Characterization of the Marine Sponge Amphimedon compressa Microbiome Across a Spatial Gradient

Renee Michelle Potens
Nova Southeastern University, rp641@nova.edu

Follow this and additional works at: https://nsuworks.nova.edu/occ_stuetd

Part of the Biodiversity Commons, Environmental Microbiology and Microbial Ecology Commons, Genetics Commons, Laboratory and Basic Science Research Commons, Marine Biology Commons, Molecular Genetics Commons, and the Oceanography and Atmospheric Sciences and Meteorology Commons

Share Feedback About This Item

NSUWorks Citation

This Thesis is brought to you by the HCNSO Student Work at NSUWorks. It has been accepted for inclusion in HCNSO Student Theses and Dissertations by an authorized administrator of NSUWorks. For more information, please contact nsuworks@nova.edu.
Characterization of the Marine Sponge *Amphimedon compressa* Microbiome Across a Spatial Gradient

By

Renee Michelle Potens

Submitted to the Faculty of
Nova Southeastern University Halmos College of Natural Science and Oceanography
in partial fulfillment of the requirements for the degree of Master of Science with a specialty in:

Biological Science

Nova Southeastern University

April 2016
Thesis of
RENEE MICHELLE POTENS
Submitted in Partial Fulfillment of the Requirements for the Degree of

Masters of Science:

Biological Science

Nova Southeastern University
Oceanographic Center
Halmos College of Natural Science and Oceanography

April 2016

Approved:
Thesis Committee

Major Professor : _____________________________

Jose Lopez, Ph.D.

Committee Member : ___________________________

Robert Smith, Ph.D.

Committee Member : ___________________________

George Duncan, Ph.D.
TABLE OF CONTENTS

1.0 ACKNOWLEDGMENTS ........................................................................ iv
2.0 ABSTRACT ......................................................................................... v
3.0 LISTS................................................................................................. vi
  3.1 List of Figures ................................................................................... vi
  3.2 List of Tables .................................................................................... ix
  3.3 List of Abbreviations ........................................................................ x
4.0 INTRODUCTION ................................................................................... 1
  4.1 Significance of Sponges ..................................................................... 1
  4.2 High-Throughput Sequencing ........................................................... 5
  4.3 16S rRNA ......................................................................................... 8
  4.4 Metagenomics and Microbiomes ...................................................... 9
  4.5 Microbial Symbionts ....................................................................... 13
  4.6 *Amphimedon compressa* Spicule Taxonomy .................................... 15
  4.7 *Amphimedon compressa* Duchassaing & Michelotti, 1864 ............. 16
5.0 OBJECTIVES AND HYPOTHESES .................................................. 18
  5.1 Objectives ....................................................................................... 19
  5.2 Hypotheses ...................................................................................... 19
6.0 MATERIALS AND METHODS ............................................................. 20
  6.1 Sponge Sample Collection ............................................................... 20
  6.2 *Amphimedon compressa* Spicule Taxonomy .................................... 22
    6.2.1 Individual Spicule Taxonomy ...................................................... 22
    6.2.2 Pseudoskelton Preparation ......................................................... 22
    6.2.3 Microscopy ................................................................................. 23
  6.3 Microbial DNA Extraction/Isolation ................................................ 23
  6.4 Illumina High-Throughput Metagenomic Sequencing ....................... 24
  6.5 Earth Microbiome Project Acquisition of Microbiome Data ............... 24
  6.6 Data Analysis .................................................................................. 26
    6.6.1 Metadata ..................................................................................... 26
    6.6.2 Analytical Platform ..................................................................... 27
    6.6.3 Analysis Categories ................................................................... 28
    6.6.4 Analysis of Biotic Data ............................................................... 29
    6.6.5 Operational Taxonomic Units ...................................................... 29
    6.6.6 Rarefaction Analysis .................................................................. 30
    6.6.7 ANOVA for OTU Richness ........................................................... 31
    6.6.8 Alpha Diversity .......................................................................... 31
    6.6.9 Inverse Simpson ........................................................................ 32
    6.6.10 Tukey for Multiple Comparisons of Means ................................ 32
    6.6.11 Beta Diversity ........................................................................... 33
    6.6.12 Bray-Curtis Dissimilarity .......................................................... 33
    6.6.13 Non-metric Multidimensional Scaling ....................................... 34
    6.6.14 Heatmap ................................................................................... 34
    6.6.15 Simper Similarities .................................................................... 34
    6.6.16 Analysis of Abiotic Factors ....................................................... 35
7.0 RESULTS .......................................................................................... 36
1.0 ACKNOWLEDGEMENTS

Thanks to my major professor Joe Lopez who first worked with me as an undergraduate and then took me on as his graduate student. Completion of this thesis was possible through his guidance and mentorship. My other two committee members, Robert Smith and George Duncan, shared their expertise with me, greatly improving the quality of my work. Special thanks to Cole Easson for teaching me about advanced statistical analyses and their specific applications in my thesis. Alexandra Campbell welcomed me into the lab on my first day, mentored me and showed me all the basic technical laboratory skills necessary for this work. She has been a good friend and sounding board through this challenging process. Data from the Earth Microbiome Project (EMP) provided the basic samples for this thesis. Lucas Moitinho who processed the raw sequence files created the OTU files for the EMP. Sponge samples were collected by the Nova Southeastern University Oceanographic Center CRAAM lab (South Florida) and the University of Alabama Thacker lab (Panama). Sponge identification expert Cristina Diaz showed me how to extract sponge spicules and process them for taxonomic verification. My lab mates, past and present, helped me develop as a scientist and provided assistance and support for this project. My friends and family were there for me when times were tough, and made the journey possible and enjoyable. Their kindness and support will never be forgotten.

This thesis is dedicated to two very special people, my Nana who was always there for me; and my father, who will always have a special place in my heart. Although they are no longer here to witness my success, I know they would be happy and proud to see the fruition of my dedication, perseverance and hard work.
Diverse and biologically important microbial communities (microbiomes) are symbiotic within marine sponges. In this study, the microbiome of Amphimedon compressa from three sample locations (Broward and Dade Counties, Southeast Florida, USA and the Southern Caribbean, Bocas del Toro, Panama) is characterized using 16S rRNA Illumina sequencing. The predominant taxa are Proteobacteria and Cyanobacteria, as expected for Low Microbial Abundance sponges, accounting for over 53% of the total microbiome community. The numbers of Operational Taxonomic Units (OTUs) decrease from Broward County (2,900) to Dade County (2,300) and then Bocas del Toro (1,200). The correlates to a decreasing north-south gradient of sponge microbiome richness and diversity. Sponge microbiome richness and Alpha diversity are nearly identical from the two closest locations (37 km), both in Southeast Florida (Tukey HSD/ANOVA; p=0.999). However Panama sponge microbiome richness and Alpha diversity are distinctly lower, with the primary driver being distance, ~1,850 km from Southeast Florida. Abiotic factors driving this trend of decreased richness and diversity include increased temperature, and deceased salinity in relation to precipitation-based seasons. Sponge microbiome Beta diversity as determined by Bray-Curtis Dissimilarity and Non-Metric Multidimensional Scaling documents the clustering of Panama samples as distinct from the Broward and Dade County samples. In a seasonal comparison, Broward County sponge microbiome richness (p=0.026, r²=0.92) and Alpha diversity (p=0.007, r²=0.98) are significantly different, documenting robust effects of temperature. This comparison confirms lowest microbiome OTU diversity in the season with highest precipitation and highest temperatures of 29.8 °C. These results are consistent with prior studies that report decreasing microbiome OTU richness and diversity under conditions of environmental stress such as decreased salinity and increased temperatures.
3.0 LISTS

3.1 List of Figures


FIGURE 5: Sample DCN31: 3/17/2011; oxea diactinal monaxial spicule of host sponge *A. compressa*, 1000x magnification with compound microscope.

FIGURE 6: Rarefaction curve for all sites and samples (Broward County, Florida, USA (n=4); Dade County, Florida, USA (n=4); Bocas del Toro, Panama (n=5).

FIGURE 7: Boxplot of microbiome OTU richness of host sponge *A. compressa* per collection site, with a marginal non-significant difference among sites, ANOVA $p=0.098$.

FIGURE 8: Regression analysis of microbiome OTU richness of host sponge *A. compressa* per temperature, with a marginal non-significant difference $p=0.078$, $r^2=0.26$.

FIGURE 9: Regression analysis of microbiome OTU richness of host sponge *A. compressa* per temperature, with significant differences $p=0.040$, $r^2=0.33$.

FIGURE 10: Boxplot of microbiome OTU richness of host sponge *A. compressa* per precipitation-based seasons, with a significant difference, ANOVA $p=0.021$.

FIGURE 11: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per collection site, with marginal non-significant differences among sites, ANOVA $p=0.081$.

FIGURE 12: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature, with a marginal non-significant difference $p=0.059$, $r^2=0.22$.

FIGURE 13: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature, with significant differences $p=0.041$, $r^2=0.27$. 
FIGURE 14: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per precipitation-based seasons, with significant differences among sites, ANOVA *p*=0.019.

FIGURE 15: Bray-Curtis Dissimilarity cluster dendrogram of host sponge *A. compressa* microbiome Beta OTU diversity per collection site. Values closer to 0.0 indicate the least dissimilarity (most similar) and values closer to 1.0 indicate the most dissimilarity (least similar).

FIGURE 16: Non-Metric Multi-Dimensional Scaling ordination plot per collection site for all thirteen of microbiome OTU Beta diversity of host sponge *A. compressa* microbiome samples. Samples located within ellipses are the most similar to each other.

FIGURE 17: Heatmap of microbiome species Beta diversity of host sponge *A. compressa* for the first fifty OTUs for taxonomic analysis of all three collection sites (Dade County, Florida, USA-Broward County, Florida, USA-Bocas del Toro, Panama). Complete with dendrogram, characterizes the square-root transformed relative abundance of taxa.

FIGURE 18; A, B, C: Relative Species Abundance histograms of host sponge *A. compressa* microbiome for the first top eight OTUs in order from highest to lowest abundance of all three collection sites (Broward County, Florida, USA-Dade County, Florida, USA-Bocas del Toro, Panama). The two most abundant identical taxa from all three locations are *Proteobacteria* (Phylum) and *Synechococcaceae* (Phylum *Cyanobacteria*).

FIGURE 19: Regression analysis of microbiome OTU richness of host sponge *A. compressa* per salinity in pair-wise comparison between Dade and Bocas del Toro, with a marginal non-significant difference *p*=0.095 *r*=0.25.

FIGURE 20: Boxplot of microbiome OTU richness of host sponge *A. compressa* per calendar-based seasons, with a marginal non-significant difference in wet and dry seasons between the site pair Broward and Bocas del Toro, Tukey *p*=0.07.

FIGURE 21: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per site, with a marginal non-significant difference between the site pair Dade and Bocas del Toro, ANOVA *p*=0.078.

FIGURE 22: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per site, with a significant difference between the site pair Broward and Bocas del Toro, ANOVA *p*=0.030.

FIGURE 23: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature in pair-wise comparison between Broward and Bocas del Toro, with a significant difference *p*=0.028 *r*=0.45.
FIGURE 24: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per salinity in pair-wise comparison between Dade and Bocas del Toro, with a marginal non-significant difference $p=0.088 \ r^2=0.27$.

FIGURE 25: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per salinity in pair-wise comparison between Broward and Bocas del Toro, with a significant difference $p=0.061 \ r^2=0.33$.

FIGURE 26: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per calendar-based seasons, with a marginal non-significant difference between the site pair Dade and Bocas del Toro, ANOVA $p=0.087$.

FIGURE 27: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per precipitation-based seasons, with a marginal non-significant difference between the site pair Dade and Bocas del Toro, ANOVA $p=0.078$.

FIGURE 28: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per precipitation-based seasons, with a significant difference between the site pair Broward and Bocas del Toro, ANOVA $p=0.022$.

FIGURE 29; A, B, C: Bray-Curtis Dissimilarity cluster dendrograms of microbiome Beta OTU diversity of host sponge *A. compressa* for the pairwise analysis of the three location comparisons (A): Dade County, Florida, USA-Broward County, Florida, USA; (B): Dade County, Florida, USA-Bocas del Toro, Panama; (C): Broward County, Florida, USA-Bocas del Toro, Panama. Larger values indicate the least dissimilarity (most similar) and smaller values indicate the most dissimilarity (least similar). Clustering follows a spatial latitudinal gradient (Dade County, Florida, USA- Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama).

FIGURE 30; A, B, C: Non-metric Multidimensional Scaling (NMDS) ordination plot of microbiome species diversity of host sponge *A. compressa* for the pairwise analysis of the three location comparisons (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama). Samples located within ellipses are the most similar to each other. Calculated cluster similarity distances are represented by lines.

FIGURE 31: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature in pair-wise comparison between Broward County, Florida, USA samples on a temporal scale, with a significant difference $p=0.026 \ r^2=0.92$.

FIGURE 32: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature in single site analysis of Broward County, Florida, USA samples on a temporal scale, with a significant difference $p=0.007 \ r^2=0.98$. 
FIGURE 33: Bray-Curtis Dissimilarity cluster dendrogram of microbiome Beta species diversity of host sponge *A. compressa* for the single site analysis of Broward County, Florida, USA on a temporal scale.

FIGURE 34: Non-metric Multidimensional Scaling (NMDS) ordination plot of microbiome species Beta diversity of host sponge *A. compressa* for the single site analysis of Broward County, Florida, USA. Samples located within ellipses are the most similar to each other. Calculated cluster similarity distances are represented by lines and follows the Bray-Curtis dendrogram.

3.2 List of Tables

**TABLE 1:**
The host sponge *A. compressa* metadata mapping file with parameters under investigation, including “Study Sample ID”, which will be used for the remainder of the analysis. The mapping file was generated for microbiome analysis with corresponding columns using the extracted raw Earth Microbiome Project microbiome data integrated in a matrix table. Abiotic variables are used for examination for OTU richness, Alpha, and Beta diversity.

**TABLE 2:** Summary of number of reads for microbiome of host sponge *A. compressa* with averages for all three collection sites; Broward County, Florida, USA; Dade County, Florida, USA and Bocas del Toro, Panama.

**TABLE 3:** Taxonomic classification summary table of the eight most abundant microbes of host sponge *A. compressa*. Earth Microbiome Project microbial OTUs are identified above a one percent threshold by location.
3.3 List of Abbreviations

ANOVA: Analysis of Variance
BC2: Broward County Second Reef
BCD: Bray-Curtis Dissimilarity
CDAB: Coral Disease Associated Bacteria
CRRAM: Coral Reef Restoration, Assessment, and Monitoring Laboratory
DC2: Dade County Second Reef
DNA: Deoxyribonucleic Acid
EMP: Earth Microbiome Project
EPA: Environmental Protection Agency
FC: Fecal Coliform
FIB: Fecal Indicator Bacteria
FIU: Florida International University
gDNA: Genomic Deoxyribonucleic Acid
HCNSO: Halmos College of Natural Sciences and Oceanography
HGT: Horizontal Gene Transfer
HMA: High Microbial Abundance
HTS: High-Throughput Sequencing
LMA: Low Microbial Abundance
MDS: Multidimensional Statistical Scaling
NCBI: National Center for Biotechnology Information
NMDS: Non-Metric Multidimensional Scaling
NSU: Nova Southeastern University
NSUOC: Nova Southeastern University Oceanographic Center
OTU: Operational Taxonomic Unit
PCR: Polymerase Chain Reaction
ppt: Parts Per Thousand
RPM: Revolutions Per Minute
rRNA: Ribosomal Ribonucleic Acid
RSMAS: Rosenstiel School of Marine and Atmospheric Science
SCUBA: Self Contained Underwater Breathing Apparatus
SBS: Sequencing by Synthesis
SECREMP: Southeast Florida Coral Reef Evaluation and Monitoring Project
SST: Sea Surface Temperature
USA: United States of America
V: Hypervariable regions
WSSF: Winter Spring Summer Fall
4.0 INTRODUCTION

4.1 Significance of Sponges

Sponges (phylum Porifera, class Demospongiae) are ancient organisms and the most primitive of metazoans in the evolutionary tree of life, with fossils dating back to the Late Precambrian period (Yin et al., 2015). Sponges are filter feeders, filtering large volumes of seawater, approximately 24,000 L/kg/day, contributing to relatively high concentrations of microorganisms within the sponge compared to the surrounding seawater (Negandhi et al., 2010, Thomas et al., 2010).

Sponges are known to support highly diverse microbial communities that can compose their biomass at densities up to 3-4 orders of magnitude greater than microbe density in seawater. Over 28 bacterial phyla have been described as associated with sponges, with Proteobacteria being the dominant phylum (Hentschel et al. 2012). Sponge microbiomes are often “sponge-enriched” by bacteria that are found in relatively high abundances within the sponge compared to much lower abundances or absence from adjacent water and sediments (Moitinho-Silva et al 2014). Since the sponges’ primary mode of feeding it through filtration of water, many organisms of the microbiome that are found in relatively low percentages may be considered “food”. Additionally, a number of sponges that host photosymbionts in which their energy can be achieved from photosynthesis along with filter feeding (Erwin and Thacker 2011).

Filtering these large volumes of seawater leaves the expelled water nearly sterile, with the sponges accumulating highly diverse and abundant microbial communities within their tissues, that can account for 40-60% of their biomass (Fieseler et al., 2006; Fieseler
et al., 2007; Hentschel et al., 2002; Kennedy and Marchesi, 2007; Schmitt et al., 2012; Webster et al., 2008; Webster et al., 2010; Wehrl et al., 2007). Nearshore benthic habitats of southeast Florida support a wide variety of invertebrate species with high diversity and biomass. Sponges are an important contributor to the ecological function of these communities that have significant economic and esthetic value (Lindeman et al. 2009; Rützler 2012).

Tropical marine sponges share similar environmental requirements and benthic habitats with scleractinian corals, the primary builders of tropical coral reefs (Negandhi et al., 2010). Sponges are important components of these communities and play a crucial role in nutrient regeneration, primary production through their photosynthetic microbial symbionts, and antimicrobial activity used for anti-fouling against predators (Erwin et al., 2012; Huang et al., 2008; Kelly et al., 2003; Newbold et al., 1999; Schmitt et al., 2012; Stabili et al., 2012; Webster, 2007). They support a phenomenal biodiversity of species, residing in the sponge tissues, providing them irreplaceable protection and refuge from predation as they use the sponges as protection (Reaka-Kudla, 1997).

