13, P16, P17 sites in 2021. Along with Bacteria, Archaea play major roles in geochemical and microbial community processes and serve as useful indicators for reef and water quality health (Glasi et al, 2017).

Similar to 2020, port sediment microbiomes of 2021 still showed a predominance of Desulfobacterales (10.4% P, 0.1% R), Gammaproteobacteria incertae sedis (3.9 P, 1.7% reef), and Deferribacteres incertae sedis (1.1% P, 0.33 % R). Also, the composite P data appears to have 4-5x more Bacterioales/Bacteroidetes (a fecal indicator bacteria) and Anaerolineales (2.1 % P, 0.5% R) compared to R site communities.

In 2021, similar to 2020 and in contrast to port sites, reef communities continued to show a greater abundance of Oceanospirillales, Planctomycetales (3.0 % P, 4.7% R) , Desulfobacterales, Oxyphotobacteria_u_o, Rhodospirillales, Nitrosopumilales (2.3% P, 3.2% R), and the E01-9c-26 marine group, Xanthomonadales and Cellvibrionales were common to both reef and port sites.

Noticeably abundant in the 2021 reef compared to the 2021 port, Oxyphotobacteria comprise variable families of cyanobacteria, and recent studies have shown that certain Oxyphotobacteria may associate positively with specific coral symbiont Symbiodiniaceae communities (Quigley et al 2020). Conversely, the uncharacterized cyanobacterium taxon referred to as “Familyi” which showed high abundance levels at several 2020 reef sites, did not rise to the same levels in 2021 (see far right -and side of heatmap, Figure 8).

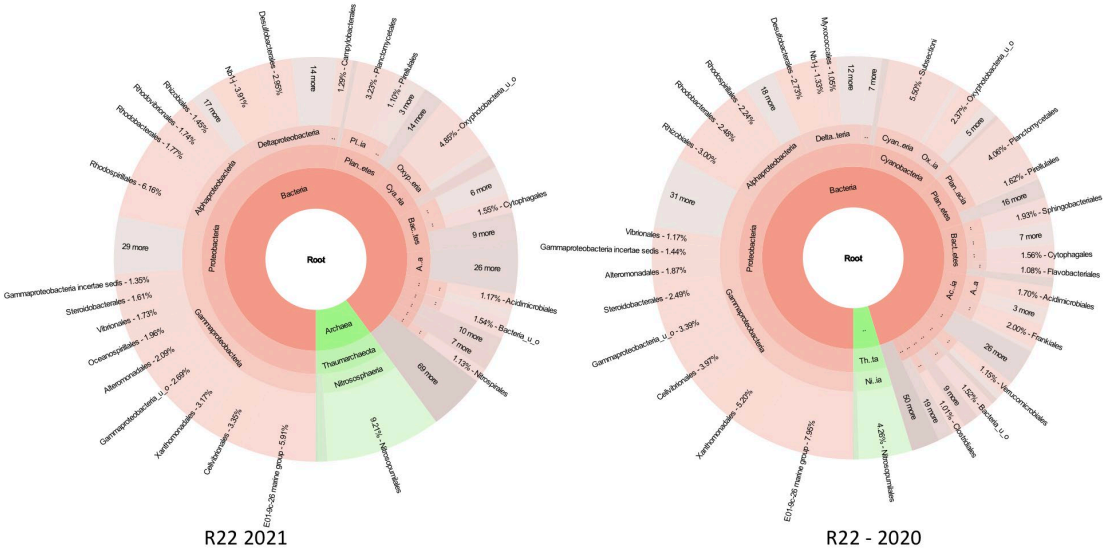


Figure 7 - *Representative sunburst plots showing bacterial taxa at the Order level for Reef site, R22 for both 2021 and 2020.*

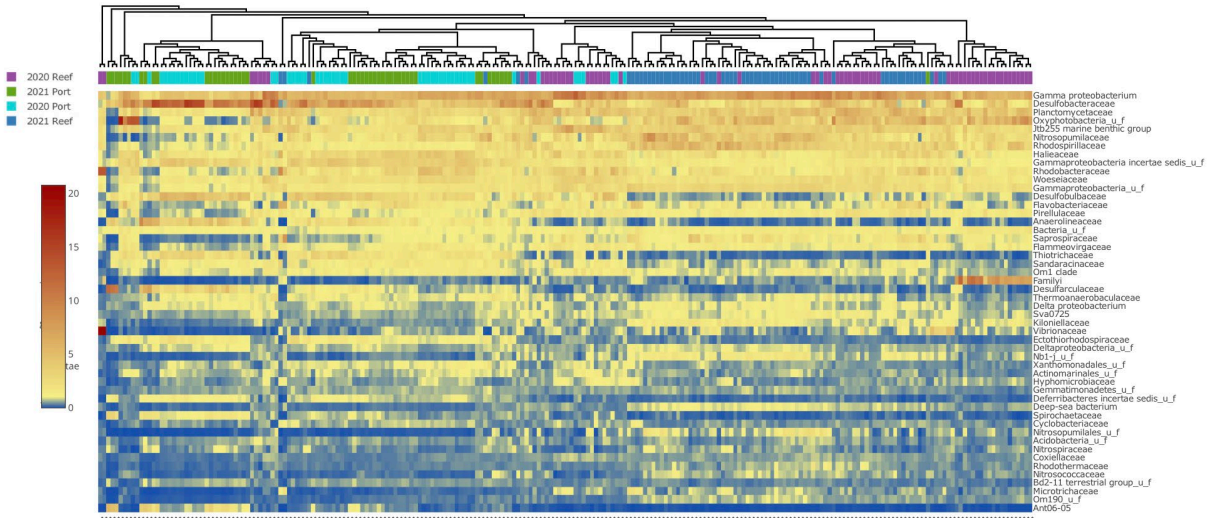


Figure 8 - *Heat Map at the Family level for 2021 microbiomes. Warmer colors indicate greater abundances.*