Sponges assist the reef structure through preventing bioerosion (McLean and Yoshioka, 2008). Sponges’ proficient filtration capabilities have a major influence on marine microbial communities and the coral reef systems in which they inhabit (Massaro et al., 2012; Pantile and Webster, 2011; Simister et al., 2012), clearing bacteria and debris from the water column, thereby not only reducing concentrations of pathogens increasing water clarity, and improving general water chemistry (Duckworth et al., 2006). Sponges can serve as an important bioindicators of reef habitat health (Webster et al., 2008). These sessile invertebrates can live many years and have the capacity to act as indicators of the
accumulation of anthropogenic pollutants (Selvin et al., 2009) such as Fecal Indicator Bacteria (FIB) and Fecal Coliforms (FC), which can be used for monitoring developmental water quality (Anderson et al., 2005; Stabili et al., 2008). Water quality is highly dependent on land use and influenced by changes to the watershed. Anthropogenic impacts have accelerated due to extensive regional population growth, and associated human by-products. Watershed manipulation, pesticide and nutrient runoff from agricultural practices, landfill leachates, and sewage plants, may cause detrimental effects. This can result in algal blooms (LaPointe et al., 2005), hypersalinity, seagrass die-offs, loss of fish species, and pollution problems (Caccia and Boyer, 2005; Caccia and Boyer 2007). In long term studies, sponge declines could accelerate declines of coral reef systems (Stabili et al., 2012; Wulff, 2006b) since they help to remove coral pathogens (Webster et al., 2008) by acting as a bioaccumulator for Coral Disease-Associated Bacteria (CDAB) (Negandhi et al., 2010; Webster and Taylor, 2012).

Thermal stress depresses sponge pumping activity (Massaro et al., 2012) which can result in the deterioration of sponge health, affecting its defenses against predation, fouling, and disease. This may result in decreased populations of this important community component and reductions in ecosystem functionality. (Webster and Blackall, 2009). Elevated seawater temperatures disrupt the symbiotic relationship between the microbes and their sponge hosts (Pantile and Webster, 2011; Stabili et al., 2012; Webster and Taylor, 2012, Thomas et al., 2010), causing suppression of proper symbiont functioning, reducing host fitness and increasing susceptibility to disease. This thermal stress may ultimately cause expulsion of the symbionts and the potentially dangerous harbored pathogens into the water column (Fan et al., 2013; Simister et al., 2012; Webster et al., 2008).
Under demanding physiological conditions such as increased temperatures, elevated nutrients and reduced water flow, sponges are no longer able to filter pathogenic marine bacteria and become incapable of controlling their proliferation throughout the coral reef ecosystem, increasing the vulnerability of the coral reef fauna to microbial attack. Shifts in sponge microbial composition preceding disease development indicate these disruptions of host-microbe symbiotic functions while approaching thermal thresholds are a major cause of the decline of marine sessile invertebrates (Stabili et al., 2012). It has been reported that even temperature elevation of as little as 2°C causes a dramatic shift in the sponge symbiont microbial communities, allowing aggressive foreign microbial populations to outcompete the native bacterial species and proliferate (Fan et al., 2013; Massaro et al., 2012; Pantile and Webster, 2011; Simister et al., 2012; Webster, 2007; Webster et al., 2008) (Figure 4; Webster et al., 2008).

With such narrow thermal thresholds, the initial stress-response of sponges can first be detected through changes to the sponge molecular systems and pathways. This affects overall fitness and physiologically compromises the sponge, allowing pathogenic and opportunistic microbial colonization, causing declines in sponge health and eventual cellular necrosis in as little as three days (Fan et al., 2013; Pantile and Webster, 2011; Simister et al., 2012). Furthermore, microbes from thermally affected sponges have sequences analogous to previously documented diseased and bleached corals and known coral pathogens (Webster et al., 2008).

With sponge microbes being sensitive to rapid environmental perturbations and with predictions that ocean temperatures will exceed the conditions for coral reefs to flourish within the next century with an increase of sea surface temperature (SST) of 4°C
(Pantile and Webster, 2011), sponges can be ideal early bioindicators of ecological stressors affecting coral reef habitat health and can serve as monitors of incipient trauma within the ocean’s valuable bionetwork (Fan et al., 2013; Simister et al., 2012; Stabili et al., 2012).

4.2 High-Throughput Sequencing

Marine bacteria have been notoriously difficult to culture in vitro (Fieseler et al., 2006; Fieseler et al., 2007; Kennedy and Marchesi, 2007; Thomas et al., 2012), and until the introduction of these molecular techniques many microbes could only be studied using blind black-box-techniques restricted to only measurements of enzymatic processes (Kemp and Aller, 2004; Knight et al., 2012). With recent 16S rRNA High-Throughput Sequencing (HTS) molecular techniques, microbes can now be identified without prior or direct knowledge of their morphology, physiology, or ecology and a valuable tool for comparing microbial community structures (Werner et al., 2012).

The advent of sophisticated molecular methods such as ribosomal RNA (rRNA) techniques, resulted in a surge of scientific investigations revolutionizing our understanding of microbial biodiversity (Kemp and Aller, 2004; Knight et al., 2012; Mardis, 2007; Webster et al., 2001). HTS of the 16S rRNA gene as a bacterial evolutionary marker is commonly used for bacterial diversity studies (Logares et al., 2012; Mardis, 2008; Thomas et al., 2012), allowing rapid, accurate microbial identification (Althoff et al., 1998; Logares et al., 2012; Mardis, 2008). This innovative technique is a valuable tool for comparing microbial community structures (Bartram et al., 2011; Gilbert et al., 2010a; Gilbert et al., 2010b; Mardis, 2007; Thomas et al., 2012; Tringe and Hugenholtz, 2008; Vasileiadis, et al., 2012; Werner et al., 2012; Zaneveld et al., 2010).
HTS became commercially available in 2005, resulting in a tremendous impact on the acceleration in the evolving field of genomic research (Shendure and Ji, 2008; Werner et al., 2012). HTS is rapidly becoming the standard for advancements in the fields of microbial ecology, evolution, and diversity (Morozova and Marra, 2008; Thomas et al., 2012), while elucidating microbial ecology studies in complex microbial environments. Our understanding of the vast taxonomic composition of the microbial world is now substantially revolutionized (Bartram et al., 2011; Caporaso et al., 2010; Caporaso et al., 2012; Gilbert et al., 2010a; Knight et al., 2012; Logares et al., 2012; Mardis, 2007; Mardis, 2008; Tringe and Hugenholtz, 2008; Vasileiadis et al., 2012).

Genetic sequencing is the procedure of determining the precise order of nucleotides in a DNA or RNA sample. The Illumina sequencing platform is rapidly becoming the most successful and widely adopted HTS technology worldwide. Illumina uses the Sequencing by Synthesis (SBS) approach, a cyclic-array technique where reagents maintain a massively parallel sequencing method that detects single bases as they are incorporated into growing DNA or RNA strands (Thomas et al., 2012). First, single-stranded DNA fragmented molecules are ligated on a flow cell followed by primer addition and amplified with polymerase so that cluster bridges are amplified, forming the template for the synthesis of their complementary strands. To determine the sequence, four types of differently colored fluorescently labeled. Reversible Terminator bases (RT-bases) are simultaneously added (Logares et al., 2012; Morozova and Marra, 2008). RT-bases are nucleotides that are chemically blocked at the 3′-OH end so that each incorporation of a RT-base is restricted. All four RT-bases are present during each sequencing cycle, minimizing incorporation bias. The RT-bases are imaged by camera as RT-bases are added,
after which the terminal 3’ blocker is chemically cleaved from the DNA or RNA and non-incorporated nucleotides are washed away, allowing incorporation of the next RT-base by DNA polymerase for sequence determination. DNA or RNA chains are extended one nucleotide at a time in cycles, with this process repeated until the full DNA or RNA molecule is sequenced (Mardis, 2007; Mardis, 2008; Shendure and Ji, 2008).

The automation of Illumina sequencing makes it possible to sequence numerous oligonucleotide chains at once and obtain sequencing data rapidly (Morozova and Marra, 2008). HTS by the Illumina sequencing platform is extensively applied to metagenomic studies (Mardis, 2007; Mardis, 2008) having capabilities now approaching the generation of more than 150-bp (base pair) reads (Thomas et al., 2012; Werner et al., 2012) and generating up to 600 Gb in approximately ten days (Logares et al., 2012). 16S rRNA gene sequencing with the Illumina superior platform facilitates affordable and rapid results with consistent reproducibility between replicates (Bartram et al., 2011; Caporaso et al., 2012; Vasileiadis et al., 2012).

The impact of 16S rRNA gene diversity screening by HTS has immensely widened the scope of metagenomic analysis and provides exceptional insight into global ecosystems (Gilbert et al., 2010a), by massively increasing throughput while improving cost effectiveness of DNA sequencing by several orders of magnitude (Mardis, 2007; Mardis, 2008; Morozova and Marra, 2008; Shendure and Ji, 2008; Thomas et al., 2012; Tringe and Hugenholtz, 2008; Vasileiadis et al., 2012; Werner et al., 2012). With the now extensive amount of 16S rRNA gene libraries obtained through HTS, there is a substantial amount of information accumulated on the bacterial diversity in environmental systems, leading to the discovery of an unexpected abundance of groups that were previously unknown or
relatively rare (Bartram et al., 2011; Caporaso et al., 2012; Lazarevic et al., 2009; Logares et al., 2012; Kemp and Aller, 2004; Knight et al., 2012; Schmitt et al., 20129). These 16S rRNA gene libraries can be extremely helpful in identifying the associated microbial diversity within sponges, while providing insight into their taxonomy and ecology (Webster et al., 2001). Previous 16S rRNA studies and sequencing have indicated that sponges harbor host-specific microbial symbionts (Lopez et al., 2008), along with potential bacterial pathogens in the marine sponge *A. compressa* along with CDAB (Negandhi et al., 2010). In addition, 16S rRNA sequence analytical methods have been previously used to determine fecal contamination using coliform FIB (Leskinen et al., 2010; Kildare et al., 2007).

4.3 16S rRNA

The 16S rRNA gene is a housekeeping gene that seldom undergoes Horizontal Gene Transfer (HGT) and that evolves independently of ecological diversification making it an exceptional marker for microbial genomic evolution. Containing both fast and slow evolving regions, the 16S rRNA gene can be used to determine relationships among taxa at differing phylogenetic depths. The 16S rRNA gene contains hypervariable (V) regions that are commonly applied to analytical methodologies. The V regions can provide OTU-specific signature sequences, and is becoming the standard for reliable microbial classification and identification (Gilbert et al., 2010a; Lazarevic et al., 2009; Tringe and Hugenholtz, 2008; Werner et al., 2012; Zaneveld et al., 2010), with the V4 region of the 16S rRNA gene demonstrating an overall superior performance for microbial classification
and phylogenetic High-Throughput Sequencing (HTS) metagenomic studies (Vasileiadis et al., 2012).

4.4 Metagenomics and Microbiomes

The Earth hosts a richness of single-celled life, >10^{30} microbial cells, with marine microbes present at billions of cells per liter in seawater. Microorganisms were the first organisms to evolve on our planet, and they still account for the majority of functional and essential contributors to our planet’s ecosystems and biosphere (Logares et al., 2012). For example, marine microbes are responsible for up to 98% of the ocean’s primary productivity. For over 80 years it has been recognized that the majority of microorganisms cannot be cultured in a laboratory, constraining our understanding of the diversity and interdependencies of Earth’s microbial ecosystems. Of these complex and poorly understood ecosystems, the world’s oceans pose a significant challenge to microbial oceanographers to more accurately incorporate the details of microbial diversity, physiology, metabolism and ecology. Marine ecosystems, being complex and dynamic, further confounds our ability to understand how marine microbiota mediate biogeochemical processes (DeLong and Karl, 2005; Gilbert et al., 2010a; Gilbert et al., 2010b; Knight et al., 2012; Larsen et al., 2011).

Metagenomic studies are invaluable to study microorganisms that are unculturable in a laboratory (Fieseler et al., 2007; Vasileiadis et al., 2012; Zaneveld et al., 2010), especially since only an estimated 1% of the microorganisms present in a specific habitat can be recovered and cultured. Metagenomics is the study of the sequencing-based characterization of DNA and/or RNA isolated from a mixed population obtained from its
natural habitat (Fieseler et al., 2006; Kennedy and Marchesi, 2007; Mardis, 2011; McMurdie and Holmes, 2013; Thomas et al., 2012). Metagenomics is the direct genetic analysis of genomes contained within environmental derived samples (Logares et al., 2012; Thomas et al., 2012), and allows us to explore the vast microbiome diversity on Earth. Through this technique we can further comprehend the who, what, when, where, why and how of microbial communities (Mardis, 2007; Mardis, 2008; Mardis, 2011). Metagenomic comparative analyses of entire microbial assemblages can provide larger-scale patterns of habitat-specific correlations that might otherwise be missed in studies of individual species, where dynamic microbial populations and environments are variable in space and time (Bartram et al., 2011; DeLong and Karl, 2005; Gilbert et al., 2010a; Gilbert et al., 2010b; Knight et al., 2012; Tringe and Hugenholtz, 2008).

In previous studies, metagenomics provided invaluable insights into the functional diversity and taxonomic fluctuations of marine bacteria. Marine bacteria demonstrate seasonal patterns in diversity, with numerous environmental factors suggested as influences (DeLong and Karl, 2005). Dramatic shifts in community diversity composition could result from changes in salinity and temperature, with clear seasonal and/or biogeographical trends (Gilbert et al., 2010a; Knight et al., 2012; Larsen et al., 2011).

Metagenomics centers on the 16S ribosomal RNA (rRNA) genes that are useful in inferring phylogenetic relationships, metabolic and functional traits independent of cultivation (Fieseler et al., 2006; Fieseler et al., 2007; Thomas et al., 2012; Vasileiadis et al., 2012). Metagenomics, along with High-Throughput Sequencing (HTS), can offer us information about the vast taxonomic and metabolic diversity of the microbial world of environmentally derived samples (Bartram et al., 2011; McMurdie and Holmes, 2013),
providing exceptional insight into Earth’s global ecosystems (Logares et al., 2012; Tringe and Hugenholtz, 2008), and refining our understanding of microbial and biogeochemical processes of our ocean systems (Knight et al., 2012; Larsen et al., 2011). This can be of great importance for future climate predictions of a warmer, more acidic ocean due to accelerated anthropogenic impacts and how our present day microorganism ‘genotypes’ respond and interpret the ocean’s ‘phenotypic’ variables (DeLong and Karl, 2005; Gilbert et al., 2010a; Gilbert et al., 2010b; Mardis, 2008).

Recognizing the importance of a multi-environmental survey of microbial diversity, an international initiative “Earth Microbiome Project” was implemented. The pursuit of EMP is to systematically characterize global microbial ecosystems, based on their taxonomic and functional biodiversity. EMP focuses on global environmental microbial ecology and emphasizes the importance of standardizing the protocols used to generate and analyze data between studies, to minimize bias associated with different material extraction techniques, analytical methods, and core-data quality control and analysis. EMP is a multidisciplinary effort to identify and categorize the various microbial populations of the Earth, identify and categorize their functions in various habitats and niches, and deduce the contributions they make to the planet’s various ecosystems. This is achieved by using metagenomics, metatranscriptomics, and amplicon sequencing of samples to construct a global metagenomic model of the earth’s microbial communities. It merges aspects of biogeochemistry, microbiology, protein-enzyme interaction and transcriptional feedback, for understanding ecology on local, regional, national, continental and global scales. EMP requires acquisition and appropriate organization of the metadata that accompanies every sequence generated, to develop a comprehensive understanding of
a particular environment. This puts the sequence data into context, and allows comprehension of critical microbial environmental processes over a vast range of spatial and temporal scales. Furthermore, data collected by the EMP promotes open access research, which is made publicly available for use in scientific research, education, and conservation, facilitating multidisciplinary cooperation across funding agencies and scientific research areas (Knight et al., 2012).

Symbiotic microbial communities in sponges are important components of benthic marine ecosystems. This project will enhance our understanding of the effects of environmental variables on these communities. Establishing a baseline of the distinctive *A. compressa* microbial symbionts and harbored microbes with their fluctuations in response to seasonal shifts while comparing the microbial communities of differing geographical gradients can provide new insights into coral ecosystem health. Since *A. compressa* is known to harbor CDAB, FIB, and FC, documenting the microbial communities within *A. compressa* can serve as a significant bioindicators of natural and anthropogenic impacts. Sponge concentrations of these environmentally significant microbes allows for their detection at levels much lower than is possible from the water column. This can have scientific significance in the future identification of alien and pathogenic microbial invasions, possibly serving as an early warning system for deleterious ecological changes that could affect the fragile South Florida coral reef ecosystem.

Beyond these benefits, reporting these findings to the Southeast Florida Coral Reef Evaluation and Monitoring Project (SECREMP), the results of this study can provide local and federal resource managers the status of the sampled microbial communities of Southeast Florida coral reef ecosystems for future monitoring.

12
4.5 Microbial Symbionts

A bacterial Phylum “Poribacteria” has been recognized due to the holobiont relationship between sponges and their sponge-specific microbial symbiont communities (Hentschel et al., 2006). It has been established by bacterial biodiversity studies that sponge-specific symbiotic microbes are in low abundance in the ambient surrounding seawater (Erwin 2012; Hentschel et al., 2002; Thomas et al., 2010; Webster and Taylor, 2012). Sponges harbor consortia of symbiotic microorganisms that are phylogenetically distinct from those in the environment and that are host sponge-specific, which exceed concentrations two to four orders of magnitude higher than environmental (Fieseler et al., 2006; Kennedy and Marchesi, 2007; Schmitt et al., 2012; Simister et al., 2012; Webster et al., 2008; Webster et al., 2010; Wehrl et al., 2007) (Appendix 1; Wehrl et al., 2007).

Microbial symbionts among related sponges host species-specific microbial phylotypes, even over large biogeographical distances (Fieseler et al., 2006; Thomas et al., 2010; Webster and Blackall, 2009; Webster et al., 2010; Webster and Taylor, 2012). However, there are distinctions between tropical and sub-tropical populations suggesting that there are environmental effects on the relationship with evidence that specific microbial lineages are ubiquitous in sponges from different oceans and that host-phylogenetic clades are more similar to each other than to types from other locations (Webster et al., 2008). This observation of widespread among-species symbiont specificity is evidence of a long established coevolved status of these microbial communities (Schmitt et al., 2012; Wilkinson, 1984; Wulff, 2006a).

Sponges can differentiate between sustenance bacteria that they digest by phagocytosis, and their own bacterial symbionts, demonstrated by the massive amounts of
microbes processed from seawater for nutrition while maintaining their own specific symbiotic community (Thomas et al., 2010). Sponge secondary metabolites produce specific antimicrobial chemical defenses that are an advantage over broad spectrum toxins, inhibiting foreign microbial attachment and interfering with general microbial colonization, indicating a targeted approach and that symbiont integration is highly sponge-specific (Kelly et al., 2003; Kelly et al., 2005). Secondary metabolites produced by sponges have anti-fouling, anti-predator and other allelopathic effects on benthic invertebrates (Engel and Pawlik, 2000). Not only do sponges strongly demonstrate consistency of symbiotic bacteria over time, but even after periods of starvation they retain their specific symbionts, which are obtained through maternal vertical transmission mechanisms present in highly coevolved host-microbe associations (Sharp et al., 2007; Wehrl et al., 2007; Wulff, 2006a). Consequently, sponges maintain an overall stability of specific microbial communities, resulting in distinct symbiotic communities. (Erwin et al., 2012; Fieseler et al., 2006; Hentschel et al., 2002; Kennedy and Marchesi, 2007; Simister et al., 2012; Schmitt et al., 2010; Webster et al., 2008; Webster et al., 2010; White et al., 2012).

Seasonality can have significant effects on sponge microbiomes, with shifts in several bacterial taxa being associated with high seasonal variability between communities sampled during spring and fall seasons. Additionally, lower rates of growth were observed during winter months in comparison to summer months (Leong et al., 2010; Kahn et al., 2012) as well as inter-annual changes in food supply (Leys and Lauzon, 1998) and changes in water flow and depth (Duckworth et al., 2004). Location of the microbes within a sponge is further evidence of symbiosis, indicated by the tissue depth of the internal microbial communities, being located within the inner most layer of the mesophyl matrix (Althoff et
These specific symbiotic sponge microbes provide benefits to their host sponges such as nutrient acquisition, UV radiation protection, nitrogen fixation/nitrification, and production of secondary metabolites (Li, 2009; Negandhi et al., 2010; Schmitt et al., 2010; Webster et al., 2008). Furthermore, syntheses of sponge secondary metabolites are of great pharmacological interest, gaining considerable attention as a rich source of new drug candidates and biotechnological applications (Fieseler et al., 2007; Hentschel et al., 2002; Kennedy and Marchesi, 2007).

There is a clear distinction between HMA sponges and LMA sponges. The HMA sponges contain large amounts of microbes in the reproductive stages where LMA sponges appear to be void of bacteria. LMA sponges have lower abundances and diversity of microbes than HMA sponges and the microbes in LMA sponges are only found within certain locations within the sponge and are not equally dispersed throughout the mesohyl as demonstrated in HMA sponges. Sponge metabolic processes also differ; HMA sponges are more influenced by microbes than LMA sponges and HMA sponges have a more intricate aquiferous system with much slower pumping rates than LMA sponges. Furthermore there is a difference in the chemistry between the types of sponges. HMA sponges have polyketide synthase (PKS) genes where as LMA sponges do not. Additionally HMA sponges demonstrate fatty acid profiles not found in LMA sponges (Giles et al., 2013).

4.6 *Amphimedon compressa* Spicule Taxonomy

*A. compressa* is classified as a Demospongiae that secretes siliceous spicules in the mesohyl layer by specialized sclerocyte cells. Spicules interlock with each other forming
three-dimensional structures resulting in a pseudoskeleton, providing a rigid framework which allows sponges to grow upwards while facilitating proper water exchange with minimal metabolic energy and aiding in catching prey. Spicules perform essential structural and functional roles in sponges, forming a framework for spongin fibers. Additionally, they are used to determine taxonomic relationships as they are relatively consistent within classes (Uriz et al., 2003) (Imsiecke et al., 1995).

*A. compressa* is in the family Haplosclerida that all produces diactinal spicules that are homogenously distributed throughout the skeleton. Spicules are considered megascleres, with oxea that are slightly bent, characterized by a simple cylinder with dual pointed ends (oxea=pointed ends, diactinal=dual identical ends, monaxons=single cylinder). Microscleres are absent. Distinctive features of spicules in *Amphimedon* include slightly bent diactinal oxeas with modified ends that are 106-158 μm long, 3-5 μm in diameter, and with the absence of microscleres. Spicules are abundant in a feathery spongin matrix with openings 90-300 μm in diameter (Desqueyroux-Faundez and Valentine, 2002; Rigby and Boyd, 2004).

### 4.7 *Amphimedon compressa* Duchassaing & Michelotti, 1864

Commonly known as the erect rope sponge, *A. compressa* is found throughout the Greater Caribbean including South East (SE) Florida, USA, the Bahamas, the Greater and Lesser Antilles and the Caribbean coast of South and Central America. When observed on nearshore reefs of SE Florida, it is generally less than 30 cm in length and several centimeters in diameter, although it is reported to reach 1m in size. Their tissue is soft and flexible and generally a vibrant red color (Zea et al., 2009).
A. compressa is in the class Demospongiae, order Haplosclerida and family Niphatidae. It is one of the species in the genus Amphimedon described from the Caribbean. Several additional putative species are listed based on a variety of morphological distinctive characters, but are not yet described (Zea et al., 2009). This sponge was originally described as Spongia rubens, and formerly referred to as Haliclona rubens in the scientific literature, but has since been taxonomically reassigned (World Porifera Database).

A. compressa is a Low Microbial Abundance (LMA) sponge (Negandhi et al., 2010), which have a tendency to have microbiomes with lower Phylum diversity than High Microbial Abundance (HMA) sponges. LMA sponges are typically dominated by the Phyla Cyanobacteria and Proteobacteria (Croue et al., 2013, Giles et al., 2013).

There is little information on reproduction in A. compressa, however a closely related species Amphimedon queenslandica from Australia has been studied extensively in this regard. Like many sponges, A. queenslandica is a hermaphroditic spermcast spawner. Spawn is released into the water column and fertilization occurs in brood chambers within the maternal sponge. Larvae are retained through the initial stages of development until their release into the water column (Maritz et al., 2010). Sponges have important asexual phases as well, often reproducing via fragmentation, budding or gemmule (packets of cells in a protective covering) formation. However, it is through sexual reproduction that planktonic larvae are formed, and these are important in dispersal via currents. Also, sponge microbiomes are often vertically transmitted from maternal parent to larva during brooding (Hentschel et al., 2012).
5.0 OBJECTIVES AND HYPOTHESES

Global climate change represents an increasing and significant threat to coral reef ecosystems. Significant impacts on marine microbial diversity, could negatively affect functional symbiosis, thus reducing fitness of host invertebrates (Massaro, 2012). Previous studies using 16S rRNA analysis to detect shifts in symbiotic microbial community structures have documented that marine sponges are experiencing significant declines through elevated temperature-induced diseases (Fan, et al., 2013; Pantile and Webster, 2011; Stabili et al., 2012). Sponges harbor ecologically labile consortia of symbiotic microorganisms (Althoff et al., 1998; Erwin et al., 2010; Thomas, 2010; Schmitt et al., 2012; Simister et al., 2012; Webster et al., 2008; Webster et al., 2010; Wehrl et al., 2007). Primary goals of this study are to identify the baseline of *A. compressa* stable symbionts that are distinct from the water column and sediment microorganisms, and document and compare fluxes of harbored microbes over seasonal and geographic gradients.

Sites with accelerated declining water quality in South Florida are primarily adjacent to metropolitan areas, and are associated with anthropogenic impacts and pollution. After a century of extensive regional population growth, the South Florida marine waters of Miami-Dade County are exposed to significant watershed output, with documented periods of environmentally declining water quality and even toxic pollutant levels. (Caccia and Boyer, 2005; Caccia and Boyer, 2007). The recreational waters of the Florida Keys have shown increased deterioration of coral reef health and declining water quality, associated with the movement of enteroviruses from septic tanks into coastal waters as detected by Polymerase Chain Reaction (PCR) methods (Donaldson, 2003). *A. compressa* is known to harbor and act as a reservoir for microbes including CDAB, FIB
and FC such as *Escherichia coli* (Negandhi *et al*., 2010), with fecal contamination determined previously by 16S rRNA sequences and PCR analytical methods (Leskinen *et al*., 2010; Kildare *et al*., 2007). This study will investigate the presence and quantities of CDAB, FIB, and FC harbored in *A. compressa* that can then be used to monitor water quality and provide information concerning coral reef habitat health.

5.1 Objectives

**Objective 1:** Determine if there are differences in *A. compressa* sponge microbiome OTU richness (numbers of OTUs) across spatial and temporal gradients.

**Objective 2:** Determine if there are differences in *A. compressa* sponge microbiome OTU diversity (numbers of OTUs and numbers of individuals within each OTU) across spatial and temporal gradients.

**Objective 3:** Determine if abiotic factors are associated with *A. compressa* sponge microbiome trends of richness and diversity differences.

5.2 Hypotheses

**H1:** There will not be differences in *A. compressa* sponge microbiome OTU richness across spatial and temporal gradients.

**H2:** There will be differences in *A. compressa* sponge microbiome OTU diversity across spatial and temporal gradients.

**H3:** Abiotic factors will be associated with trends of *A. compressa* sponge microbiome OTU richness and diversity.
6.0 MATERIALS AND METHODS

6.1 Sponge Sample Collection

Working in collaboration with Nova Southeastern University Oceanographic Center’s Coral Reef Restoration, Assessment, and Monitoring (CRRAM) laboratory, approximately 6cm³ *Amphimedon compressa* sponge tissue samples containing microbial communities were collected by SCUBA from two locations on the South Florida second reef; ten samples from one site in Broward county (BC2) (Latitude: 26° 09.597’ N, Longitude: 080° 04.950’ W) and ten samples from one site in Dade County (DC2) (Latitude: 25° 50.520’ N, Longitude: 080° 05.704’ W) (n=20) (FIGURE: 1).

BC2 replicate samples were taken from ten individuals approximately every three months consecutively for a fifteen month period (n=60) (9/3/2010, 11/9/2010, 3/1/2011, 5/10/2011, 9/1/2011, 11/10/2011), and DC2 replicate samples taken from ten individuals approximately every three months consecutively for a twelve month period (n=40) (9/3/2010, 12/6/2010, 3/17/2011, 5/9/2011). The sampled individual sponges were tagged so each replicated sample taken over time so consistent samples can be obtained from the identical individual sponge for a seasonal studies and for further metagenomic studies.

The Bocas del Toro, Panama samples were collected from five separate individuals all from the same location (9° 21.1002’ N, -82° 15.57’ W) and date (7/20/2012) by the University of Alabama’s Department of Biology Thacker lab (n=5) (FIGURE: 2).

6.2 *Amphimedon compressa* Spicule Taxonomy

Spicule identification was performed to confirm the host sponge *A. compressa* taxonomy. Sub-samples ~0.5 cm$^3$ of each *A.n compressa* sponge sample ($n=20$) were sectioned from the primary sample with sterile scalpel and forceps in a sterile petri dish and prepared using two methods, one for observation of individual spicules and the other for observation of the intact pseudoskeleton. All spicule preparation and identification methods were assisted by Dr. Maria Cristina Diaz PhD., sponge taxonomy expert at Nova Southeastern University (NSU) Halmos College of Natural Sciences and Oceanography (HCNSO) (Diaz, 2007).

6.2.1 Individual Spicule Preparation

*A. compressa* sponge sub-samples were treated with 1 mL 100% household bleach solution, approximately 5% sodium hypochlorite and 0.03% sodium hydroxide in a 2.0 mL microcentrifuge tube. This dissolves sponge tissue leaving only the spicules, allowing them to be viewed under a compound microscope. This was assisted by Dr. Cristina Diaz PhD., sponge taxonomy expert at Nova Southeastern University (NSU) Halmos College of Natural Sciences and Oceanography (HCNSO).

6.2.2 Pseudoskeleton Preparation

*A. compressa* sponge sub-samples were sliced approximately 0.5 mm thick by hand with a sterile scalpel, placed intact on a sterile glass slide, and dehydrated with 1 ml of 100% EtOH. The alcohol was applied to the sample under a fume hood and then allowed to evaporate for approximately 24 hours. Tissue was then dissolved with 100% household
bleach solution, with 1 mL of approximately 5% sodium hypochlorite and 0.03% sodium hydroxide, followed by evaporation over 24 hours. Samples were then infiltrated with 1 mL xylene and coverslips affixed on the slides with 1 mL Permount® which were allowed to cure for approximately 24 hours.

6.2.3 Microscopy

A. compressa sponge spicules and pseudoskeleton slides were observed with a compound microscope at 100, 400, and 1000x magnifications. An integrated digital camera produced the images.

6.3 Microbial DNA Extraction/Isolation

For this study, four A. compressa sponge samples from one individual N50 from location BC2 (n=4) and four A. compressa sponge samples from two individuals N31 and N32 from location DC2 (n=4) from sample dates (BC N50: 3/1/2011, BC N50: 5/10/2011, BC N50: 9/1/2011, BC N50: 11/10/2011; DC N31: 3/17/2011, DC N31: 5/9/2011; DC N32: 12/6/2010, DC N32: 5/9/2011) (APPENDIX: 1). The A. compressa sponge tissue harboring the microbial communities was extracted by conducting the “squeeze method” (Lopez, unpublished data; Oceanographic Center Microbiology Lab Manual; Accessed 2013). This method allows for the extraction of A. compressa tissue containing the microbial cells using a lysis buffer and centrifugation technique. Approximately 1.5 cm³ A. compressa sponge sub-sample was saturated in 2.0 µl 4°C cell lysis buffer in a sterile petri dish. Using a sterile scalpel and forceps, the tissue was pulverized and squeezed against the bottom of the petri dish, expelling the sponge tissue and microbial cells. The
supernatant was collected by pipette and transferred a 2.0 ml microcentrifuge tube, then centrifuged at 10,000 RPM for 1 minute. The supernatant is decanted and the pellet is retained. The microcentrifuge tube was centrifuged again at 10,000 RPM for 1 minute and the remaining supernatant decanted from the pellet.

All microbial DNA isolations of *A. compressa* sponge samples were conducted using the Earth Microbiome Project (EMP) protocol, performed with the UltraClean MoBio Power Soil DNA Isolation Kit® per manufacturer’s instructions.

### 6.4 Illumina High-Throughput Metagenomic Sequencing

The extracted and isolated microbiome DNA from *A. compressa* sponge samples were sent for sequencing on the Illumina HiSeq platform in collaboration with the EMP. EMP amplified and sequenced the V4 region of the bacterial/archaeal 16S rRNA gene using the primer set 515F and 806R, followed by linking Golay barcoded primer sets.

### 6.5 Earth Microbiome Project Acquisition of Microbiome Data

Thirteen *A. compressa* sponge sequenced data sets were assimilated from the open source EMP, in which eight were submitted by Nova Southeastern University’s (NSU) Halmos College of Natural Sciences and Oceanography (HCNSO) laboratory of Microbiology and Genetics and five were submitted by the University of Alabama’s Department of Biology. Four samples are complete seasons from one Broward County, Florida, USA BC2 individual (N50.3.1.11.BC.1019585, N50.5.10.11.BC.1019840, N50.9.1.11.BC.1020044, N50.11.10.11.BC.1020037), four are from two Dade County, Florida, USA DC2 individuals each from two sample dates (N31.3.17.11.DC.1020439, N31.3.17.11.DC.1020439,

The three sampling sites are at the following locations; Bocas del Toro, Panama (9° 21.1002’ N, -82° 15.57’ W), Broward County, Florida, USA (26° 09.597’ N, 080° 04.950’ W) and Dade County, Florida, USA (25° 50.520’ N, 080° 05.704’ W). The geographic distances between these sites range from 37 km (Broward County – Dade County, Florida) to 1,839 km (Dade County, Florida – Bocas del Toro, Panama), and 1,875 km (Broward County, Florida – Bocas del Toro, Panama).

The raw microbiome data used for this study is available from EMP, to be integrated into the form of an Operational Taxonomic Unit (OTU) matrix table. Processing of the raw sequence data to OTUs was performed by Lucas Moitinho using platform mother v.1.31.2 based on a 97% sequence similarity through database Silva, and further taxonomic classification was performed on RDP and Greengenes. OTUs with a single sequence (singletons) from all samples were removed along with samples containing less than 500 sequences. Quality control of the raw sequences were trimmed to a minimum length of 100, and then aligned and screened with a start of 1968 and an end of 4411.
Chimeras were detected and removed, then trimmed for taxonomic classification with a start of 11894 and an end of 25319. The sequences were then classified on Silva with a cutoff of 60, and then clustered at a 0.03 cutoff. A matrix was developed, containing the number of sequences in each OTU per sample, removing OTUs with just one sequence assigned across all the samples, and removing samples with less than 500 sequences. The OTU representative sequences were then trimmed using both RDP and Greengenes for taxonomic classification, then the taxonomies and OTUs were integrated into a single matrix table which was used for this study (L. Moitinho, 2014; Unpublished data).

6.6 Data Analysis

6.6.1 Metadata

A metadata mapping file was created using Microsoft Excel and saved in .csv format, necessary for importation into R Studio. Metadata categories investigated for this study were “SampleID” (identification of specific A. compressa Microbiome sample for analysis), “Collection_Site” (Broward County, Florida, USA; Dade County, Florida, USA; Bocas del Toro, Panama), “Collection_Date” (MM/DD/YY), “Temp_C” (SST in degrees C), “Seasons” (calendar-based four seasons: winter/summer/spring/fall (WSSF)), “Season2” (tropical climate-based two seasons: Wet/Dry), and “Salinity_ppt” (ppt = parts per thousand). The two different seasonal parameters were based on season WSSF as astronomical boundaries (solstices and equinoxes: Winter = 22 Dec – 21 Mar, Spring = 22 Mar – 21 June, Summer = 22 June – 21 September, and Fall = 22 September – 21 Dec.) and wet/dry season corresponding to tropical precipitation patterns (South Florida: Wet; June-Oct. and Dry; Nov.-May)/ (Panama: Wet; May – Nov. and Dry; Dec. – April) (STRI
– Climate). Since both South Florida and Bocas del Toro Panama follow tropical precipitation climate patterns, the latter seasonal analysis is relevant to data interpretation. The mapping file is also used for abiotic variables that are used for examination for OTU richness, Alpha, and Beta diversity (TABLE: 1).