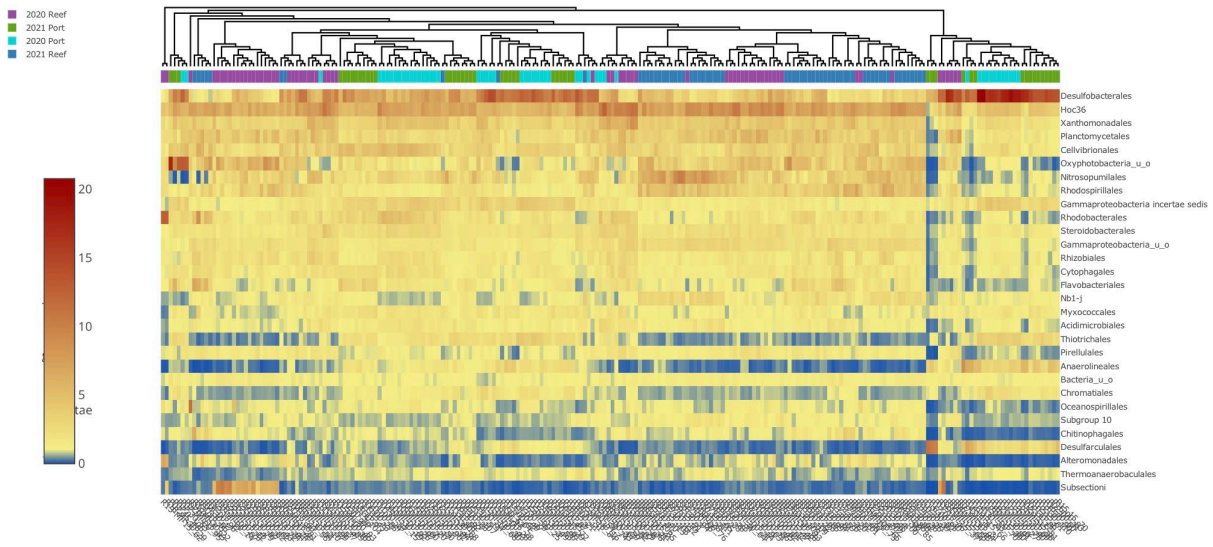


Figure 9 - *Heat Map at the Ordinal level for 2021 microbiomes. Warmer colors indicate greater abundances.*

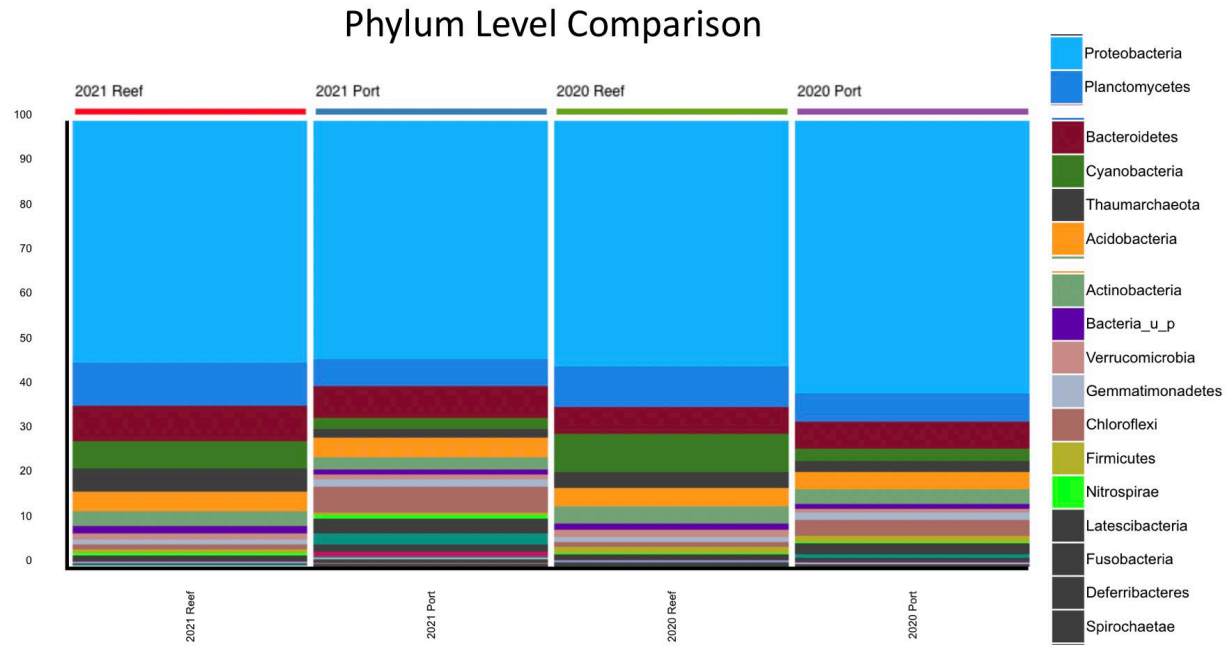


Figure 10 - *Aggregate Stacked bar chart at the Phylum level for all 2020 Port (N=54) and Reef (N=63), and 2021Port (N=51) and Reef (N=62) samples.*

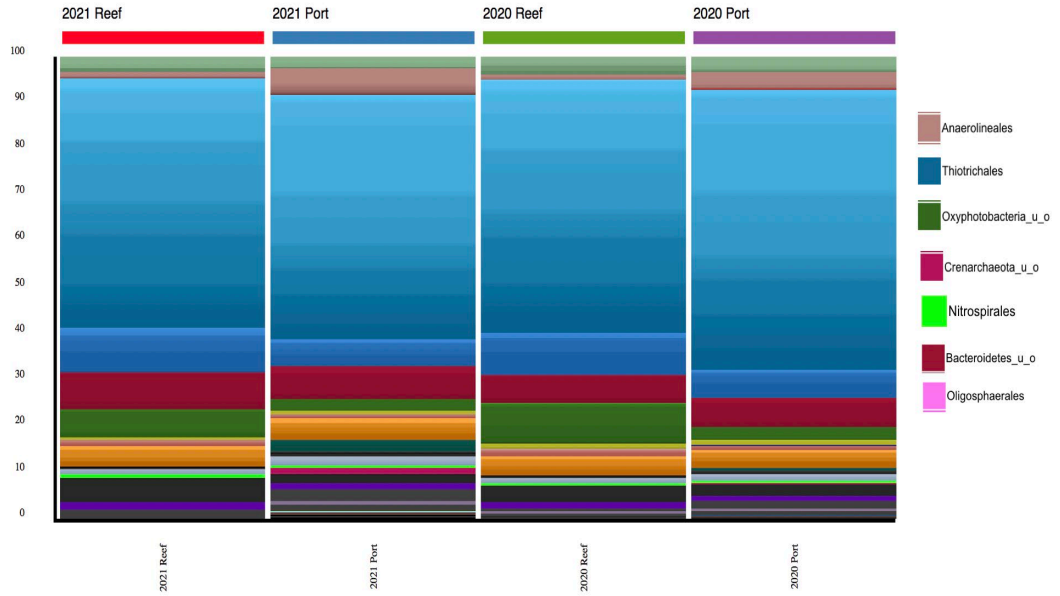


Figure 11 - *Aggregate Stacked bar chart at the Order level for all 2020 Port (N=54) and Reef (N=63), and 2021Port (N=51) and Reef (N=62) samples. Other color codes are keyed at the Cosmos website, or in the appendices of this report.*