<table>
<thead>
<tr>
<th>“EMP SampleID”</th>
<th>“Study ID”</th>
<th>“Collection_Site”</th>
<th>“Collection_Date”</th>
<th>“Temp_C”</th>
<th>“Salinity_ppt”</th>
<th>“Seasons”</th>
<th>“Season2”</th>
</tr>
</thead>
<tbody>
<tr>
<td>N30.3.11.LBC1.101 9585</td>
<td>BC N50: 5/1/2011</td>
<td>BC Florida, USA</td>
<td>3.1.11</td>
<td>23.7</td>
<td>35.1</td>
<td>Winter</td>
<td>Dry</td>
</tr>
<tr>
<td>N30.5.10.LBC1.10 19940</td>
<td>BC N50: 5/10/2011</td>
<td>BC Florida, USA</td>
<td>5.10.11</td>
<td>28.4</td>
<td>36.5</td>
<td>Spring</td>
<td>Dry</td>
</tr>
<tr>
<td>N30.5.11.LBC1.102 6044</td>
<td>BC N50: 9/1/2011</td>
<td>BC Florida, USA</td>
<td>9.1.11</td>
<td>29.8</td>
<td>35.4</td>
<td>Summer</td>
<td>Wet</td>
</tr>
<tr>
<td>N30.11.LBC1.104200037</td>
<td>BC N50: 11/10/2011</td>
<td>BC Florida, USA</td>
<td>11.10.11</td>
<td>25.1</td>
<td>35.3</td>
<td>Fall</td>
<td>Dry</td>
</tr>
<tr>
<td>N31.3.17.LDC1.10 28439</td>
<td>DC N31: 3/17/2011</td>
<td>DC Florida, USA</td>
<td>5.9.11</td>
<td>22.8</td>
<td>35.3</td>
<td>Winter</td>
<td>Dry</td>
</tr>
<tr>
<td>N31.5.5.LDC1.102 0371</td>
<td>DC N31: 5/9/2011</td>
<td>DC Florida, USA</td>
<td>3.17.11</td>
<td>24.3</td>
<td>35.3</td>
<td>Spring</td>
<td>Dry</td>
</tr>
<tr>
<td>N32.12.6.DC1.10 28311</td>
<td>DC N32: 12/6/2010</td>
<td>DC Florida, USA</td>
<td>12.6.10</td>
<td>24.51</td>
<td>36.09</td>
<td>Fall</td>
<td>Dry</td>
</tr>
<tr>
<td>N32.5.5.LDC1.101 9961</td>
<td>DC N32: 5/9/2011</td>
<td>DC Florida, USA</td>
<td>5.9.11</td>
<td>24.3</td>
<td>35.3</td>
<td>Spring</td>
<td>Dry</td>
</tr>
<tr>
<td>P12x145.1020431</td>
<td>PC145: 7/20/2012</td>
<td>Bocas del Toro, Panama</td>
<td>7.20.12</td>
<td>28.5</td>
<td>32.1</td>
<td>Summer</td>
<td>Wet</td>
</tr>
<tr>
<td>P12x147.10195513 5</td>
<td>PC147: 7/20/2012</td>
<td>Bocas del Toro, Panama</td>
<td>7.20.12</td>
<td>28.5</td>
<td>32.1</td>
<td>Summer</td>
<td>Wet</td>
</tr>
<tr>
<td>P12x149.1020354</td>
<td>PC149: 7/20/2012</td>
<td>Bocas del Toro, Panama</td>
<td>7.20.12</td>
<td>28.5</td>
<td>32.1</td>
<td>Summer</td>
<td>Wet</td>
</tr>
<tr>
<td>P12x150.1020217</td>
<td>PC150: 7/20/2012</td>
<td>Bocas del Toro, Panama</td>
<td>7.20.12</td>
<td>28.5</td>
<td>32.1</td>
<td>Summer</td>
<td>Wet</td>
</tr>
<tr>
<td>P12x151.1020099</td>
<td>PC151: 7/20/2012</td>
<td>Bocas del Toro, Panama</td>
<td>7.20.12</td>
<td>28.5</td>
<td>32.1</td>
<td>Summer</td>
<td>Wet</td>
</tr>
</tbody>
</table>

TABLE 1: The host sponge A. compressa metadata mapping file with parameters under investigation, including “Study Sample ID”, which will be used for the remainder of the analysis. The mapping file was generated for microbiome analysis with corresponding columns using the extracted raw Earth Microbiome Project microbiome data integrated in a matrix table. Abiotic variables are used for examination for species richness, Alpha, and Beta diversity.

6.6.2 Analytical Platform

A. compressa sponge microbiome data was analyzed using the R Studio package “picante” (Phylocom, Integration, Community Analyses, Null-models, Traits, and Evolution) in R tools for integrating phylogenies and ecology; Version 1.6-2 Date 2014-
03-05 (Kembel et al. 2010; 2014). ‘Picante’ includes the following libraries: ape (Analyses of Phylogenetics and Evolution), vegan (Community Ecology Package), permute (Functions for Generating Restricted Permutations of Data), lattice (Trellis Graphics for R), nlme (Linear and Nonlinear Mixed Effects Models), and ggplot (An Implementation of the Grammar of Graphics).

6.6.3 Analysis Categories

For this microbiome study two types of categories were used. The first type of analysis was a comparison and statistical testing of all three locations simultaneously. This consisted of *A. compressa* sponge samples from Broward County, Florida, USA, (BC N50: 3/1/2011, BC N50: 5/10/2011, BC N50: 9/1/2011, BC N50: 11/10/2011) from one location (n=4), Dade County, Florida, USA, (DC N32: 12/6/2010, DC N31: 3/17/2011, DC N31: 5/9/2011; DC N32: 5/9/2011) from two locations (n=4) and Bocas del Toro, Panama, (PC145: 7/20/2012, PC147: 7/20/2012, PC149: 7/20/2012, PC150: 7/20/2012; PC151: 7/20/2012) from one location (n=5). The total number of samples for this microbiome characterization study is n=13.

The second study, using the identical samples, was used to conduct pairwise evaluations for a more in-depth microbiome comparison and to investigate latitudinal/spatial trends between the three locations (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama).
6.6.4 Analysis of Biotic Data

The analyses performed were: Counts and comparisons of OTUs, Rarefaction analysis, Analysis of Variance (ANOVA) for OTU richness, Tukey for multiple comparisons of means, Inverse Simpson for Alpha OTU diversity, Bray-Curtis Dissimilarity (BCD) to calculate Beta diversity comparisons, ADONIS for statistical analysis of beta diversity, Non-metric Multidimensional Scaling (NMDS) ordination distance metric, and Simper similarity percentages pair-wise comparisons combined with a taxonomy matrix. For the continuous variables (eg: temperature, salinity) the same analyses were performed using regression analysis, to infer relationships between the independent and dependent variables.

6.6.5 Operational Taxonomic Units

In this microbiome study, OTUs were sequenced from thirteen *A. compressa* sponge samples. Bacteria do not follow the same biological species concept as eukaryotes due to a variety of unique challenges to traditional interpretation, including horizontal gene transfer (HGT). Typically, species determination in bacteria requires a fine-scale analysis of physiological characteristics such as biochemical reactions and culturing. The use of OTUs in this study partitions bacteria into taxonomic units that represent various taxonomic levels (Sneath and Sokal, 1973).

In general, OTUs determine bacterial identity based on sequence divergence. The 16S rRNA gene is found in all bacterial species and has regions that are highly conserved and other regions that are quite variable. For microbiome studies the variable V4 region is targeted by the Illumina platform. Bacteria with more similarities in the variable region are
more closely related than those with greater differences and are clustered together based on their sequence similarities. When there is greater than 97% similarity among 16S rRNA V4 region sequences, the organisms are considered to be from the same taxonomic unit.

6.6.6 Rarefaction Analysis

Rarefaction assesses OTU richness from sampling “depth”. Depth refers to the improvement in correctly representing diversity and the numbers of samples as they increase. A rarefaction curve is a technique of comparing the profile of the curve rather than absolute number of OTUs, being able to compare OTU richness between different data sets with different sample sizes and OTU diversity. The curves accelerate at first, indicating the most abundant OTU have been identified, and then the curve plateaus as the rarest OTUs continue to be sampled and diversity decelerates. This allows us to determine if sampling is sufficient to correctly determine Alpha diversity, the OTU diversity within one site that also incorporates population levels.

Rarefaction was used to standardize the OTU datasets from the three different locations to the lowest number of reads for adequately comparing OTU richness and further downstream diversity analyses (Gotelli, et al. 2001). This was necessary due to two main factors. Firstly, the number of reads was considerably skewed from the three locations, lowest in Bocas del Toro, Panama (9,410) and highest in Broward County, Florida, USA (53,744). This large range of reads poses a problem for valid OTU richness and diversity comparisons of the three locations. Secondly, with large amounts of data associated with High-Throughput sequencing, as resampling increases it is likely to continue to keep encountering extremely rare taxa and singletons. This is particularly important in this study
due to the first two taxa accounting for 30.0-58.0% and 4.3-22.6% of the total host sponge microbiome respectively. After the first eight OTUs the taxa in all three locations falls below 1.0%, indicating continued sequencing depth would be related to rare taxa.

6.6.7 ANOVA for OTU Richness

OTU richness was analyzed by all metadata parameters: collection site, collection date, sea surface temperature (SST) in degrees Celsius, salinity in parts per thousands (ppt), seasons (calendar-based), and seasons (precipitation-based). This determines if there are differences in numbers of OTUs based on each of the different parameters.

6.6.8 Alpha Diversity

OTU diversity comprises two components; OTU richness and OTU evenness. The total number of OTUs present, without knowing the relative abundances (proportions) or diversity (distribution) of each OTU, is defined as OTU richness. Diversity indices account for the number of different OTU in a community, while also considering how evenly individual OTUs are distributed in the community under analysis. Alpha diversity incorporates population levels with OTU diversity within a particular site or location. Two indices commonly used to determine Alpha diversity methods include Shannon and Inverse Simpson. It is the latter that incorporates the important ecological contribution of population levels, and the one that will be used in this study.
6.6.9 Inverse Simpson

Inverse Simpson is dominance based, giving more weight to the most common OTUs where rare OTUs have a lesser effect on diversity. For the Inverse Simpson index 1.0 means there is no diversity, and 10.0 means maximum diversity. The similarity index values increase as diversity increases. The Inverse Simpson formula is the inverse (1/D) of

\[ D = \sum p_i^2 \] (Inside R-Forum).

For this study, the Inverse Simpson index was used to evaluate the microbiome diversity of *A. compressa*, due to the high abundance domination of two OTU taxa.

Inverse Simpson indices were determined for all *A. compressa* microbiome samples \((n=13)\) with respect to metadata parameters \((n=6)\) including collection site, collection date, SST in degrees Celsius, salinity in parts per thousands (ppt), seasons (calendar-based), and seasons (precipitation-based). This generates an interpretation of alpha OTU diversity for the sponge microbiome. Effects of each abiotic factor on alpha diversity was determined among all sites \((n=3)\).

6.6.10 Tukey for Multiple Comparisons of Means

A Tukey Honest Significant Difference (HSD) Multiple Comparisons of Means conducts all pair-wise comparisons among independent variables to determine which groups are different from one another. This is a multiple comparisons pair-wise test, analyzing which metadata sets are responsible for differences in OTU richness as determined by ANOVA. The Tukey test was used to compare of each of the three different collection sites for OTU richness.
6.6.11 Beta Diversity

Beta diversity is the differentiation between habitats/biological community compositions among environmental gradients. Beta diversity compares the Alpha diversity among sites, allowing for meaningful comparisons of Alpha diversity among different site pairs to assess compositional dissimilarity. For this study Bray-Curtis Dissimilarity (BCD), ADONIS, Non-Metric Multidimensional Scaling (NMDS), and Simper Similarities are utilized.

6.6.12 Bray Curtis Dissimilarity

The Bray-Curtis Dissimilarity (BCD) index determines the Beta diversity of count-data between two or more sites. This index incorporates elements of OTU richness (number of OTUs) and number of individuals (instances of each OTU) and compares them among site pairs. BCD has a scale between 0.0-1.0, where 0.0 means the two sites have the same composition (that is they share all the OTUs and are least dissimilar), and 1.0 means the two sites do not share any OTU (most dissimilar). At sites with where BCD is intermediate (e.g. BCD = 0.25) this index differs from other commonly used diversity indices.

ADONIS is a Beta diversity dissimilarity function consisting of an R implementation of a PERMANOVA multivariate pairwise factorial design. The ADONIS test consists of combining two “treatments” or “mixed effects” variables, indicating if there an interaction between them. Conducting the ADONIS function is important for identifying where significance occurs when studying various mechanisms of different environmental variables, indicating where there is an interaction.
6.6.13 Non-metric Multidimensional Scaling

Non-metric Multidimensional scaling (NMDS) method used to signify and visualize Beta dissimilarities (compositional differences) in a dataset defined by BCD, while also indicating outliers using two axes. The first axis explains the maximum amount of OTU abundance in the data per sites, the second explains the second most amount of OTU abundance. This is useful in the interpretation of BCD indices. The purpose of NMDS is to calculate a distance matrix used to produce a graphical interpretation in rank order, so the dissimilarity distances of variables can be visualized. Data sets that are closer together are considered to be less dissimilar than those farther apart. Before conducting ordination, a stressplot is run to test the robustness and goodness of fit of the data.

6.6.14 Heatmap

A Heatmap was generated in R “picante”, with the OTUs on the x-axis and the sample IDs and a BCD dendrogram on the y-axis. It is graphical representation of the top fifty OTUs of the microbiome within A. compressa, characterized in matrix form. The darker colors are representative of the higher abundance of OTUs and the lighter colors representative of the lower abundance of OTUs. The dendrogram joins the clusters of samples by relative abundance similarity while identifying the location. A square-root transformation function of the values were used to normalize color ramp.

6.6.15 Simper Similarities

The Simper similarity percentage table was created in R “picante” for a pair-wise comparisons between the three locations (Bocas del Toro, Panama-Dade County, Florida,
USA; Dade County, Florida, USA-Broward County, Florida, USA; Broward County, Florida, USA- Bocas del Toro, Panama). Using the location independent variables, Simper was used to measure the contributions of specific OTUs to the overall BCD. The Simper data combined with the EMP OTU matrix table was used to generate the identification of the top fifty bacterial OTUs against the Green Genes database to study variations between the different sampling sites.

6.6.16 Analysis of Abiotic Factors

Abiotic factors considered in the *A. compressa* sponge microbiome study include: Collection site to investigate a latitudinal gradient of 37 km between Dade County, Florida, USA and Broward County, Florida, USA; 1839 km between Dade County, Florida, USA and Bocas del Toro, Panama; and 1875 km between Broward County, Florida, USA and Bocas del Toro, Panama. Collection date to investigate each sample microbiome independent of seasons. SST in degrees Celsius to investigate the effect of temperature on sample microbiomes. Salinity in parts per thousands (ppt) to investigate the effect of salinity on sample microbiomes. Calendar-based seasons to investigate the effect of traditional seasons on the effects of sample microbiomes. Precipitation-based seasons to investigate the effect of tropical seasons on the effects of sample microbiomes (geographic distances: Google Earth. March 31, 2011. December 20, 2015).
7.0 RESULTS

7.1 Sponge Taxonomy by Spicule Analysis

Sponge spicules exhibit characteristics consistent with documented taxonomy of *Amphimedon compressa*, demonstrating slightly curved spindles, pointed at both ends with a central ridge. Their size is approximately 120 μm. Their spicule type is classified as oxea diactinal monaxons, an identical pointed ended spicule with a single axis (*FIGURES: 3, 4, 5*) (*SUPPLEMENTARY: 1*). Spicules interlock with each other forming three-dimensional structures resulting in a pseudoskeleton. The samples used in this study confirm the taxonomic identification as *A. compressa* sponges, further endorsed by Dr. Cristina Diaz PhD., sponge taxonomy expert at NSU HCNSO.


FIGURE 5: Sample DC N31: 3/17/2011; oxea diactinal monaxial individual spicule of host sponge *A. compressa*, 1000x magnification with compound microscope.
7.2 Data Analysis with R

7.2.1 Triple Site Analysis

Site analysis of the three *A. compressa* host sponge collection sites (Broward County, Florida, USA; Dade County, Florida, USA; Bocas del Toro, Panama) was investigated to determine microbiome OTU abundances, richness and diversity on a spatial scale.

7.2.1.1 Operational Taxonomic Units

The total number of reads of the of the thirteen *A. compressa* sponge samples used for this microbiome study is 284,832. The average number of reads of the three locations is 21,910, with the largest average number of reads in Broward County, Florida, USA totaling 34,955. The second largest average number of reads is in Dade County, Florida, totaling 21,405. The least amount of average number of reads is in Bocas del Toro, Panama totaling 11,879. (TABLE: 2).

7.2.1.2 Rarefaction Analysis

A rarefaction analysis curve was generated, representing the thirteen *A. compressa* host sponge sample microbiomes used in this microbiome study (Broward County, Florida, USA (*n*=4); Dade County, Florida, USA (*n*=4); Bocas del Toro, Panama (*n*=5)). The reads were rarefied to the lowest number (9,410; Panama) to standardize the data for valid OTU richness and diversity analyses. Although the rarefaction curve did not reach an asymptote associated with sufficient sequencing depth, taxon rich High-Throughput rarely reaches a horizontal curve. Any further sequencing depth would continue to recover additional rare
### TABLE 2: Summary of number of reads for microbiome of host sponge *A. compressa* with averages for all three collection sites; Broward County, Florida, USA; Dade County, Florida, USA and Bocas del Toro, Panama.

<table>
<thead>
<tr>
<th>Study Sample ID</th>
<th>Total Number of Reads per Sample</th>
<th>Total Average Number of Reads BC2 South Florida</th>
<th>Average Number of Reads DC2 South Florida</th>
<th>Average Number of Reads PC Panama</th>
<th>Difference of Number of Reads BC2 vs DC2</th>
<th>Difference of Number of Reads BC2 vs PC</th>
<th>Difference of Number of Reads DC2 vs PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCN31: 3/17/2011</td>
<td>23831</td>
<td>21910</td>
<td>N/A</td>
<td>21405</td>
<td>N/A</td>
<td>13550</td>
<td>N/A</td>
</tr>
<tr>
<td>DCN31: 5/9/2011</td>
<td>20719</td>
<td>21910</td>
<td>N/A</td>
<td>21405</td>
<td>N/A</td>
<td>13550</td>
<td>N/A</td>
</tr>
<tr>
<td>DCN32: 12/6/2010</td>
<td>19400</td>
<td>21910</td>
<td>N/A</td>
<td>21405</td>
<td>N/A</td>
<td>13550</td>
<td>N/A</td>
</tr>
<tr>
<td>DCN32: 5/9/2011</td>
<td>21669</td>
<td>21910</td>
<td>N/A</td>
<td>21405</td>
<td>N/A</td>
<td>13550</td>
<td>N/A</td>
</tr>
<tr>
<td>DCN32: 11/10/2011</td>
<td>22145</td>
<td>21910</td>
<td>34955</td>
<td>N/A</td>
<td>N/A</td>
<td>13550</td>
<td>10031</td>
</tr>
<tr>
<td>DCN50: 2/1/2011</td>
<td>27135</td>
<td>21910</td>
<td>34955</td>
<td>N/A</td>
<td>N/A</td>
<td>13550</td>
<td>10031</td>
</tr>
<tr>
<td>DCN50: 5/10/2011</td>
<td>36796</td>
<td>21910</td>
<td>34955</td>
<td>N/A</td>
<td>N/A</td>
<td>13550</td>
<td>10031</td>
</tr>
<tr>
<td>DCN50: 9/1/2011</td>
<td>53744</td>
<td>21910</td>
<td>34955</td>
<td>N/A</td>
<td>N/A</td>
<td>13550</td>
<td>10031</td>
</tr>
<tr>
<td>PC145: 7/20/2012</td>
<td>12210</td>
<td>21910</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>11879</td>
<td>N/A</td>
</tr>
<tr>
<td>PC147: 7/20/2012</td>
<td>10223</td>
<td>21910</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>11879</td>
<td>N/A</td>
</tr>
<tr>
<td>PC149: 7/20/2012</td>
<td>13709</td>
<td>21910</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>11879</td>
<td>N/A</td>
</tr>
<tr>
<td>PC150: 7/20/2012</td>
<td>9410</td>
<td>21910</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>11879</td>
<td>N/A</td>
</tr>
<tr>
<td>PC151: 7/20/2012</td>
<td>13841</td>
<td>21910</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>11879</td>
<td>N/A</td>
</tr>
<tr>
<td>Total Number of Reads</td>
<td>284,832</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Microbial taxa and singletons. Since previous microbiome studies have determined that a minimum threshold of 6,000 reads is sufficient, rarefying the reads to 9,410 is adequate to validate the sequencing depth (FIGURE 6).
7.2.1.3 OTU Richness

Collection site demonstrated marginal non-significance of *A. compressa* sponge microbiome OTU richness with ANOVA $p=0.098$. There is no significance at $\alpha=0.05$, but with a $p$-value of 0.098, there is only a small, 9.8% chance of rejecting a true null hypothesis. The highest to lowest mean of OTUs is for Broward County, Florida, USA at
approximately 2,900; Dade County, Florida, USA at approximately 2,300 and Bocas del Toro, Panama at approximately 1,200 (FIGURE: 7).