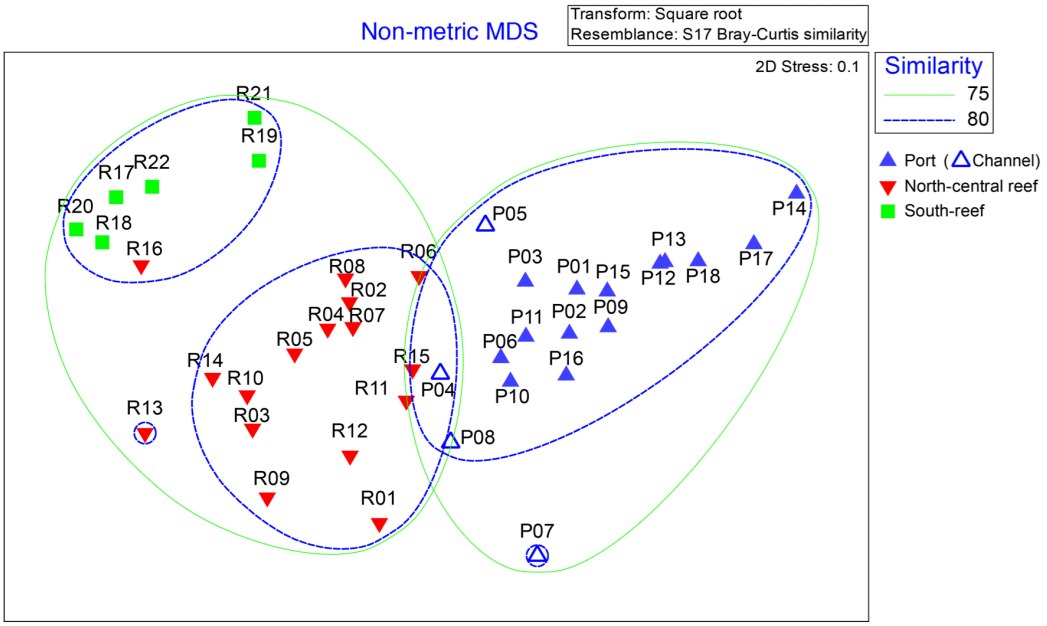


Figure 12 - *NMDS plot of both R and P samples from 2020. The points reflect an average of the replicates taken for each sample site. Note R sites cluster into two.*

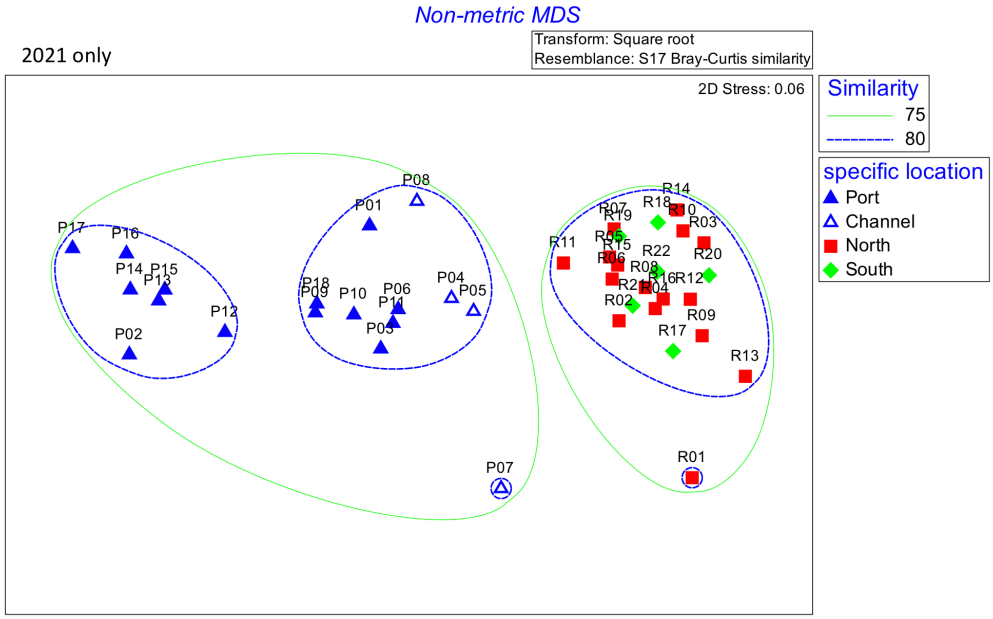


Figure 13 - *NMDS plot of both R and P samples from 2021. Similar to Figure 12 the points reflect an average of the replicates taken for each sample site.*

Potential Pathogens

As in Phase I 2020, some pathogenic and enteric taxa repeatedly occur in the current Phase II sediment dataset, although their relative abundances remain relatively low. More potential human pathogens appear in port sediments than reef sediments. These include

Staphylococcus, Streptococcus, and some *Vibrio. Enterococcus* was not as prevalent in this 2021 dataset. Also, potentially pathogenic microbial taxa occurred at both P and R sites. This was not unexpected, as fecal indicator bacteria (FIB) do appear higher in port vs reef sediments.

The results can continue to be compared with other recent surveys from the same Broward county area (Aranda et al, 2016; Campbell et al, 2015; O'Connell et al, 2018). We cannot make predictions on what diseases would follow from microbial activities or changes in abundances if and when specific communities were disturbed and relocated to different parts of the port, reef or beyond.

As indicated in Phase I, predicting which bacteria could cause harm or be involved in future disease is beyond the scope of this report, and requires a more specific experimental design and identification of targets (e.g. human, reef organisms, other microbial ecosystems etc.). For example, the pathogen for coral Stony Coral Tissue Loss Disease (SCLTD) still remains equivocal though recent progress in identifications have been made (Paul et al, 2019). Rosales et al (2020) indicated that bacteria in the *Rhizobiales* and *Rhodobacterales* orders may be associated with SCLTD. However, these taxa also appear to be very common in the sediments analyzed in this study. In the port for example, *Rhodobacterales* ranges from 1.0 – 9.1% at almost all reef sites, while it is lower in the port but still ranging from 1 – 2% across most samples. Becker et al. (2021) more recently identified some bacteria that could be associated with SCTLD, such as *Cohaesibacter*, *Algicola*, *and Thalassobius*. These taxa can also be found in the Phase II sediments dataset, but compared to *Rhizobiales* and *Rhodobacterales* orders, appear present in both reef and port sites at relatively low abundance. *Algicola* was present at multiple reef and port sites, mostly below 0.01%, except notably at R14 and R15 where it rose to 0.12.

Moreover, the 16S primers used here survey the large bacterial composition, but cannot address other microbial components such as viruses, protozoans or fungi likely to also be in these samples. However, the data represent valuable information which could be viewed as a baseline, relatively "undisturbed" state of bacterial communities.

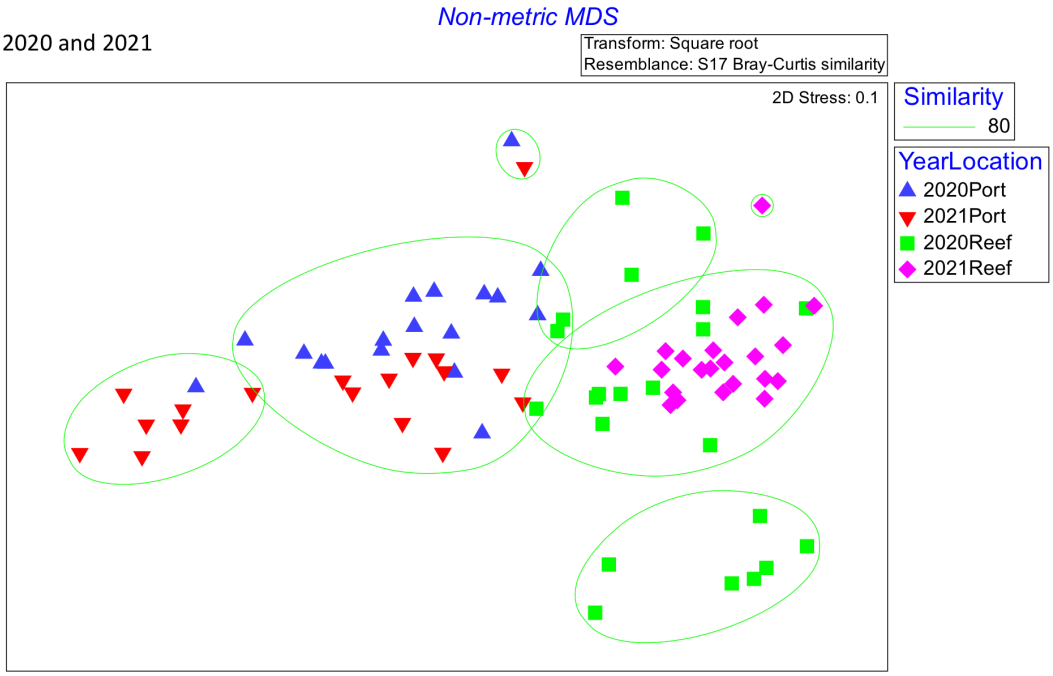


Figure 14 - *NMDS plot of combined 2020 and 2021 port and reef microbial community data using Bray-Curtis similarities. The low stress value correlates with greater accuracy of the two-dimensional representation.*

By using comprehensive spatial and temporal microbiome characterizations like this, sources of reef threats may be identified and conveyed to local and regional resource managers.

3.1.3 Community-Specific Patterns

3.1.3.1 Cluster Analyses

In a multidimensional space principle coordinates (or components) analysis (PCoA) was performed on the 2020 data last year. However, non-metric multidimensional scaling (NMDS) is a similar method of non-parametrically visualizing levels of similarity of individual cases of datasets. Similar to PCoA, NMDS graphs exhibit the 16S data in a two-dimensional clustering using Bray-Curtis distances. The 2020 16S results had showed significant clusters of separate port and reef communities. The NMDS plot of Figure 12 shows a reanalysis of the 2020 data, which was generated after the Phase I report and reflects that reef communities could actually be partitioned into north and southern reef sectors. Again, each point on the plot represents thousands of 16S sequences. Also, more support is shown for the intermediate position of channel sites – P04 – P07. These sites geographically straddle Port and Reef areas and likely have the most water flow (disturbances).

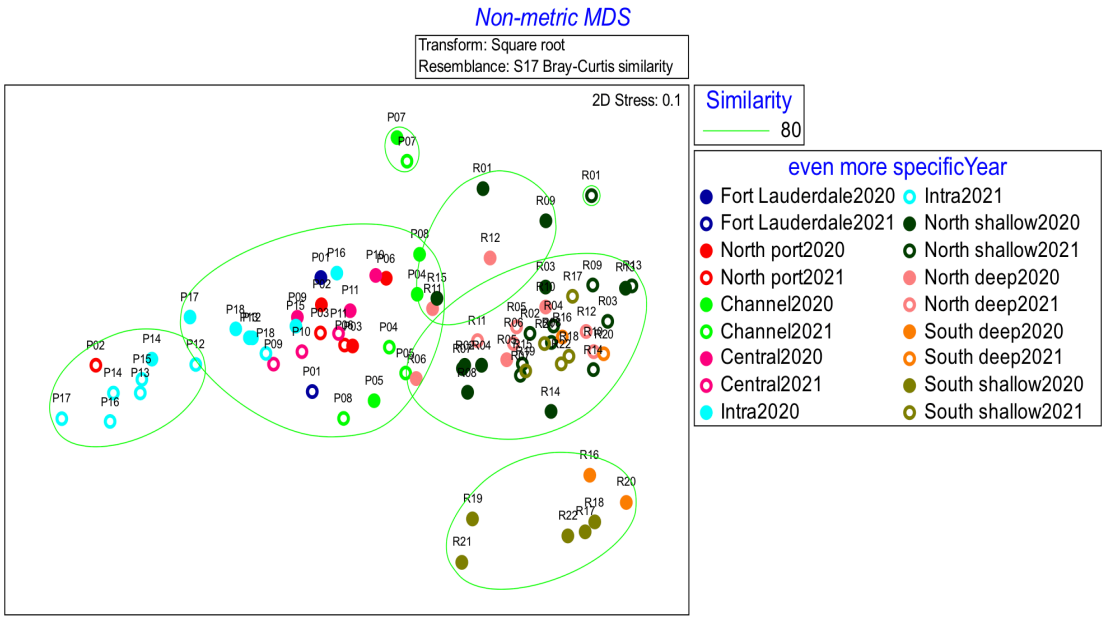


Figure 15. *NMDS plot of combined 2020 and 2021 port and reef microbial community data using Bray-Curtis similarities. More details are shown for each location and type of site.*

Two distinct clusters representing port and reef samples appear again in the NMDS analyses of 2021 datasets (Figure 13). Similar to last year, some microbiomes appear correlated to their geographic location. For example, the P sites within the inlet channel (P4, P5, P7, and P8) tended to overlap with the reef sites. Interestingly, however, port communities now appear partitioned into two distinct clusters which was not observed in 2020. Moreover, the previous northern and southern reef clusters seen in the 2020 datasets are not recapitulated in the 2021 dataset. Rather Figure 13 shows that all R sites cluster strongly with each other except for one outlier (R01). Figure 14 shows the possible effects of time across both P and R communities. As the 2020 and 2021 datasets are combined and analyzed as a single dataset, the clustering changes from the two distinc reef groups to a single group. Figure 15 shows a more detailed NMDS plot and indicates that sample depth did play a factor in clustering.

The NMDS and cluster analysis indicated that Intracoastal sites P12-P17 and north port site P02 in 2021 clustered separately from other port sites compared to 2020. The remainder of the port sites clustered into a second distinct group. While there was a distantly different cluster of samples from “south reef” in 2020, all reefs sites now clustered together in one group in 2021. Analysis of similarity (ANOSIM) below indicated which of these clusters were statistically significant, as well as confirming trends in both the port and reef samples were different between 2020 and 2021.

3.1.3.2 Canonical Correspondence Analysis (CCA) and Non-Multidimensional Scaling (NMDS)

As in Phase I, sediment samples were analyzed for Trace metals (see Table 4), Total Phosphorus and Total Carbon/Total Nitrogen. When trying to determine and explain the environmental drivers which may explain microbiome patterns, these would be the only eligible parameters to apply. These were the only data that correspond to the microbiome sequences. Canonical Correspondence analysis (CCA) was applied to the 16S data and shown in Figure 16.