![Richness per Sites](image)

**FIGURE 7**: Boxplot of microbiome OTU richness of host sponge *A. compressa* per collection site, with a marginal non-significant difference among sites, ANOVA $p=0.098$.

Collection date demonstrated no significance of *A. compressa* sponge microbiome OTU richness with ANOVA $p=0.112$.

Sea surface temperature (SST) in degrees Celsius demonstrated marginal non-significance of *A. compressa* sponge microbiome OTU richness with Regression analysis $p=0.078$, $r^2=0.26$. There is a trend of decreasing OTU richness with increasing sea surface
temperature. Although not significant with a p-value of 0.078, there is only a small, 7.8% chance of rejecting a true null hypothesis. (FIGURE: 8).

![OTU Richness per Temperature](image)

**FIGURE 8:** Regression analysis of microbiome OTU richness of host sponge *A. compressa* per temperature, with a marginal non-significant difference $p=0.078$, $r^2=0.26$.

Salinity in parts per thousand (ppt) demonstrated significance of *A. compressa* sponge microbiome OTU richness with Regression analysis $p=0.040$, $r^2=0.33$. There is a trend of increasing OTU richness with increasing sea surface salinity (FIGURE: 9).
Calendar-based seasons (winter/summer/spring/fall (WSSF)) demonstrated no significance of *A. compressa* sponge microbiome OTU richness with $p=0.16$.

Precipitation-based seasons (wet/dry) demonstrated a significant difference of *A. compressa* sponge microbiome OTU richness with ANOVA $p=0.021$ (FIGURE: 10) (APPENDIX: 2).

**FIGURE 9**: Regression analysis of microbiome OTU richness of host sponge *A. compressa* per temperature, with significant differences $p=0.040$, $r^2=0.33$. 
For Tukey’s HSD for multiple comparisons of means there are no significant differences in microbiome OTU richness among all *A. compressa* sponge collection site pairs (Broward County, Florida, USA-Bocas del Toro, Panama, *p*=0.149; Dade County, Florida, USA-Bocas del Toro, Panama, *p*=0.143; and Dade County, Florida, USA-Broward County, Florida, USA, *p*=0.999). There is no significance at *α*=0.05, but with a *p*-value of 0.15 and 0.14 for the two Florida-Panama comparisons, there is only a small, 14% or 15% chance rejecting a true null hypothesis. There is clearly no significant difference (*p*=0.999).

**FIGURE 10:** Boxplot of microbiome OTU richness of host sponge *A. compressa* per precipitation-based seasons, with a significant difference, ANOVA *p*=0.021.
in microbiome OTU richness between the Broward and Dade County samples (FIGURE: 7). For pair-wise comparisons of Calendar-based seasons (spring-fall, summer-fall, winter-fall, summer-spring, winter-spring, winter-summer) there is no significant differences with all $p>0.10$, yet for the pair-wise comparison between precipitation-based seasons (wet/dry) there is a significant difference of 0.021. (FIGURE: 10) (APPENDIX: 3).

### 7.2.1.4 Alpha Diversity - Inverse Simpson

There are no significant differences in microbiome OTU Alpha diversity among all *A. compressa* sponge collection sites (ANOVA, $p=0.081$). There is marginal non-significance at $\alpha=0.05$, but with a $p$-value of 0.081, there is only a small, 8.1% chance rejecting a true null hypothesis. The highest diversity is at Dade County; the second highest diversity is at Broward County; and the least diversity is at Bocas del Toro (FIGURE: 11).

There are no significant differences in microbiome OTU diversity among all *A. compressa* sponge collection dates ($p=0.594$).

There is a marginal non-significant difference in microbiome OTU diversity among all *A. compressa* sponges related to SST in degrees Celsius with Regression analysis $p=0.059$, $r^2=0.22$. There is a trend of decreasing OTU Alpha diversity with increasing sea surface temperature. There is no significance at $\alpha=0.05$, but with a $p$-value of 0.059, there is only a small, 5.9% chance rejecting a true null hypothesis. The highest to lowest diversity levels follow an inverse relationship with temperature, with highest levels at 23.7 °C and lowest levels at 29.8 °C (FIGURE: 12).
FIGURE 11: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per collection site, with marginal non-significant differences among sites, ANOVA $p=0.081$. 
There is a significant difference in microbiome OTU diversity among all *A. compressa* sponge salinity (ppt) with Regression analysis $p=0.041$, $r^2=0.27$. There is a trend of increasing OTU Alpha diversity with increasing salinity. With significance, on a scale of 1.0-10.0 the highest to lowest in diversity is within 35.3 ppt at approximately 8.8; 35.1 ppt at approximately 8.6; 36.09 ppt at approximately 6.5; 36.5 ppt at approximately 5.8; 35.4 ppt at approximately 5.0 and 32.1 ppt at approximately 3.0 (FIGURE: 13).

**FIGURE 12:** Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature, with a marginal non-significant difference $p=0.059$, $r^2=0.22$. 
There is no significant differences in microbiome OTU diversity among all A. compressa sponge calendar-based seasons (winter/summer/spring/fall (WSSF)) (ANOVA $p=0.120$). On a scale of 1.0-10.0 the highest to lowest in diversity is within spring at approximately 7.0; fall at approximately 6.8; winter at approximately 5.8; and summer at approximately 3.8.

There is a significant difference in microbiome OTU diversity among all A. compressa sponge precipitation-based seasons (wet/dry) (ANOVA $p=0.019$). The highest
is during the dry season and lowest diversity is during the wet season. (FIGURE: 14) (TABLE: 1) (APPENDIX: 4).

7.2.1.5 Beta Diversity

7.2.1.5.1 Bray-Curtis Dissimilarity

According to the BCD results for Beta diversity in *A. compressa* sponge microbiome communities, clustering in this study is generally following geographical gradients. The range of least dissimilarity (most similar) to highest dissimilarity (least
similar) is from 0.0-1.0. The samples are split into two main groups; one of these groups includes all five samples from Bocas del Toro, Panama (PC145: 7/20/2012, PC147: 7/20/2012, PC149: 7/20/2012, PC150: 7/20/2012, PC151: 7/20/2012) and one sample from Dade County, Florida, USA (DC N31: 3/17/2011). The other group includes the remaining three samples from Dade County, Florida, USA (DC N32: 12/6/2010, DC N31/N32: 5/9/2011) and all four samples from Broward County, Florida, USA (BC N50: 3/1/2011, BC N50: 5/10/2011, BC N50: 9/1/2011, BC N50: 11/10/2011) (FIGURE: 15).

**FIGURE 15:** Bray-Curtis Dissimilarity cluster dendrogram of host sponge *A. compressa* microbiome Beta OTU diversity per collection site. Values closer to 0.0 indicate the least dissimilarity (most similar) and values closer to 1.0 indicate the most dissimilarity (least similar).
7.2.1.5.2 ADONIS

The BCD ADONIS test of the interaction of variables (collection site, collection date, temperature, salinity, calendar-based seasons, and precipitation-based seasons) were tested pair-wise in differing combinations. Significance from highest to lowest were: temperature and precipitation-based seasons \((p=0.001)\), temperature and calendar-based seasons \((p=0.003)\), and collection site and temperature \((p=0.041)\). Marginally non-significant from highest to lowest were: salinity and calendar-based seasons \((p=0.059)\), salinity and temperature \((p=0.069)\), and collection site and salinity \((p=0.081)\). On an individual basis, both collection site and salinity demonstrated high significance \((p \leq 0.010)\) and both calendar-based seasons and precipitation-based seasons demonstrated significance \((p \leq 0.050)\) (APPENDIX: 5).

7.2.1.5.3 Non-Metric Multi-Dimensional Scaling

Previous to creating a Non-Metric Multi-Dimensional Scaling (NMDS) plot, a stressplot was conducted to test goodness of fit. The stress plot indicated the data passed a robust test with a non-metric fit of \(r^2=0.998\) and a liner fit of \(r^2=0.992\) (APPENDIX: 6).

NMDS was used to determine dissimilarity of \(A. \ compressa\) sponge microbiome communities from three sampling locations (Broward County, Florida, USA; Dade County, Florida, USA: Bocas del Toro, Panama). The y-axis (NMDS2) has a scale of -1.0 – 1.0. Calculated cluster similarity distances are represented by lines that represent 95% confidence limit with samples that are most similar grouped together in ellipses. To identify the samples to their location on the ellipse two other NMDS plots were created, one with points and the other with sample IDs (APPENDIX: 7).
The most dissimilar (least similar) are the Bocas del Toro, Panama samples that are distinct from both Broward County, Florida, USA; Dade County, Florida, USA. The most similar sites (least dissimilarity) are the two South Florida, USA sites (Broward County, Dade County).

FIGURE 16: Non-Metric Multi-Dimensional Scaling ordination plot per collection site for all thirteen of microbiome OTU Beta diversity of host sponge *A. compressa* microbiome samples. Samples located within ellipses are the most similar to each other.

### 7.2.1.5.4 Heatmap

The Heatmap generated gives a distinguishing visual interpretation of the relative abundances (square-root transformed) of the microbiome within the host sponge *A. compressa* characterized by the top fifty OTUs. The BCD dendrogram identifies the individual samples per location, while demonstrating clustering of samples by Beta diversity. The dendrogram includes all thirteen samples form the three collection locations, with a clustering scale from 0.0-1.0. The values closer 0.0 indicate the least dissimilarity.
(most similar), and the values closer to 1.0 indicate the most dissimilarity (least similar) (FIGURE: 17). When used in conjunction with the taxonomic table, the specific samples by location and collection date can be further investigated.

![Bray-Curtis Dissimilarity Dendrogram](image)

**FIGURE 17:** Heatmap of microbiome species Beta diversity of host sponge *A. compressa* for the first fifty OTUs for taxonomic analysis of all three collection sites (Dade County, Florida, USA-Broward County, Florida, USA-Bocas del Toro, Panama). Complete with dendrogram, characterizes the square-root transformed relative abundance of taxa.

For the most abundant OTU, Phylum *Proteobacteria*, on the first main branch; the most abundance is in Bocas del Toro, Panama (58.2%) with PC 150: 7/20/2012 and PC 151: 7/20/2012 having the highest abundance in comparison to the other three Panama samples, while clustering in the least dissimilarity overall. The next branch of the least dissimilarity joins Panama sample PC 147: 7/20/2012, followed by joining Dade County sample DCN31: 3/17/2011; this outlier can demonstrate Beta diversity dissimilarity along the spatial gradient. The next branch joins the remaining closely related Panama samples PC 149: 7/20/2012 and PC145: 7/20/2012 the first main branch of the dendrogram.

For the most abundant OTU, Phylum *Proteobacteria*, on the second main branch; the clustering of least dissimilarity is for Broward County samples BCN50: 9/1/2011 and

For the second most abundant OTU, Phylum *Cyanobacteria*, on the first main branch the least abundance is in all of the Panama samples (4.3%). Conversely, on the second main branch the most abundance is considerably higher in in South Florida, USA, (22.6% Broward County, 16.9% Dade County), containing all the northern most samples with the exception of one Dade County sample (DCN31: 3/17/2011) that clustered with Panama.


Only the two top taxa *Proteobacteria* and *Cyanobacteria* are in high abundance in *A. compressa* with most of the South Florida samples having them in similar relative high abundances while in the Panama samples the single taxon *Proteobacteria* having relative high abundance. The remaining six OTUs drop drastically and fluctuate in abundances between locations, with South Florida samples demonstrating the greater overall Beta diversity. After the first total OTUs all samples fall below the <1% threshold.
7.2.1.5.5 Simper Similarity Percentages

Of the top most abundant fifty OTUs of the *A. compressa* host sponge microbiome, the top most abundant twenty OTUs were generated into a table. Since the assignment of taxa from OTUs is dependent on the 97% threshold-based Greengenes database, groups were assigned to different levels of taxonomic specificity (APPENDIX: 8).

The most abundant bacterial OTUs of *A. compressa* from all three locations are dominated by two bacterial Phyla, *Proteobacteria* and *Cyanobacteria*. The highest abundance of bacterial OTU for all three locations is classified to the Phylum level as a *Proteobacteria*. This *Proteobacteria* has the highest abundance in samples from Bocas del Toro, Panama at 58.2% of the sponge’s microbiome. The *Proteobacteria* is second highest abundance in samples from Dade County, Florida, USA at 34.7% of the sponge’s microbiome. The *Proteobacteria* is least abundant in samples from Broward County, Florida, USA at 30.4% of the sponge’s microbiome. This *Proteobacteria* is following a geographical spatial gradient, highest in the southernmost location and lowest in the northernmost location. The second highest abundance of bacterial OTU for all three locations is classified to the Family level as a *Synechococcaceae*; Phylum *Cyanobacteria*. This *Synechococcaceae* is the highest abundance in samples from Broward County, Florida, USA at 22.6% of the sponge’s microbiome. The *Synechococcaceae* is second highest abundance in samples from Dade County, Florida, USA at 16.9% of the sponge’s microbiome. The *Synechococcaceae* is least abundant in samples from Bocas del Toro, Panama at 4.3% of the sponge’s microbiome. This *Synechococcaceae* is following a geographical spatial gradient, opposite than the *Proteobacteria*, highest in the northernmost location and lowest in the southernmost location. The most abundant OTU
in this study, accounting for 30-58% of the detected groups, are from the *Proteobacteria* phylum. The next most abundant OTU (family *Synechococcaceae*) is from the *Cyanobacteria*, another common sponge microbiome phylum that accounts for an additional 4.3-22.6% of the groups in this study.

Following the two most dominate bacterial OTUs across all three sites, the abundance of the remaining bacterial OTUs drops off significantly and disproportionately between locations. A bacterial OTU identified to the Order level as *Oceanospirillales* is abundant in Dade County, Florida, USA at 2.6%, however it is <1% in both Broward County, Florida, USA and Bocas del Toro, Panama. A bacterial OTU identified to the Genus level as *Prochlorococcus* is abundant in Dade County, Florida, USA at 2.6%, however it is <1% in both Broward County, Florida, USA and Bocas del Toro, Panama. A bacterial OTU identified to the Genus level as a second *Prochlorococcus* is abundant in Broward County, Florida, USA at 1.9%, however it is <1% in both Dade County, Florida, USA and Bocas del Toro, Panama. A bacterial OTU identified to the Family level as *Pirellulaceae* is abundant in Broward County, Florida, USA at 1.3%, however it is <1% in both Dade County, Florida, USA and Bocas del Toro, Panama. A bacterial OTU identified to the Genus level as *Synechococcus* is abundant in Broward County, Florida, USA at 1.2%, however it is <1% in both Dade County, Florida, USA and Bocas del Toro, Panama. The last remaining microbe identified above 1% is a bacterial OTU identified to the Family level as *Endozoicimonaceae*, which is abundant in Dade County, Florida, USA at 1.1%, however it is <1% in both Dade County, Florida, USA and Bocas del Toro, Panama.

A total of eight microbial OTUs of *A. compressa* are identified above a 1% threshold, with a Phylum level *Proteobacteria* being the most abundant across all sample
sites and a Family level *Synechococcaceae* the second most abundant across all samples. These two bacterial OTU dominate the microbiome of *A. compressa*, candidates as species specific symbionts (TABLE: 3) (FIGURE: 18) (SUPPLEMENTARY: 2).

<table>
<thead>
<tr>
<th>EMP OTU ID</th>
<th>CLASSIFICATION LEVEL</th>
<th>TAXONOMY</th>
<th>PERCENTAGE BROWARD COUNTY</th>
<th>PERCENTAGE DADE COUNTY</th>
<th>PERCENTAGE BOCAS TORO</th>
</tr>
</thead>
<tbody>
<tr>
<td>003905</td>
<td>Phylum</td>
<td><em>Proteobacteria</em></td>
<td>30.4</td>
<td>34.7</td>
<td>58.2</td>
</tr>
<tr>
<td>014935</td>
<td>Family</td>
<td><em>Synechococcaceae</em></td>
<td>22.6</td>
<td>16.9</td>
<td>4.3</td>
</tr>
<tr>
<td>001669</td>
<td>Order</td>
<td><em>Oceanospirillales</em></td>
<td>&lt;1</td>
<td>2.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>003494</td>
<td>Genus</td>
<td><em>Prochlorococcus</em></td>
<td>&lt;1</td>
<td>2.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>005974</td>
<td>Genus</td>
<td><em>Prochlorococcus</em></td>
<td>1.9</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>000275</td>
<td>Family</td>
<td><em>Pirellulaceae</em></td>
<td>1.3</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>000008</td>
<td>Genus</td>
<td><em>Synechococcus</em></td>
<td>1.2</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>000650</td>
<td>Family</td>
<td><em>Endozoicimonaceae</em></td>
<td>&lt;1</td>
<td>1.1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**TABLE 3:** Taxonomic classification summary table of the eight most abundant microbes of host sponge *A. compressa*. Earth Microbiome Project microbial OTUs are identified above a one percent threshold by location.

**FIGURE 18:** A: Relative Species Abundance histogram of host sponge *A. compressa* microbiome for the first top eight OTUs in order from highest to lowest abundance at Broward County, Florida, USA.
Dade County, Florida

Bocas del Toro, Panama

**FIGURE 18; B:** Relative Species Abundance histogram of host sponge *A. compressa* microbiome for the first top eight OTUs in order from highest to lowest abundance at Dade County, Florida, USA.

**FIGURE 18; C:** Relative Species Abundance histograms of host sponge *A. compressa* microbiome for the first top eight OTUs in order from highest to lowest abundance at Bocas del Toro, Panama.

**FIGURE 18; A, B, C:** Relative Species Abundance histograms of host sponge *A. compressa* microbiome for the first top eight OTUs in order from highest to lowest abundance of all three collection sites (Broward County, Florida, USA-Dade County, Florida, USA-Bocas del Toro, Panama). The two most abundant identical taxa from all three locations are *Proteobacteria* (Phylum) and *Synechococcaceae* (Phylum *Cyanobacteria*).
7.2.1.6 BLAST Taxonomic Investigation

Taxonomic analysis of OTUs documents eight taxa that each compose more than 1% of the total sponge microbiome community identified on the Greengenes database at a 97% identity threshold.