We again see a strong association of Total Phosphorus (TP) with port samples. In contrast to 2020, cadmium effects were less pronounced than total Carbon (TC) as a vector driving reef microbial communities.

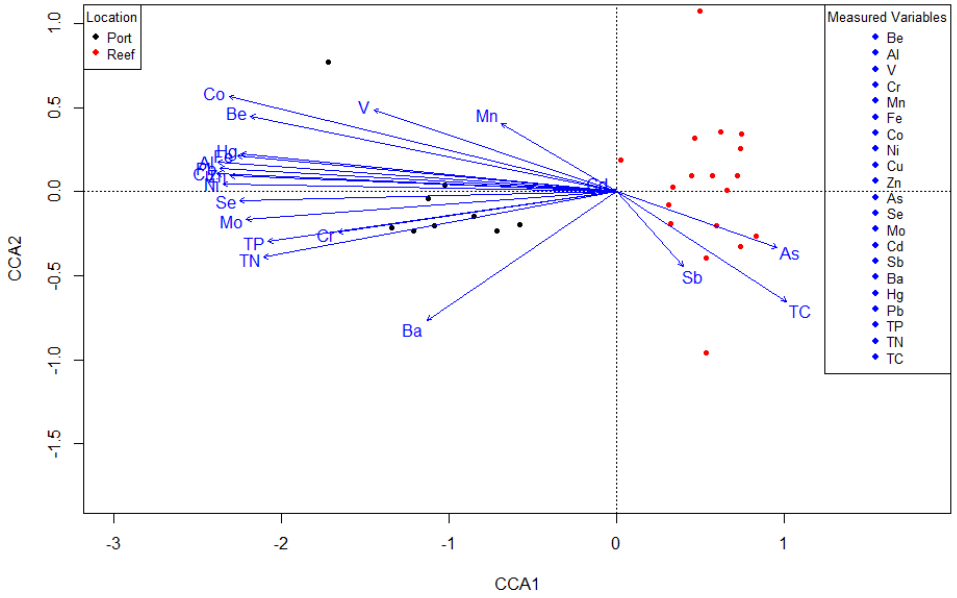


Figure 16 - *Plot of CCA analyses of 2021 the nine (x 3 replicate) port and reef sites with respect to heavy metal metadata.*

3.1.3.3 Supporting Statistics

The ANOSIM (Analysis of Similarity) test was performed on the 113 samples of 16S rRNA sequence data, whereby the R value (i.e. the strength of the factors on the samples) and P value (i.e. significance levels) were as follows:

ANOSIM to compare port and reef groups in 2020 and 2021
Statistically significant differences between all groups.
The strongest differences were between the port and reef in 2021 (R = 0.863).
Weak but significant, the trends in the port were different in 2020 and 2021 (R= 0.216).
Reef samples were more strongly different between 2020 and 2021 (R = 0.457).

ANOSIM reef only 2020-2021
There were very strong differences (R =0.830) between microbial communities in the north and south reef samples in 2020, but this was completely changed in 2021 (R=-0.1, p = 82.9). Differences between the south reef samples in 2020 and 2021 were strongly different (R = 1).

ANOSIM Port only 2020-2021
Differences between intracoastal samples in 2020 and 2021 were moderately strong (R =0.525) and significant.

Simper analysis revealed which of the ASVs are the primary drivers contributing to dissimilarities in the current data, i.e, which species and in what proportion contributes to the differences between the groups.

For comparisons between 2021 port vs reef, these ordinal level taxa appeared to be significant drivers at the p = 0.01 level: *Thiotrichales, BD7-8, Polyangiales, Ectothiorhodospirales, Steroidobacterales, Nitrosopumilales Ectothiorhodospirales, Desulfatiglandales, Ectothiorhodospirales, Ectothiorhodospirales, Nitrosopumilales.*

Only *Thiotrichaceae, Desulfobullbaceae, , Ectothiorhodospiraceae, Desulfuromonadales_u_f, Planctomycetaceae* appeared consistent with the 2020 SIMPER results.

SIMPER results for 2020 vs 2021 Port only communities also indicated that Desulfobacteraceae, Desulfobulbaceae, Anaerolineaceae, Nitrosopumilaceae, Desulfarculaceae, Rhodospirillaceae, and an unknown family of Thermoplasmatales and Crenarchaeota increased in relative abundance in 2021 (across all areas), and Rhodobacteraceae and an unknown family of Cyanobacteria decreased in 2021.

Archaea, Desulfarculaceae, and a family within the Gif9 order of Chloroflexi and order Aminicenantes increased in abundance in the intracoastal sites in 2021, while another Desulfobactereceae and unknown Cyanobacterial family decreased.

An unknown Cyanobacteria decreased and Sporichthyaceae (Actinobacteria), Nostocaeae (Cyanobacteria), Comamonadaceae (Proteobacteria) and Terrimicrobiaceae (Verrucomicrobia) were absent in the south reef samples in 2021 compared to 2020 but 2021 south reef samples increased in the Archaea Nitrosopumilaceae.

In general, the reef samples between 2020 and 2021 had an increase of Thaumarchaeota (Archaea), Rhodospirillaceae, Saprospiraceae (Bacteroidetes).

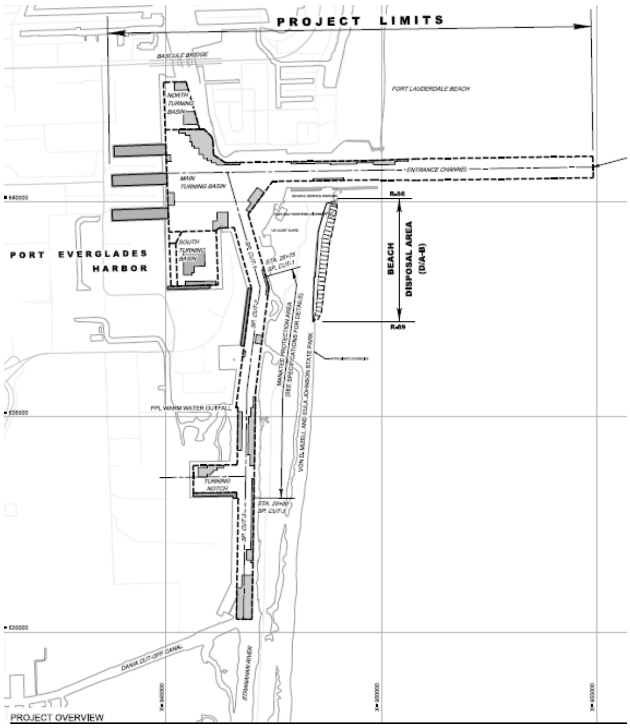


Figure 17 - *Map of sites from March 2020 Port Everglades O&M dredging derived from "June 2019 Port Everglades O&M Spillage Analysis". Dredging locations shown in grey shading.*

3.2 Conclusions and Future Directions

Marine microbes play important ecological and biogeochemical roles in the world's habitats including our oceans. They not only provide sourcing information about water masses, they may serve as indicators of degradation in water quality. They are also part of sensitive ecosystems like coral reefs and more importantly do have the potential to directly affect the health of human and marine life. This CRCP project has provided an interesting opportunity to characterize sediments from heavily built environment closely juxtaposed to a more natural (reef) habitat.

Sediments hold some of the most complex and biodiverse microbial communities ("microbiomes") among all habitats on the planet including marine sediments. Port Everglades Inlet and the adjacent Florida's Coral Reef seawater bacterioplankton microbiomes have been surveyed on a few occasions, but this is not a regular monitoring activity and previously did not include sediments. Again, it should be re-emphasized that the 16S rRNA marker used in this study represents only one gene, albeit a proven tool for many bacterial identifications to the Family level.

One primary hypothesis guiding this CRCP project has been that port and reef sediment microbiomes differ significantly by having qualitatively and quantitatively different species composition, or beta diversity, which was corroborated last year. Phase II data allowed testing for the consistency of this hypothesis across a one-year time span, albeit with a minor planned human disturbance (O&M dredging) in the port in February 2021 (Figure 17). Reef sites were generally affected by natural phenomena, and no major storm affected Broward County between the 2020 and 2021 sampling dates.

Similar to the 2020 survey, the 2021 results also point to significant differences between port and reef microbial communities, while many overlapping and common microbial taxa remain present in similar abundances. Significant variation in microbiomes still existed between some port and reef sites. The differences appear to be mildly supported based on a few taxa that drive the major differences. On the other hand, the overall similarities in microbial composition that occur between most reef and port sites, also suggests a homogenization and continual mixing of communities throughout the year.

We make cannot make recommendations regarding any specific remediation or actions against potential pathogens (human or non-human), as potential pathogens did appear in both 2020 and 2021 microbiome profiles in varying abundances. However, these data can be used as baselines for which microbes occur in both port and reef sites. Potentially pathogenic bacteria are part of the flora, and so disease etiologies may be determined by other factors besides the presence of the microbes themselves.

The next set of questions in the future could focus on whether these methods can detect and demonstrate transfer of unique taxa from one site to the next, affecting the community's composition or functions. There is always the possibility of transmission between sites due to water currents, tides and human disturbances. A computational tool that can be used to mark certain taxa as potential "sources" and "sinks" is SourceTracker, which can be applied to this data in the future. We were unable to apply the tool on the 2021 data due to an elapsing of time. Moreover, this CRCP study only sampled annually in 2020 and 2021. If funding were not an obstacle, more frequent sampling, perhaps monthly or similar to the weekly sampling of water samples by O'Connell et al (2018) could reveal other dynamics in the sediment microbiomes.

We have to keep in mind that sediments can be easily disturbed even without active dredging events. For example, the samples collected were only taken from the top 10-20cm of sediments. Large tanker ships regularly stir up sediments in the turning basin of PEI. While in the water column, disturbed microbial taxa and partial communities can be easily re-distributed to different parts of the port, re-settle at a different location, or be carried out to the adjacent reef. These movements would depend on timing of the incoming and outbound tides.

Although not a goal of this project per se, in the future we could compare the reef sediment microbiomes derived here to those from healthier reefs at different Caribbean locales, especially those not adjacent to large metropolises such as Fort Lauderdale. The comparison could provide which bacteria contribute to healthy vs deteriorated reef tracts.

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5 APPENDICES

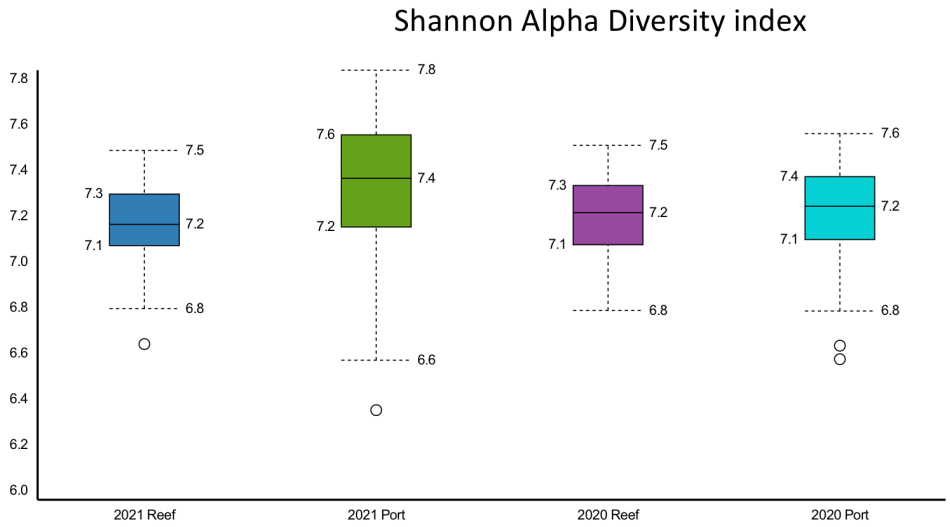
5.1 Links to Supplementary Files Stored Online

All raw data and files can be accessed online, at the following links:

1. Permanent data storage repository at - https://nsuworks.nova.edu/lopez_lab/3/

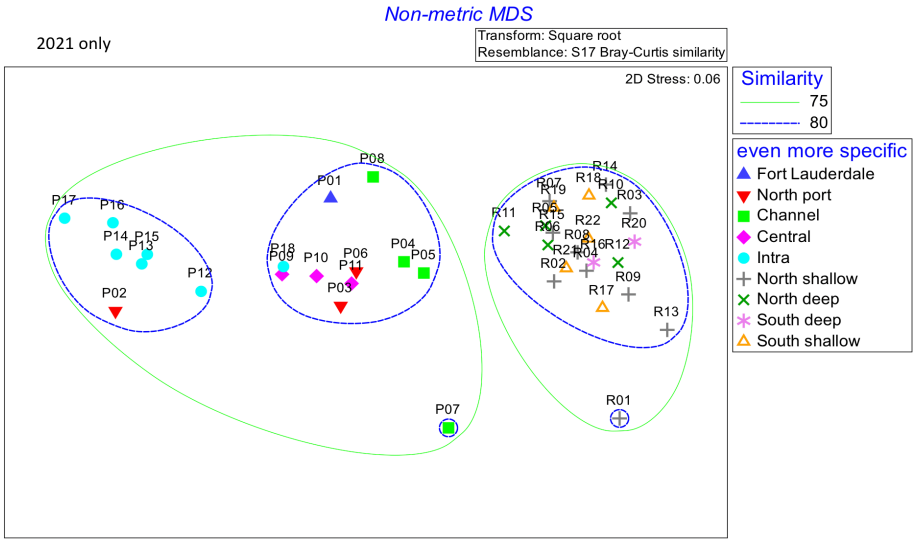
2. At the NCBI GenBank repository under BioProject #PRJNA742832). Official public release date is set for 12 months from final report, to allow for possible publication of this data.