The microbiome community of *A. compressa* is primarily composed of two bacterial taxa. Together, bacteria from the Phyla *Proteobacteria* and *Cyanobacteria* accounts for over half of the OTUs in Broward and Dade Counties (53.0% and 56.8% respectively), and nearly two-thirds of the OTUs in Panama (62.5%).

The first Phylum *Proteobacteria* (OTU number: 003905) accounts for 30.4% (Broward County), 34.7% (Dade County) and 58.2% (Panama) of the OTUs. A BLAST search was performed on this sequence and had a 100%-97% match to several other sponge derived bacterial communities. In South Florida this sequence matched a sponge microbe at 100% in *Agelas tubulata* (Negandhi *et al.*, 2010), at 100% to a sponge in *Tedania* (Lopez *et al.*, unpublished), at 100% to a gorgonian microbe in *Eunicea fusca* (Duque-Alarcon *et al.*, unpublished). One sequence match at 100% was to a sponge microbe in *Haliclona tubifera* collected in the Gulf of Mexico (Erwin *et al.*, 2011) and two sequences matched at 99% and 97% (respectively) to a sponge *betaproteobacterium* in *Crambe crambe* from two locations of in the Mediterranean Sea; Spain (Sipkema and Jaeger, unpublished) and France (Croue *et al.*, 2013).

The second Phylum *Cyanobacteria* (OTU number: 014935) accounts for 22.6% (Broward County), 16.9% (Dade County), and 4.3% (Panama) of the OTUs and is consistent with previous findings as the second most dominant Phyla in LMA sponges (Croue *et al.*, 2013; Giles *et al.*, 2013). A BLAST search was performed on this sequence
and had a 100% match to several different bodies of seawater, one sponge, and one coral. In Cuatro Cienegas Basin, Mexico this sequence matched a microbe in seawater, *picocyanobacteria* (Beltran et al., unpublished), an unculturable microbe in seawater from the Changjiang Estuary (Liu et al., unpublished), and an unculturable microbe in seawater from the Arabian Sea (Gomes et al., unpublished). In The Gulf of Mexico and Brazil this sequence matched a sponge microbe in *Hymeniacidon heliophila* (Weigel and Erwin, 2015) and in Curacao this sequence matched an unculturable microbe in a scleractinian coral (Frade, unpublished).

The third and fourth most abundant groups (OTU numbers: 001669, 003494), with much smaller OTU percentages, include bacteria from the Phyla *Proteobacteria* and *Cyanobacteria*. *Proteobacteria*, order *Oceanospirillales* occurs only in Dade County at relatively higher frequencies than the other sites (2.6%), compared to <1% in both Broward County and Panama. *Cyanobacteria*, genus *Prochlorococcus* also occurs in Dade County at 2.6%, however it is much less abundant (<1%) in both Broward County and Panama. A BLAST search was performed on the second most abundant *Proteobacteria* sequence and third most abundant overall having a 99%-100% match to several sponges from different oceans. 100% match is to *songioabacter* sp. in the host sponge *Halocordyle disticha* from Kuwait, 100% match to a *gammaproteobacterium* in the host sponge *Mycale llaxissima* from Key Largo Florida, 99% match to a *gammaproteobacterium* in the host sponge *Theonella swinhoei* from the Red Sea, 99% match to a *gammaproteobacterium* in the host sponge *Aplysina californis* from the Bahamas, 99% match to an uncultured bacterium in the host sponge *Axinella verrucosa* from the Mediterranean, and 99% match to an uncultured marine bacterium in the host sponge *Tsitsikamma favus* from Algoa Bay South
Africa (NCBI). A BLAST search was performed on the second most abundant Cyanobacteria sequence and the fourth most abundant overall having a 100% match to environmental samples, similar to the first Cyanobacteria characterized. 100% match is to a Rhodobacteraceae bacterium in seawater from the Arabian Sea, 100% match is to Prochlorococcus sp. “complete genome” in seawater (unpublished; source not identified), 100% match is to uncultured bacterium in seawater from the West Pacific, and 100% match to an uncultured Prochlorococcus in seawater from the Arabian Sea (NCBI).

The fifth, sixth, and seventh most abundant groups (OTU numbers: 005974, 000275, 000008), with much smaller OTU percentages, include bacteria from the Phylum Cyanobacteria, genus Prochlorococcus, family Pirellulaceae, and genus Synechococcus respectively, occurring only in Broward County at relatively higher frequencies than the other sites (1.9%, 1.3%, 1.2% respectively) in comparison to <1% in both Dade County and Panama. A BLAST search was performed on the fourth most abundant Cyanobacteria sequence and the fifth most abundant overall having a 100% match to two sponges and environmental samples. 100% match is to an uncultured Cyanobacterium in the host sponge Hymeniacidon heliophila from The Gulf of Mexico, 100% match is to an uncultured bacterium in a host sponge (unpublished; source not identified) from the South China Sea, 100% match to an uncultured bacterium in a hydrothermal vent (unpublished; source not identified) from the Guaymas Basin, and 100% match to an uncultured planctomycete, Hymeniacidon helioophila (unpublished; source not identified). A BLAST search was performed on the fifth most abundant Cyanobacteria sequence and the sixth most abundant overall having a 100% match to an uncultured bacterium in a hydrothermal vent (unpublished; source not identified) from the Guaymas Basin and an uncultured
planctomycete, *Hymeniacidon helioophila* (unpublished; source not identified). A BLAST search was performed on the sixth most abundant *Cyanobacteria* sequence and the seventh most abundant overall having a 100% match to an uncultured and 100% to an uncultured *cyanobacterium* from the Cochin Estuary (unpublished; source not identified) (NCBI).

Lastly, the eighth most abundant group (OTU number: 000650) with much smaller OTU percentages, include bacteria from the Phylum *Proteobacteria*, family *Endozoicimonaceae*, occurring only in Dade County at relatively higher frequencies than the other sites (1.1%), compared to <1% in both Broward County and Panama. A BLAST search was performed on the eighth most abundant sequence, a *Proteobacteria*, overall having a 100% match to three sponge samples. 100% match is to an uncultured *gammaproteobacterium* in the host sponge *Discodermia* sp. from the Bahamas (unpublished; source not identified), 100% match is to an uncultured *gammaproteobacterium* in the host sponge *Halichondria* from Japan (unpublished; source not identified), and 100% match to an uncultured *bacterium* in the host sponge *Theonella swinhoei* from the South China Sea (unpublished; source not identified) (NCBI).

### 7.2.2 Dual Site Analysis; Pairwise

Pairwise analysis of the three independent *A. compressa* host sponge collection sites (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama) was investigated to further determine microbiome abundances, richness and diversity on a finer spatial scale.
7.2.2.1 OTU Richness

In general, there were no significant differences in sponge microbiome OTU richness for all variables in all pair-wise comparisons.

There are no significant differences in pairwise comparison of microbiome OTU richness between sponges from all three site pairs; Dade-Broward (Tukey $p=0.816$). Dade-Bocas del Toro (Tukey $p=0.108$), and Broward-Bocas del Toro (Tukey $p=0.102$). The average number of OTUs is approximately 2,200 for Dade County, 2,900 for Broward County and 1,100 for Bocas del Toro.

There were no significant differences in Regression analyses of pair-wise comparisons of microbiome OTU richness based on SST in all three site pairs; Dade-Broward Counties, $r^2=0.03 p=0.316$, Dade-Bocas del Toro, $r^2=0.16 p=0.152$, and Broward-Bocas del Toro, $r^2=0.24 p=0.101$. Temperatures ranged from 32.1 ppt in Bocas del Toro to 36.5 ppt in Broward. SSTs ranged from 22.8 °C in Dade County to 29.8 °C in Broward County.

There were no significant differences in Regression analyses of pair-wise comparisons of microbiome OTU richness based on salinity in all three site pairs; Dade-Broward Counties, $r^2=0.13 p=0.674$, marginal non-significance for Dade-Bocas del Toro, $r^2=0.25 p=0.095$, and no significance between Broward-Bocas del Toro, $r^2=0.18 p=0.141$. Salinities ranged from 32.1 ppt in Bocas del Toro to 36.5 ppt in Broward. There is no significance at $\alpha=0.05$, but with a $p$-value of 0.095 in the pair-wise analysis between Dade and Bocas del Toro, there is only a small, 9.5% chance rejecting a true null hypothesis. There is a trend of increasing OTU richness with increasing salinity. (FIGURE: 19).
There were no significant differences in pair-wise comparisons of microbiome OTU richness by calendar-based seasons (WSSF) for all three site pairs; Dade-Broward; Dade-Bocas del Toro; Broward-Bocas del Toro (Tukey $p>0.100$) in all six pair-wise seasonal comparisons, (e.g. Winter-Spring, Winter-Summer, etc.).

There were no significant differences in microbiome OTU richness between the pair-wise comparison of precipitation-based seasons (wet/dry) between all three location pairs Dade-Broward (Tukey $p=0.300$), Dade-Bocas del Toro (Tukey $p=0.108$), although marginally non-significant for Broward-Bocas del Toro (Tukey $p=0.071$). There is no

**FIGURE 19:** Regression analysis of microbiome OTU richness of host sponge *A. compressa* per salinity in pair-wise comparison between Dade and Bocas del Toro, with a marginal non-significant difference $p=0.095$ $r^2=0.25$. 
significance at $\alpha=0.05$, but with a $p$-value of 0.071 in the pair-wise analysis between Dade and Bocas del Toro, there is only a small, 7.1% chance rejecting a true null hypothesis (FIGURE: 20) (APPENDIX: 9).

![Richness per Precipitation Based-Seasons](image)

**FIGURE 20:** Boxplot of microbiome OTU richness of host sponge *A. compressa* per calendar-based seasons, with a marginal non-significant difference in wet and dry seasons between the site pair Broward and Bocas del Toro, Tukey $p=0.07$.

7.2.2.2 Alpha Diversity – Inverse Simpson

There are no significant differences in microbiome OTU Alpha diversity among two of the *A. compressa* host sponge collection site pairs; Dade-Broward County, (ANOVA $p=0.999$), a marginal non-significance between Dade-Bocas del Toro (ANOVA $p=0.078$). Although marginally non-significant at $\alpha=0.05$, but with a $p$-value of 0.078 in
the pair-wise analysis between Dade and Bocas del Toro, there is only a small, 7.8% chance rejecting a true null hypothesis (FIGURE: 21).

![Boxplot of microbiome OTU Alpha diversity per site, with a marginal non-significant difference between the site pair Dade and Bocas del Toro, ANOVA p=0.078.](image)

**FIGURE 21**: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per site, with a marginal non-significant difference between the site pair Dade and Bocas del Toro, ANOVA $p=0.078$.

However there is a significant difference between collection site pair Broward-Bocas del Toro, (ANOVA $p=0.030$). The highest mean diversity is in Broward County, Florida and the lowest mean diversity is in Bocas del Toro, Panama (FIGURE: 22).
There were no significant differences in Regression analyses of two pair-wise comparisons of microbiome OTU diversity based on SST; Dade-Broward Counties, $r^2=0.09$ $p=0.552$, and Dade-Bocas del Toro, $r^2=0.15$ $p=0.162$. There was a significant difference between Broward-Bocas del Toro, $r^2=0.45$ $p=0.028$. There is a trend of decreasing OTU Alpha diversity with increasing temperature. SSTs ranged from 22.8 °C in Dade County to 29.8 °C in Broward County. (FIGURE: 23)
There were no significant differences in Regression analyses of pair-wise comparisons of microbiome OTU alpha diversity based on salinity in two site pairs; Dade-Broward Counties, $r^2=0.043\ p=0.427$, a marginal non-significance between Dade-Bocas del Toro, $r^2=0.27\ p=0.088$. Although marginally non-significant at $\alpha=0.05$, but with a $p$-value of 0.088 in the pair-wise analysis between Dade and Bocas del Toro, there is only a small, 8.8% chance rejecting a true null hypothesis (FIGURE: 24).

**FIGURE 23:** Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature in pair-wise comparison between Broward and Bocas del Toro, with a significant difference $p=0.028\ r^2=0.45$. 

![Alpha Simpson Diversity per Temperature](image_url)
There was a marginal non-significance between Broward-Bocas del Toro, $r^2=0.33$ $p=0.061$. Although marginally non-significant at $\alpha=0.05$, but with a $p$-value of 0.061 in the pair-wise analysis between Broward and Bocas del Toro, there is only a small, 6.1% chance rejecting a true null hypothesis (FIGURE: 25). There is a trend of increasing OTU Alpha diversity with increasing salinity for both pairs Dade and Bocas del Toro and Broward and Bocas del Toro.

FIGURE 24: Regression analysis of microbiome OTU Alpha diversity of host sponge $A. compressa$ per salinity in pair-wise comparison between Dade and Bocas del Toro, with a marginal non-significant difference $p=0.088$ $r^2=0.27$. 

FIGURE 25: Regression analysis of microbiome OTU Alpha diversity of host sponge $A. compressa$ per salinity in pair-wise comparison between Dade and Bocas del Toro, with a marginal non-significant difference $p=0.088$ $r^2=0.27$. 

There was a marginal non-significance between Broward-Bocas del Toro, $r^2=0.33$ $p=0.061$. Although marginally non-significant at $\alpha=0.05$, but with a $p$-value of 0.061 in the pair-wise analysis between Broward and Bocas del Toro, there is only a small, 6.1% chance rejecting a true null hypothesis (FIGURE: 25). There is a trend of increasing OTU Alpha diversity with increasing salinity for both pairs Dade and Bocas del Toro and Broward and Bocas del Toro.
There were no significant differences in pair-wise comparisons of microbiome OTU Alpha diversity by calendar-based seasons (WSSF) for all three site pairs; Dade-Broward (ANOVA $p=0.802$) in all six pair-wise seasonal comparisons, (e.g. Winter-Spring, Winter-Summer, etc.), a marginal non-significance for Dade-Bocas del Toro (ANOVA $p=0.087$) in all six pair-wise seasonal comparisons. Although marginally non-significant at $\alpha=0.05$, but with a $p$-value of $0.087$ in the pair-wise analysis between Dade and Bocas del Toro, there is only a small, $8.7\%$ chance rejecting a true null hypothesis (FIGURE: 26).

**FIGURE 25**: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per salinity in pair-wise comparison between Broward and Bocas del Toro, with non-significant difference $p=0.061$ $r^2=0.33$. 

---

![Alpha Simpson Diversity per Salinity](chart.png)
There were no significant differences between Broward-Bocas del Toro (ANOVA $p=0.117$) in all six pair-wise seasonal comparisons.

There were no significant differences in microbiome OTU Alpha diversity between the pair-wise comparison of precipitation-based seasons (wet/dry) between two location pairs Dade-Broward (ANOVA $p=0.469$), a marginal non-significance for Dade-Bocas del Toro (ANOVA $p=0.078$) although marginally non-significant at $\alpha=0.05$, but with a $p$-value of 0.078 in the pair-wise analysis between Dade and Bocas del Toro, there is only a small, 7.8% chance rejecting a true null hypothesis (FIGURE: 27).

FIGURE 26: Boxplot of microbiome OTU Alpha diversity of host sponge *A. compressa* per calendar-based seasons, with a marginal non-significant difference between the site pair Dade and Bocas del Toro, ANOVA $p=0.087$. 
There was a significant difference in the third pair, Broward-Bocas del Toro (ANOVA $p=0.022$) (FIGURE: 28) (APPENDIX: 10).
7.2.2.3 Beta Diversity

7.2.2.3.1 Bray-Curtis Dissimilarity

Bray-Curtis Dissimilarity was analyzed for each of the three independent A. compressa host sponge microbiome pairwise collection sites (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama) with clustering in this study following a spatial latitudinal gradient.

FIGURE 28: Boxplot of microbiome OTU Alpha diversity of host sponge A. compressa per precipitation-based seasons, with a significant difference between the site pair Broward and Bocas del Toro, ANOVA $p=0.022$. 
For collection pair site one (Dade County, Florida, USA-Broward County, Florida, USA), the scale of least dissimilarity (most similar) to highest dissimilarity (least similar) is from 0.0-1.0. The samples are split into two main groups; one of these groups includes two samples each from both Dade County, Florida, USA and Broward County, Florida, USA (DC N32: 5/9/2011; DC N50: 3/1/2011) with the other group including the remaining six samples each from both Dade County, Florida, USA and Broward County, Florida, USA (DC N31: 5/9/2011; DC N31: 3/17/2011; DC N32: 12/6/2010; BC N50: 11/10/2011; BC N50: 5/10.2011; BC N50: 9/1/2011).

For collection pair site two (Dade County, Florida, USA- Bocas del Toro, Panama), the scale of least dissimilarity (most similar) to highest dissimilarity (least similar) is from 0.0-1.0. The samples are split into two main groups; one of these groups includes two samples both Dade County, Florida, (DC N31: 5/9/2011; DC N32: 5/9/2011) with the other group including the remaining seven samples each from both Dade County, Florida, USA and Bocas del Toro, Panama (DC N31: 3/17/2011; DC N32: 12/6/2010; PC145: 7/20/2012; PC147: 7/20/2012; PC149: 7/20/2012; PC150: 7/20/2012; PC151: 7/20/2012).

For collection pair site three (Broward County, Florida, USA-Bocas del Toro, Panama), the scale of least dissimilarity (most similar) to highest dissimilarity (least similar) is from 0.0-1.0. The samples are split into two main groups; one of these groups includes all five samples form Bocas del Toro, Panama (PC145: 7/20/2012; PC147: 7/20/2012; PC149: 7/20/2012; PC150: 7/20/2012; PC151: 7/20/2012) with the other group including all four samples from Broward County, Florida, (BC N50: 3/1/2011; BC N50: 5/10/2011; BC N50:9/1/2011; BC N50: 11/10/2011) (FIGURE 29; A, B, C).
FIGURE 29; A: Bray-Curtis Dissimilarity dendrogram for collection pair site one (Dade County, Florida, USA-Broward County, Florida, USA)
FIGURE 29; B: Bray-Curtis Dissimilarity dendrogram for collection pair site two (Dade County, Florida, USA-Bocas del Toro, Panama)
The largest significance in microbiome Beta diversity is between Broward County, Florida, USA and Bocas del Toro, Panama; the two sites that have the largest geographic distance (1,875 km) among the three sampling locations. The second largest significance...
in microbiome Beta diversity is between Dade County, Florida, USA and Bocas del Toro, Panama; sites separated by slightly less geographic distance (1,839 km). The least Beta diversity is between Dade County, Florida, USA and Broward County, Florida, USA, the closest two sampling sites (37 km).

7.2.2.3 Pair-wise NMDS

Non-metric Multidimensional Scaling (NMDS) was analyzed for each of the three independent *A. compressa* host sponge microbiome pairwise collection sites (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama) The NMDS procedure produces an ordination based on a dissimilarity matrix. Calculated cluster similarity distances are represented by lines that represent 95% confidence limit with samples that are most similar grouped together in ellipses.