5.2 Supplementary Figures and Tables

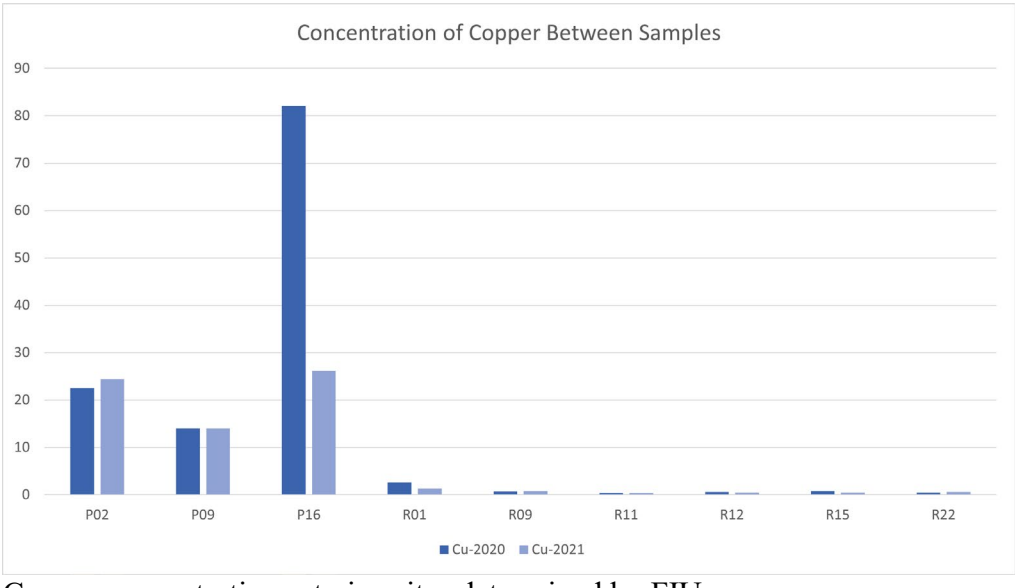


Cohorts	Statistic	P-value
2021 Reef ↔ 2021 Port	-3.4513	0.0006
2021 Port ↔ 2020 Reef	2.6022	0.0093
2021 Port ↔ 2020 Port	2.2124	0.0269

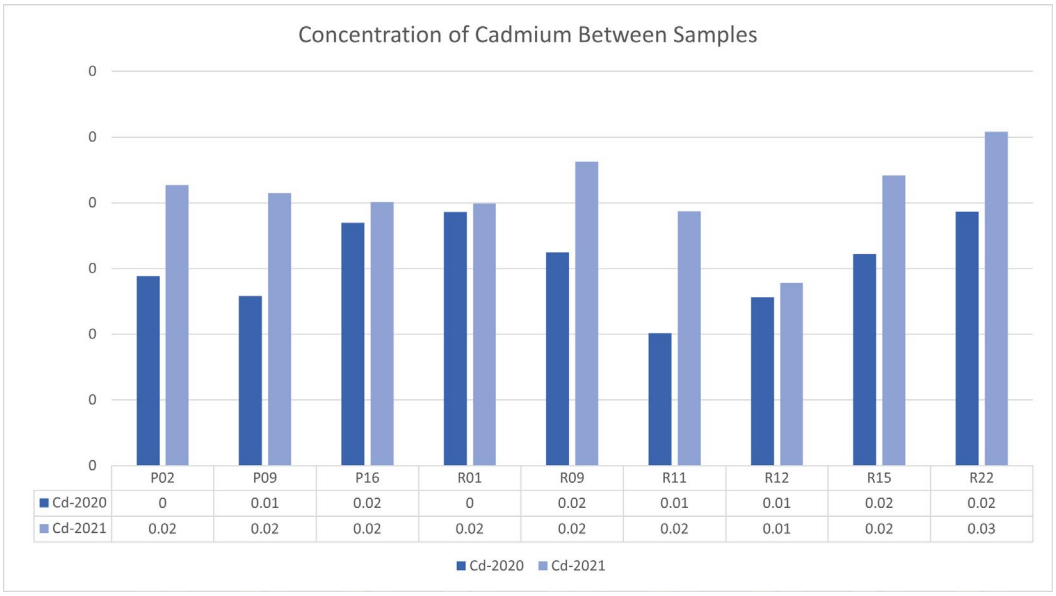
Shannon alpha diversity index with p values. Patterns and significance agree with the Chao I index.



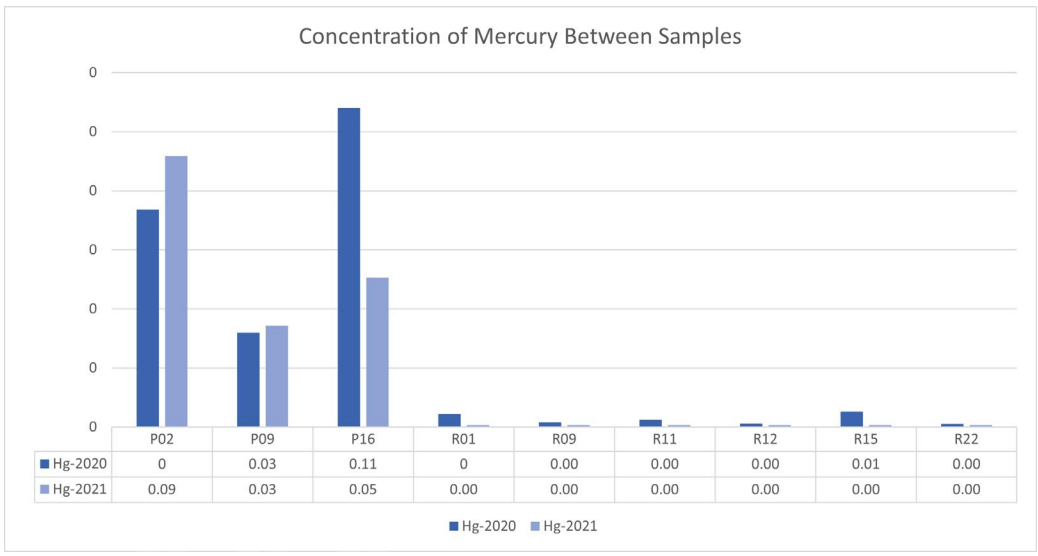
NMDS plot showing Phase II microbiomes and geographic locations.



Copper concentrations at nine sites determined by FIU



Cadmium concentrations at nine sites determined by FIU



Mercury concentrations at nine sites determined by FIU

5.3 R Code Used for 2021 Statistical Analyses in Vegan

R Code for Report:

```
setwd("C:/Users/cmb08/Desktop/PEI")
PEI_Abundances <- read.delim("C:/Users/cmb08/Desktop/PEI/PEI_Abundances.txt",
row.names=1)
#Used the “Import Dataset” option the load the rest of the data
metadata <-Metadata
t.dat<- as.data.frame(t(PEI_Abundances))
dat<-t.dat
common.rownames <- intersect(rownames(dat),rownames(metadata))
dat <- dat[common.rownames,]
metadata <- metadata[common.rownames,]
all.equal(rownames(dat), rownames(metadata), ignore.row.order = TRUE)
#TRUE
ASV.abund<-which(colSums(dat)>2)
dat.dom<-dat[,ASV.abund]
library(vegan)
library(base)
dat.pa<-decostand(dat.dom, method ="pa")
dat.ASVs.05per<-which(colSums(dat.pa) > (0.05*nrow(dat.pa)))
dat.05per<-dat.dom[,dat.ASVs.05per]
library(phyloseq)
library(ggplot2)
library(qiime2R)
library(dplyr)
library(microbiome)
library(RColorBrewer)
taxonomy = read.table(file= "Cat_Taxonomy.txt", header = TRUE, sep ="\t", row.names
= 1)
phy_tree=qza_to_phyloseq(tree="take-two.rooted-tree.qza")
ASV.UF = otu_table(as.matrix(dat), taxa_are_rows=FALSE)
tax.UF = tax_table(as.matrix(taxonomy))
meta.UF = sample_data(metadata)
taxa_names(tax.UF)
taxa_names(ASV.UF)
taxa_names(phy_tree)
physeq = phyloseq(ASV.UF,tax.UF,meta.UF,phy_tree)
sample_sums(physeq)
write.csv(sample_sums(physeq), file = "SampleReads.2021.csv", row.names = TRUE)
mean(sample_sums(physeq))
#Mean=52710.95
min(sample_sums(physeq))
#Min=4464
max(sample_sums(physeq))
#Max=106300
```

```
sd(sample_sums(physeq))
#SD=22841.98
physeq
#58189 taxa and 113 samples


#Alpha Diversity Figure
rich = estimate_richness(physeq)
rich
write.csv(rich, file = "Richness.2021.csv", row.names = TRUE)
mean(rich$Observed)
#Mean=1412.566
min(rich$Observed)
#Min=245
sd(rich$Observed)
#SD=518.8601


#ASVs
any(taxa_sums(physeq) == 0)
#TRUE

#Plot 1
plot_richness(physeq, x="Description")+ geom_boxplot()

#Saved as "Alpha Diversity 2021"

#Plot 1.5
plot_richness(physeq, x="Description", measures = c("Observed","Chao1", "Shannon",
"InvSimpson"))+ geom_boxplot()
#Saved as "Alpha Diversity_simplified 2021"

#NMDS
dat.ra<-decostand(dat.05per, method = "total")
dat.rat <- as.data.frame(t(dat.ra))
View(dat.rat)
write.table(dat.rat, "dat.rat.2021.txt", sep="\t",row.names = T)
dat.ra<-decostand(dat.05per, method = "total")
##ANOSIM
ano = anosim(dat.ra, metadata$Description, distance = "bray", permutations = 9999)
ano
#R: 0.88
#Significance: 1e-04 aka p value
#Location significantly impacts the microbiome of the sediment. (p=1e-04)
```

```
#Adonis - Analysis of variance using distance matrices
dat.bc.dist<-vegdist(dat.ra, method = "bray")
adonis(dat.bc.dist~Description, data = metadata)
#See image #saved as adonis output 2021
#The location description counts for 31% of the variance in the dataset. (R2=0.30916,
p=0.001)

#Create NMDS Chart
comm.bc.mds<-metaMDS(dat.ra,trace=FALSE, trymax=100)
comm.bc.mds
#stress= 0.09731676 (very good!)
#how much stress your data went through to become a 2D plot.
stressplot(comm.bc.mds)
#Saved as stressplot

mds.fig<-ordiplot(comm.bc.mds, display="sites")
ordiellipse(mds.fig,metadata$Description,label=F,conf=0.95,col=c("black","red"))
#adjust colors,pch=20 make it bullet points
points(mds.fig,"sites", pch=20, col= "black", select = metadata$Description == " Port")
points(mds.fig,"sites", pch=20, col= "red", select = metadata$Description == " Reef")
#Add Stress Value
text(1.1,1.0, "Stress = 0.0973", cex=0.7)
#Add Legend

legend("topleft",legend= c("Port","Reef"),
       title = "Location",
       col=c("black","red"),
       pch=19, cex=0.8)
#Saved as NMDS_2021



#see if there are differences
#PerMANOVA

library(RVAideMemoire)

pairwise.perm.manova(dat.bc.dist,metadata$Description)
#p-value=0.001

#SIMPER
dat.simp<-simper(dat.ra, metadata$Description, permutations = 999)
sink("Simper_PEI.csv")
summary(dat.simp)
sink()
```

```
#CCA
setwd("C:/Users/cmb08/Desktop/PEI")
metadata <-metadata_cca
dat.ra<-elements

library(CCA)

library(vegan)

set.seed(42); env.cca<-
cca(dat.ra~Be+Al+V+Cr+Mn+Fe+Co+Ni+Cu+Zn+As+Se+Mo+Cd+Sb+Ba+Hg+Pb+TP
+TN+TC, data =metadata)


env.cca
vif.cca(env.cca)
#ended removing some variables because that had no values, they were: ...
set.seed(42);lwr<- cca(dat.ra~1, data=metadata)
lwr
#Using a forward selecting model, must keep our set seed

set.seed(42);mods.all<-ordiR2step(lwr, scope = formula(env.cca))
mods.all
vif.cca(mods.all)
#Be=138.886170;TP=25.467769; Mn=26.143080; Co=229.565987; V=71.956519;
Cd=7.369238; Pb=215.348469
R2.adj.all<-RsquareAdj(mods.all)
R2.adj.all
#R2 value is 0.9991338, all very correlated.

mods.all$anova
#Be had a R value of 0.63371 (which was the lowest one).
# Add extra space to right of plot area; change clipping to figure; used to add legend to
outside the plot area
par(mar=c(5.1, 4.1, 4.1, 8.1), xpd=TRUE)
#Plot CCA
windows(10,7)
cca.p <- plot(mods.all,type = "none")
cca.p <- plot(mods.all,ylim=c(-1.8,1), type = "none")
#Adjust plot point shapes and colors
points(cca.p, "sites", col=metadata$Description, pch=16)
points(cca.p, "sites", col= as.numeric(metadata$Description), pch =
as.numeric(metadata$Description))
points(mds.fig,"sites", pch=20, col= "black", select = metadata$Description == " Port")
points(mds.fig,"sites", pch=20, col= "red", select = metadata$Description == " Reef")
```

```
ef.all<-
envfit(cca.p,metadata[,c("Be","Al","V","Cr","Mn","Fe","Co","Ni","Cu","Zn","As","Se","
Mo","Cd","Sb","Ba","Hg","Pb","TP","TN","TC")])
plot(ef.all)
egend("topleft",legend= c("Port","Reef"),
      title = "Location",
      col=c("black","red"),
      pch=19, cex=0.8)
#Saved as CCA_2021
```