In the comparison between Dade County, Florida, USA-Bocas del Toro, Panama, there are two groups. In the first group, all five samples from Bocas del Toro, Panama (PC145: 7/20/2012, PC147: 7/20/2012, PC149: 7/20/2012, PC150: 7/20/2012, PC151: 7/20/2012) are grouped together. In the second group, all samples from Dade County, Florida, USA (DC N31: 5/9/2011, DC N31/N32: 5/9/2011, DC N32: 12/6/2010) and four samples from Bocas del Toro, Panama (PC145: 7/20/2012, PC147: 7/20/2012, PC150: 7/20/2012, PC151: 7/20/2012) are grouped together, and one from Bocas del Toro, Panama (PC149: 7/20/2012) is not included in this group. The x-axis has a scale of -1.0 – 1.0 and the y-axis has a scale of -1.0 – 1.0.

In the comparison between Broward County, Florida, USA-Bocas del Toro, Panama, there are two groups. In the first group, all four samples from Broward County, Florida, USA (BC N50: 3/1/2011, BC N50: 5/10/2011, BC N50: 9/1/2011, BC N50: 11/10/2011) are grouped together. In the second group, all five samples from Bocas del Toro, Panama (PC145: 7/20/2012, PC147: 7/20/2012, PC149: 7/20/2012, PC150: 7/20/2012, PC151: 7/20/2012) are grouped together. The x-axis has a scale of -1.0 – 1.0 and the y-axis has a scale of -1.0 – 1.0 (FIGURE 30; A, B, C).

7.2.3 Single Site Analysis; Broward County, Florida, USA

Single site analysis of the one most northern A. compressa host sponge collection site (Broward County, Florida, USA) was investigated to further determine microbiome abundances, richness and diversity on a temporal scale. The Broward County, Florida, USA samples were taken from the same sponge four times during one year. This data is
used to determine if there are seasonal differences in microbiome OTUs over one complete season.

FIGURE 30; A: Non-metric Multidimensional Scaling (NMDS) ordination plot for collection pair site one (Dade County, Florida, USA-Broward County, Florida, USA)
FIGURE 30; B: Non-metric Multidimensional Scaling (NMDS) ordination plot for collection pair site two (Dade County, Florida, USA-Bocas del Toro, Panama)
Of the parameters tested (collection date, SST in degrees Celsius, salinity (ppt), calendar-based seasons (winter/summer/spring/fall (WSSF)), and precipitation-based seasons (wet/dry), only SST demonstrated significance in OTU richness (Regression
$p=0.026, r^2=0.92$), with decreasing OTU richness with increasing temperature (FIGURE: 31) (APPENDIX: 11).

FIGURE 31: Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature in pair-wise comparison between Broward County, Florida, USA samples on a temporal scale, with a significant difference $p=0.026$ $r^2=0.92$.

7.2.3.2 Alpha Diversity – Inverse Simpson

Of the parameters tested (collection date, SST in degrees Celsius, salinity (ppt), calendar-based seasons (winter/summer/spring/fall (WSSF)), and precipitation-based seasons (wet/dry), only SST demonstrated significance in OTU Alpha diversity (Regression $p=0.007$, $r^2=0.98$), with decreasing OTU richness with increasing temperature (FIGURE: 32) (APPENDIX: 12).
7.2.3.3 Beta Diversity

7.2.3.3.1 Bray-Curtis Dissimilarity

According to the BCD results for Beta diversity in *A. compressa* host sponge microbiome communities, clustering in this study is generally following temperature trends related to seasonal collection dates. The range of least dissimilarity (most similar) to highest dissimilarity (least similar) is from 0.0-1.0. The samples are split into two main groups; one of these groups includes BCN50:11/10/2011 and BCN50: 3/1/2011 which are from the two lower temperatures (winter and fall respectively); and the other group

**FIGURE 32:** Regression analysis of microbiome OTU Alpha diversity of host sponge *A. compressa* per temperature in single site analysis of Broward County, Florida, USA samples on a temporal scale, with a significant difference $p=0.007 \quad r^2=0.98$. 
includes BCN50: 5/10/2011 and BCN50: 9/1/2011 which are from the two higher temperatures (spring and summer respectively) (FIGURE: 33).

**FIGURE 33**: Bray-Curtis Dissimilarity cluster dendrogram of microbiome Beta species diversity of host sponge *A. compressa* for the single site analysis of Broward County, Florida, USA on a temporal scale.

### 7.2.3.3.2 Non-metric Multidimensional Scaling

According to the NMDS Beta diversity in *A. compressa* host sponge microbiome communities, samples that are most similar are grouped together in ellipses. Ellipses in this study are generally following temperature trends related to seasonal collection dates. The
samples are split into two main groups; one of these groups includes BCN50: 11/10/2011 and BCN50: 3/1/2011 which are from the two lower temperatures (winter and fall respectively); and the other group includes BCN50: 5/10/2011 and BCN50: 9/1/2011 which are from the two higher temperatures (spring and summer respectively) The NMDS is consistent with the BCD dendrogram. (FIGURE: 34).

**FIGURE 34:** Non-metric Multidimensional Scaling (NMDS) ordination plot of microbiome species Beta diversity of host sponge *A. compressa* for the single site analysis of Broward County, Florida, USA. Samples located within ellipses are the most similar to each other. Calculated cluster similarity distances are represented by lines and follows the Bray-Curtis dendrogram.
8.0 DISCUSSION

This study documents differences in microbiome communities within the sponge *Amphimedon compressa* among three locations, two in South Florida, USA (Broward County and Dade County), and one in Bocas del Toro, Panama. There were four samples from the same individual taken seasonally from Broward County, four samples taken from two individuals from different time periods in Dade County, and five samples taken from different individuals taken at an identical time period from Bocas del Toro (n=13). The spatial gradient covers an approximately 1,875 km maximum latitudinal distance. Environmental parameters were analyzed to determine if they could be drivers of observed differences in OTU richness (numbers of OTUs) and OTU diversity (number of OTUs, incorporating population evenness). These variables included collection site, collection date, sea surface temperature (SST), salinity (ppt), calendar-based seasons and precipitation-based seasons. This study investigated different microbiome OTU richness and diversity in *A. compressa* microbiomes across a geographic spatial gradient. In addition, important differences in microbiome OTU richness and diversity were observed on a temporal scale.

Comparisons were made by three different approaches, used to pinpoint where the OTU differences were and what are the driving forces. The first comparison was all three locations simultaneously, to investigate a large spatial gradient as a single element. This was used to outline OTU differences while determining which parameters required closer examination. The second comparison was to investigate all three locations pair-wise to distinguish which location(s) and parameter(s) are contributing to the OTU differences. The third comparison was to investigate a single location which had complete seasonal
sampling, used to determine any parameter(s) driving OTU differences on a temporal scale. The final investigation was to determine which OTUs, representing microbiome taxa, were dominant in \textit{A. compressa}. The taxa were investigated to determine if \textit{A. compressa} has core microbes, possible symbionts, and differences in abundances by location.

For Broward County, Dade County and Panama there were respectively 34,955, 21,405, 11,879 reads that correspond to approximately 2,900, 2,300 and 1,200 OTUs. This correlates to a north-south gradient of OTU richness with highest levels in South Florida, USA and the lowest levels in Panama.

Sole reliance on \textit{p}-values for data interpretation has merit, but (Nuzzo, 2014) provides compelling arguments to consider a broader interpretation of the data and not to strictly adhere to them. There are instances in this study where analyses result in \textit{p}-values having an \( \alpha \)-value between \( p=0.05 \) and 0.10, where there is only a small 10\% chance rejecting a true null hypothesis. This study took into consideration trends and consistency of results that are close to significant at \( \alpha=0.10 \) as a threshold.

For OTU richness, there were no significant differences among the three sample locations when investigated simultaneously (ANOVA; \( p=0.098 \)). Although marginally non-significant, the trend of OTU richness followed the spatial trend in the north-south gradient from highest to lowest, with Panama having substantially less OTU richness than the two South Florida locations that are closer in proximity to each other (OTU richness from north to south: Broward; 1,350, Dade; 1,300, Bocas del Toro; 1,050). When a Tukey HSD test was used for OTU pairwise richness comparisons of the three sample locations, a similar result was found. The South Florida locations, in close proximity to each other, demonstrate high non-significance of OTU richness (Dade-Broward: Tukey HSD;
The $p$-value is nearly equivalent to 100% non-significance. Yet when Panama is investigated pairwise to the South Florida locations, there was a trend of differences in OTU richness, with Panama not only having lower OTU richness but also driving spatial differences (Panama-Dade: Tukey HSD; $p=0.143$; Panama-Broward: Tukey HSD; $p=0.149$). Although not significant, the $p$-values of Panama in combination with the South Florida locations were nearly equivalent to each other, with only a 0.6% difference. This indicates location is an important spatial driver of OTU richness differences.

OTU richness in the triple site analysis was further investigated for SST, salinity, calendar-based seasons, and precipitation-based seasons. Of these parameters, SST demonstrated marginal non-significance (Regression; $p=0.078$, $r^2=0.26$), salinity demonstrated significance (Regression; $p=0.040$, $r^2=0.33$), and precipitation-based seasons demonstrated significance (ANOVA; $p=0.021$). OTU richness demonstrated a trend of decreased richness with increased temperature, increased richness with increased salinity, and decreased richness related to the wet season (increased precipitation and deceased salinity). Although the $r^2$-values are below 50.0%, this is due to large variance of the Panama samples.

In the pair-wise, one by one analyses of locations (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama), OTU richness did not demonstrate significance but did demonstrate trends similar to the triple site analyses. The two South Florida locations in close proximity demonstrated high non-significance (Dade-Broward: HSD Tukey; $p=0.816$), with Panama influencing differences (Panama-Dade: HSD Tukey; $p=0.108$; Panama-Broward: HSD Tukey; $p=0.102$). Although not significant,
the \( p \)-values of Panama in combination with the South Florida locations are nearly equivalent to each other, with only a 0.6% difference with the two adjacent South Florida locations being 81.6% similar. This indicates location is an important spatial driver of OTU richness differences.

OTU richness in the pair-wise analysis of SST, salinity, and precipitation-based seasons followed the same trend where the two South Florida locations (Dade-Broward) in close proximity demonstrate high non-significance SST (Regression; \( p=0.316, r^2=0.03 \)), salinity (Regression; \( p=0.674, r^2=0.13 \)), and precipitation-based seasons (\( p=0.300 \)). The same trend of Panama influencing differences is seen in pair-wise comparisons (Dade-Bocas del Toro) SST (Regression; \( p=0.152, r^2=0.16 \)), salinity (Regression; \( p=0.095, r^2=0.25 \)), and precipitation-based seasons (Tukey; \( p=0.108 \)). For the second pair-wise spatial comparison Panama is once more demonstrating a trend of influencing differences (Broward-Bocas del Toro) SST (Regression; \( p=0.101, r^2=0.03 \)), salinity (Regression; \( p=0.141, r^2=0.18 \)), and precipitation-based seasons (Tukey; \( p=0.071 \)). Although not significant, the \( p \)-values of Panama in combination with the South Florida locations are nearly equivalent to each other. Similar to the triple site analyses, the pair-wise tests demonstrated similarities in \( p \)-values whereby Panama is influencing OTU richness differences while the South Florida locations that are in close proximity to each other have higher \( p \)-values indicating less differences in OTU richness. Generally, OTU richness demonstrated a trend of decreased richness with increased temperature, increased richness with increased salinity, and decreased richness related to the wet season (increased precipitation and decreased salinity).
In the single site analyses of Broward County, the only parameter demonstrating significant OTU richness was SST (Regression; \( p=0.026, r^2=0.92 \)). In the location with a complete seasonal study the low \( p \)-value and high \( r^2 \) value indicated a strong significance of OTU change of richness dependent on temperature with a robust correlation.

OTU diversity is a type of Alpha diversity that relates to the combined interactions of the number of OTUs (richness) and relative population numbers in each OTU (evenness).

For OTU Alpha diversity there were marginally non-significant differences among the three sample locations when investigated simultaneously (ANOVA; \( p=0.080 \)), with higher diversity in South Florida than Panama. The Alpha diversity was marginally non-significant between locations for SST (Regression; \( p=0.059, r^2=0.22 \)), significant for salinity (Regression; \( p=0.041, r^2=0.27 \)), and significant for precipitation-based seasons (ANOVA; \( p=0.019 \)), with higher Alpha diversity in South Florida than Panama. The trend of OTU Alpha diversity again follows the spatial trend from the north-south gradient from highest to lowest, with Panama having less OTU Alpha diversity than the two South Florida locations who are in a closer proximity to each other.

In the pair-wise, one by one analyses of locations (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama), OTU Alpha diversity did not demonstrate significance for comparisons between Broward-Dade for location (ANOVA; \( p=0.999 \)), SST (Regression; \( p=0.552, r^2=0.09 \)), salinity (Regression \( p=0.427, r^2=0.04 \)), and precipitation-based seasons (ANOVA; \( p=0.469 \)). For the site pair Dade-Panama there were marginally non-significant differences for location (ANOVA; \( p=0.078 \)), SST (Regression;
p=0.162, r^2=0.15), salinity (Regression; p=0.088, r^2=0.27), and precipitation-based seasons (ANOVA; p=0.078). Yet there were significant differences for comparisons between Broward-Panama for location (ANOVA; p=0.030), SST (Regression; p=0.028, r^2=0.45), marginal non-significance for salinity (Regression; p=0.088, r^2=0.27), and significance for precipitation-based seasons (ANOVA; p=0.022). Similar to the triple site analyses, the pair-wise tests demonstrated similarities in p-values where OTU Alpha diversity differences on a spatial gradient, where South Florida locations in close proximity have higher p-values indicating less differences in OTU Alpha diversity. Generally, OTU Alpha diversity demonstrated a trend of decreased Alpha diversity between adjacent locations and increased Alpha diversity between locations with a larger geographical distance.

In the single site analyses of Broward county, the only parameter demonstrating significant OTU Alpha diversity was SST (Regression; p=0.007, r^2=0.98). In the location with a complete seasonal study the low p-value and high r^2 value indicated a strong significance of OTU change of Alpha diversity dependent on temperature with a robust correlation.

Beta diversity compares the Alpha diversity among sites, allowing for important comparisons of Alpha diversity among different site pairs. In general, the Panama *A. compressa* microbiome Beta diversity is distinct from the microbiome Beta diversity in sponges sampled from Broward and Dade Counties. This is consistent with the expectation that the greater geographic distance and indicate environmentally driven differences in parameters that can lead to greater differences in microbiome Beta diversity within the same species of the host sponge.
OTU Beta diversity for the three sample locations when investigated simultaneously demonstrates BCD and NMDS clustering of the South Florida samples distinct from the Panama samples, with the exception of one outlier from Dade clustering with the Bocas del Toro samples. For Beta diversity pair-wise ADONIS comparisons of parameters, there was marginal non-significance for location in relation to salinity (ADONIS; $p=0.081$), salinity in relation to SST (ADONIS; $p=0.069$), and salinity in relation to precipitation-based seasons (ADONIS; $p=0.059$). There is significance in differences for location in relation to SST (ADONIS; $p=0.040$), SST in relation to salinity (ADONIS; $p=0.003$), and SST in relation to precipitation-based seasons (ADONIS; $p=0.001$). This corresponds with trends in OTU richness and OTU Alpha diversity supporting that location, SST, salinity, and precipitation-based seasons were drivers of differences of the host sponge microbiome.

OTU Beta diversity for the pair-wise one by one analyses of locations (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama), demonstrated no clear clustering of the South Florida samples in BCD and NMDS analyses. Dade-Bocas del Toro demonstrated clustering on two of the three branches with no clear clustering on the third branch. Broward-Panama demonstrated clear clustering on two distinct branches.

OTU Beta diversity in the single site analyses of Broward County, demonstrates BCD and NMDS clustering associated to SST, with distinct branches of lower and higher temperatures.

*A. compressa* host sponge harbors two dominant Phyla of microbes, *Proteobacteria* and *Cyanobacteria*, which account for over 50% of the total microbiome community. This
is consistent with previous studies documenting these Phyla as the primary microbes of LMA sponges which could be sponge specific core microbes or potential symbionts. The *Proteobacteria* has the highest abundance in samples from Bocas del Toro at 58.2%, the second highest abundance in samples from Dade at 34.7%, and is least abundant in samples from Broward at 30.4%. Following a spatial south-north gradient, highest in Bocas del Toro to lowest in Broward County, the South Florida samples exhibit similar abundances that are both nearly double than in Panama. This *Proteobacteria* is solely found in marine sponges so can be theorized to be a sponge specific core microbe classified at the Phylum level.

The second highest microbe abundance is classified as a *Cyanobacteria*. This *Cyanobacteria* has the highest abundance in samples from Broward at 22.6%, the second highest abundance in samples from Dade at 16.9%, and is least abundant in samples from Bocas del Toro at 4.3%. Following a spatial north-south gradient, highest in Broward County to lowest in Bocas del Toro, the South Florida samples exhibit similar abundances, each as much as five times higher than in Panama. This is following a geographical spatial gradient, opposite than the *Proteobacteria*. This *Cyanobacteria* is solely found in marine environments. Being the most abundant Phyla found in marine environments it is theorized to not be sponge specific but a transient taxa found naturally in high abundances. The remaining microbial taxa rapidly drop in abundances and fall below a 1.0% threshold after the first eight.
9.0 CONCLUSIONS

Marine sponges harbor vast abundances of important ecological, biotechnological, and pharmaceutical microbes which can only be characterized with High-Throughput sequencing. Understanding of their microbiome is invaluable for scientific research to establish how marine microbiota mediate biogeochemical processes and what factors regulate shifts of the microbiome. This study has established two dominant microbial taxa from three sample locations on a large spatial geographical gradient from Southeast Florida, USA to the Southern Caribbean, Panama. The two dominant microbial taxa of the marine sponge *Amphimedon compressa* are identical, having been characterized to the Phylum level as a *Proteobacteria* and a *Cyanobacteria*, compromising approximately 53% of the total sponge microbiome.

Differences in the *A. compressa* microbiome richness and diversity are primarily driven by location, where the Panama samples are dissimilar from the South Florida samples, and the South Florida samples are nearly identical in composition. These results support the interpretation that Panama *A. compressa* microbiome richness and diversity is distinct from the microbiome richness and diversity in Southeast Florida.

This could possibly be attributed to not only distance and proximity but also to water flow. Panama is mostly restricted in water flow in a semi-enclosed lagoon, compared to the South Florida locations which are in open ocean water currents. Further studies could determine if this factor is indeed a vital driving force.

A trend of decreased richness and diversity is related to increased temperature and deceased salinity in relation to high precipitation. Although this study is supported by previous research, this study was limited in sample size and complete seasons. Future
studies could investigate with greater resolution the abiotic factors that determine possible mechanisms for variation in richness and diversity. Additional studies incorporating a larger number of samples and also incorporating samples taken at different time points in complete seasons would help determine how sponge microbiomes are affected by precipitation and at different salinity levels and temperatures.
10.0 APPENDICES

APPENDIX 1:
Summary table of collection dates and locations of South Florida USA sponge *A. compressa* samples used for DNA microbial extraction/isolation.

<table>
<thead>
<tr>
<th>Study Sample ID</th>
<th>Collection Date</th>
<th>Collection Location</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC N50: 3/1/2011</td>
<td>3-1-11</td>
<td>Broward County</td>
<td>26° 09.597’ N</td>
<td>080° 04.950’ W</td>
</tr>
<tr>
<td>BC N50: 5/10/2011</td>
<td>11-5-10</td>
<td>Broward County</td>
<td>26° 09.597’ N</td>
<td>080° 04.950’ W</td>
</tr>
<tr>
<td>BC N50: 9/1/2011</td>
<td>11-9-11</td>
<td>Broward County</td>
<td>26° 09.597’ N</td>
<td>080° 04.950’ W</td>
</tr>
<tr>
<td>BC N50: 11/10/2011</td>
<td>11-11</td>
<td>Broward County</td>
<td>26° 09.597’ N</td>
<td>080° 04.950’ W</td>
</tr>
<tr>
<td>DC N31: 3/17/2011</td>
<td>3-17-11</td>
<td>Dade County</td>
<td>25° 50.520’ N</td>
<td>080° 05.704’ W</td>
</tr>
<tr>
<td>DC N31: 5/9/2011</td>
<td>5-9-11</td>
<td>Dade County</td>
<td>25° 50.520’ N</td>
<td>080° 05.704’ W</td>
</tr>
<tr>
<td>DC N32: 12/6/2010</td>
<td>12-6-10</td>
<td>Dade County</td>
<td>25° 50.520’ N</td>
<td>080° 05.704’ W</td>
</tr>
<tr>
<td>DC N32: 5/9/2011</td>
<td>5-9-11</td>
<td>Dade County</td>
<td>25° 50.520’ N</td>
<td>080° 05.704’ W</td>
</tr>
</tbody>
</table>

**Triple Site Analyses**

APPENDIX 2:
R studio “picante” codes and ANOVA/Regression results for rarified OTU richness analyses. Parameters include: Collection Site, Collection Date, Temperature, Salinity, Calendar-Based Seasons, and Precipitation-Based Seasons.

```r
> rich.rar.aov <- aov(richness.rar ~ metadata$Collection_Site)
> summary(rich.rar.aov)

    Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Site  2 276075 138037   2.969 0.0972 .
Residuals                10 464930  46493
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> rich.rar.aov <- aov(richness.rar ~ metadata$Collection_Date)
> summary(rich.rar.aov)

    Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Date 11 739600  67236  47.87  0.112
Residuals                1   1404   1404
> rich.reg <- lm(richness.rar ~ metadata$Temp_C)
> summary(rich.reg)

Call:
  lm(formula = richness.rar ~ metadata$Temp_C)

Residuals:
   Min      1Q  Median      3Q     Max
-475.82  -78.82  101.95  147.03  242.18

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 2593.17     716.80   3.618  0.00404 **
metadata$Temp_C -52.19      26.87  -1.942  0.07818 .
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 227.3 on 11 degrees of freedom
Multiple R-squared:  0.2553, Adjusted R-squared:  0.1876
F-statistic: 3.771 on 1 and 11 DF,  p-value: 0.07818
```
> rich.reg<-lm(richness.rar~metadata$Salinity_ppt)
> summary(rich.reg)

Call:
  lm(formula = richness.rar ~ metadata$Salinity_ppt)

Residuals:
      Min       1Q   Median       3Q      Max
-396.40 -145.95    35.07   153.07   281.60

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)               -1550.93    1181.03  -1.313   0.2158
metadata$Salinity_ppt     80.17      34.48   2.325   0.0402 *

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 212.5 on 11 degrees of freedom
Multiple R-squared:  0.3296,     Adjusted R-squared:  0.2686
F-statistic: 5.407 on 1 and 11 DF,  p-value: 0.0402

> rich.rar.aov<-aov(richness.rar~metadata$Seasons)
> summary(rich.rar.aov)

Df  Sum Sq Mean Sq F value Pr(>F)
metadata$Seasons  3 310800 103600   2.167  0.162
Residuals        9 430205  47801

> rich.rar.aov<-aov(richness.rar~metadata$Season2)
> summary(rich.rar.aov)

Df  Sum Sq Mean Sq F value Pr(>F)
metadata$Season2  1 292757 292757   7.184 0.0214 *
Residuals        11 448248  40750

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> TukeyHSD(rich.rar.aov)

Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Collection_Site)

$‘metadata$Collection_Site’
              diff      lwr     upr   p adj
Broward.USA-Boca.Panama 297.65 -98.86152 694.1615 0.1488582
Dade.USA-Boca.Panama     301.40 -95.11152 697.9115 0.1430474
Dade.USA-Broward.USA     3.75  -414.20984 421.7098 0.9996666

> TukeyHSD(rich.rar.aov)

Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Seasons)

$‘metadata$Seasons’
              diff      lwr     upr   p adj
Spring-Fall -219.0000 -742.0615 504.0615 0.9306837
Summer-Fall -380.1667 -937.4498 177.1165 0.2149976
Winter-Fall  -98.5000 -781.0297 584.0297 0.9678621
Summer-Spring -261.1667 -743.7880 221.4547 0.3822136
Winter-Spring  20.5000  -602.5615 643.5615 0.9995816
Winter-Summer 281.6667  -275.6165 838.9498 0.4159631

APPENDIX 3:
R studio “picante” codes and results for Tukey HDS multiple comparisons of means of OTU richness per Collection Site, Calendar-Based Seasons, and Precipitation-Based Seasons.
APPENDIX 4:

R studio “picante” codes and results for Inverse Simpson/Regression for rarified OTU Alpha diversity analyses. Parameters include: Collection Site, Collection Date, Temperature, Salinity, Calendar-Based Seasons, and Precipitation-Based Seasons.

```r
> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
  95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Season2)
$ metadata$Season2
       diff  lwr   upr     p adj
Rain-Dry  -301.0238 -548.2115 -53.83612 0.0213949

> div.rar.aov<-aov(diversity.rar~metadata$Collection_Site)
> summary(div.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Site  2  29.36  14.679   3.264 0.0811 .
Residuals                10  44.98   4.498
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> div.rar.aov<-aov(diversity.rar~metadata$Collection_Date)
> summary(div.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Date 11  69.61   6.328   1.338  0.594
Residuals                 1   4.73   4.728

> div.reg<-lm(diversity.rar~metadata$Temp_C)
> summary(div.reg)
Call:
  lm(formula = diversity.rar ~ metadata$Temp_C)
Residuals:
   Min      1Q  Median      3Q     Max
-4.2795 -1.4301  0.7791  1.4260  3.1580
Coefficients:             Estimate Std. Error t value Pr(>|t|)
(Intercept)           20.1248     7.0064   2.872   0.0152 *
metadata$Temp_C    -0.5528     0.2627  -2.105   0.0591 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 2.222 on 11 degrees of freedom
Multiple R-squared:  0.2871, Adjusted R-squared:  0.2223
F-statistic: 4.429 on 1 and 11 DF,  p-value: 0.05912

> div.reg<-lm(diversity.rar~metadata$Salinity_ppt)
> summary(div.reg)
Call:
  lm(formula = diversity.rar ~ metadata$Salinity_ppt)
Residuals:
   Min      1Q  Median      3Q     Max
-3.0767 -1.7365 -0.7801  1.6660  3.5316
Coefficients:             Estimate Std. Error t value Pr(>|t|)
(Intercept)           -22.3446    11.9822  -1.865 0.0891 .
metadata$Salinity_ppt  0.8120     0.3498   2.321  0.0405 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 2.156 on 11 degrees of freedom
Multiple R-squared:  0.3288, Adjusted R-squared:  0.2678
F-statistic: 5.389 on 1 and 11 DF,  p-value: 0.04048

> div.rar.aov<-aov(diversity.rar~metadata$Seasons)
> summary(div.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Seasons  3  34.23  11.408  2.56   0.12
Residuals        9  40.11   4.457

> div.rar.aov<-aov(diversity.rar~metadata$Season2)
> summary(div.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Season2  1  30.43  30.427  7.622 0.0185 *
Residuals        11  43.91   3.992
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
  95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Season2)
$ metadata$Season2
       diff  lwr   upr     p adj
Rain-Dry  -301.0238 -548.2115 -53.83612 0.0213949
```
APPENDIX 5:
R Studio “picante” codes and results for Bray-Curtis dissimilarity OTU Beta diversity pair-wise comparisons of all variables explained by ADONIS. Parameters include: Collection Site, Collection Date, Temperature, Salinity, Calendar-Based Seasons, and Precipitation-Based Seasons.

```r
> print(adonis(comm.bc.dist ~ Collection_Site*Collection_Date, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Collection_Site * Collection_Date, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to Last)
  DF Sum of Sq Mean Sq F value  R^2     Pr(>F)
Collection_Site   2    0.72582 0.36291 6.0548 0.47971 0.011 *
Collection_Date   9    0.72730 0.08081 1.3482 0.48068 0.333
Residuals        12   1.51306           1.00000
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> print(adonis(comm.bc.dist ~ Collection_Site*Temp_C, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Collection_Site * Temp_C, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to Last)
  DF Sum of Sq Mean Sq  F value  R^2     Pr(>F)
Collection_Site   2    0.72582 0.36291 5.4052 0.47971 0.001 ***
Temp_C            1    0.10269 0.10269 1.5294 0.06787 0.194
Collection_Site:Temp_C 1    0.14742 0.14742 2.1957 0.09743 0.041 *
Residuals        8    0.53713 0.06714          0.35499
Total            12   1.51306           1.00000
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> print(adonis(comm.bc.dist ~ Collection_Site*Salinity_ppt, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Collection_Site * Salinity_ppt, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to Last)
  DF Sum of Sq Mean Sq  F value  R^2     Pr(>F)
Collection_Site   2    0.72582 0.36291 4.3695 0.47971 0.001 ***
Salinity_ppt     1    0.05098 0.05098 0.6139 0.03370 0.740
Collection_Site:Salinity_ppt 1    0.07180 0.07180 0.8645 0.04745 0.504
Residuals        8    0.66445 0.08306          0.43914
Total            12   1.51306           1.00000
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> print(adonis(comm.bc.dist ~ Collection_Site*Seasons, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Collection_Site * Seasons, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to Last)
  DF Sum of Sq Mean Sq  F value  R^2     Pr(>F)
Collection_Site   2    0.72582 0.36291 6.2133 0.47971 0.001 ***
Seasons           3    0.27528 0.09176 1.5710 0.18194 0.105
Collection_Site:Seasons 2    0.21991 0.10995 1.8825 0.14534 0.081 .
Residuals        5    0.29205 0.05841 0.19302
Total            12   1.51306           1.00000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```
```r
> print(adonis(comm.bc.dist ~ Collection_Site*Season2, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Collection_Site * Season2, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SumOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collection_Site</td>
<td>2</td>
<td>0.72582</td>
<td>0.36291</td>
<td>4.8240</td>
<td>0.47971</td>
<td>0.001 ***</td>
</tr>
<tr>
<td>Season2</td>
<td>1</td>
<td>0.11015</td>
<td>0.11015</td>
<td>1.4642</td>
<td>0.0172</td>
<td></td>
</tr>
<tr>
<td>Residuals</td>
<td>9</td>
<td>0.67708</td>
<td>0.07523</td>
<td></td>
<td>0.44749</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>0.00000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> print(adonis(comm.bc.dist ~ Salinity_ppt*Season2, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Salinity_ppt * Season2, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SumOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity_ppt</td>
<td>1</td>
<td>0.55172</td>
<td>0.55172</td>
<td>6.9564</td>
<td>0.36464</td>
<td>0.001 ***</td>
</tr>
<tr>
<td>Season2</td>
<td>1</td>
<td>0.16897</td>
<td>0.16897</td>
<td>2.1305</td>
<td>0.11168</td>
<td>0.046 *</td>
</tr>
<tr>
<td>Salinity_ppt:Season2</td>
<td>1</td>
<td>0.07856</td>
<td>0.07856</td>
<td>0.9905</td>
<td>0.05192</td>
<td>0.407</td>
</tr>
<tr>
<td>Residuals</td>
<td>9</td>
<td>0.71381</td>
<td>0.07931</td>
<td></td>
<td>0.47176</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>0.00000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> print(adonis(comm.bc.dist ~ Salinity_ppt*Temp_C, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Salinity_ppt * Temp_C, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SumOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity_ppt</td>
<td>1</td>
<td>0.55172</td>
<td>0.55172</td>
<td>7.1672</td>
<td>0.36464</td>
<td>0.001 ***</td>
</tr>
<tr>
<td>Temp_C</td>
<td>1</td>
<td>0.12092</td>
<td>0.12092</td>
<td>1.5708</td>
<td>0.07992</td>
<td>0.178</td>
</tr>
<tr>
<td>Salinity_ppt:Temp_C</td>
<td>1</td>
<td>0.14761</td>
<td>0.14761</td>
<td>1.9176</td>
<td>0.09756</td>
<td>0.469</td>
</tr>
<tr>
<td>Residuals</td>
<td>9</td>
<td>0.69281</td>
<td>0.07698</td>
<td></td>
<td>0.45789</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>0.00000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> print(adonis(comm.bc.dist ~ Salinity_ppt*Collection_Date, data = metadata))
Call:
adonis(formula = comm.bc.dist ~ Salinity_ppt * Collection_Date, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SumOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity_ppt</td>
<td>1</td>
<td>0.55172</td>
<td>0.55172</td>
<td>9.2049</td>
<td>0.36464</td>
<td>0.003 **</td>
</tr>
<tr>
<td>Collection_Date</td>
<td>10</td>
<td>0.90140</td>
<td>0.09014</td>
<td>1.5039</td>
<td>0.59575</td>
<td>0.296</td>
</tr>
<tr>
<td>Residuals</td>
<td>1</td>
<td>0.05994</td>
<td>0.05994</td>
<td></td>
<td>0.03961</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>0.00000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```
> print(adonis(comm.bc.dist ~ Salinity_ppt*Seasons, data = metadata))
Call: adonis(formula = comm.bc.dist ~ Salinity_ppt * Seasons, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th>DF</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity_ppt</td>
<td>1</td>
<td>0.55172</td>
<td>0.55172</td>
<td>9.4458</td>
<td>0.36464 ***</td>
</tr>
<tr>
<td>Seasons</td>
<td>3</td>
<td>0.36902</td>
<td>0.12301</td>
<td>2.1059</td>
<td>0.24389 *</td>
</tr>
<tr>
<td>Salinity_ppt:Seasons</td>
<td>3</td>
<td>0.30027</td>
<td>0.10009</td>
<td>1.7136</td>
<td>0.19845</td>
</tr>
<tr>
<td>Residuals</td>
<td>5</td>
<td>0.29205</td>
<td>0.05841</td>
<td>0.19302</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>1.00000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> print(adonis(comm.bc.dist ~ Temp_C*Collection_Date, data = metadata))
Call: adonis(formula = comm.bc.dist ~ Temp_C * Collection_Date, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th>DF</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp_C</td>
<td>1</td>
<td>0.21266</td>
<td>0.21266</td>
<td>3.5480</td>
<td>0.14055 .</td>
</tr>
<tr>
<td>Collection_Date</td>
<td>10</td>
<td>1.24046</td>
<td>0.124046</td>
<td>2.0696</td>
<td>0.81984</td>
</tr>
<tr>
<td>Residuals</td>
<td>1</td>
<td>0.05994</td>
<td>0.059938</td>
<td>0.01961</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>1.00000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> print(adonis(comm.bc.dist ~ Temp_C*Seasons, data = metadata))
Call: adonis(formula = comm.bc.dist ~ Temp_C * Seasons, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th>DF</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp_C</td>
<td>1</td>
<td>0.21266</td>
<td>0.21266</td>
<td>3.6409</td>
<td>0.14055 .</td>
</tr>
<tr>
<td>Seasons</td>
<td>3</td>
<td>0.52075</td>
<td>0.173582</td>
<td>2.9718</td>
<td>0.34417 **</td>
</tr>
<tr>
<td>Temp_C:Seasons</td>
<td>3</td>
<td>0.48761</td>
<td>0.162535</td>
<td>2.7827</td>
<td>0.32227 **</td>
</tr>
<tr>
<td>Residuals</td>
<td>5</td>
<td>0.29205</td>
<td>0.058409</td>
<td>0.19302</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>1.00000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> print(adonis(comm.bc.dist ~ Temp_C*Season2, data = metadata))
Call: adonis(formula = comm.bc.dist ~ Temp_C * Season2, data = metadata)
Permutation: free
Number of permutations: 999
Terms added sequentially (First to last)

<table>
<thead>
<tr>
<th>DF</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp_C</td>
<td>1</td>
<td>0.21266</td>
<td>0.21266</td>
<td>2.5915</td>
<td>0.14055 .</td>
</tr>
<tr>
<td>Season2</td>
<td>1</td>
<td>0.25923</td>
<td>0.25923</td>
<td>3.1590</td>
<td>0.17133 **</td>
</tr>
<tr>
<td>Temp_C:Season2</td>
<td>1</td>
<td>0.30263</td>
<td>0.30263</td>
<td>3.6879</td>
<td>0.20001 ***</td>
</tr>
<tr>
<td>Residuals</td>
<td>9</td>
<td>0.73854</td>
<td>0.08206</td>
<td>0.48811</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>1.51306</td>
<td>1.00000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
APPENDIX 6:
R Studio “picante” codes and results for Beta diversity Stressplot robust goodness of fit for downstream Beta diversity analyses.

```r
> comm.bc.mds <- metaMDS(comm, dist = "bray")
Square root transformation
Wisconsin double standardization
Run 0 stress 0.04516498
Run 1 stress 0.05400586
Run 2 stress 0.05364641
Run 3 stress 0.05659318
Run 4 stress 0.04516502
... procrustes: rmse 7.754662e-05 max resid 0.0001945433
*** Solution reached
```
**APPENDIX 7:**
R Studio “picante” codes and results for Non-metric Multidimensional Scaling (NMDS) ordination plot of distance matrices point and text plots used to identify samples for Beta diversity.
APPENDIX 8: Taxonomic classification summary table of the twenty most abundant *A. compressa* microbiomes from highest to lowest by percentages from Simper Similarities produced in R Studio “picante”.

<table>
<thead>
<tr>
<th>EMP OTU ID</th>
<th>TAXONOMY OF FIRST TWENTY OF ABUNDANCE: HIGHEST TO LOWEST</th>
<th>CONTRAST LOCATIONS</th>
<th>PERCENTAGE TOTAL OF DUAL LOCATIONS</th>
<th>PERCENTAGE FIRST LOCATION</th>
<th>PERCENTAGE SECOND LOCATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otu003905</td>
<td><strong>k__Bacteria(100);p__Proteobacteria(89);unclassified;unclassified;unclassified;unclassified</strong></td>
<td>Dade vs Broward</td>
<td>18.7</td>
<td>34.7</td>
<td>30.4</td>
</tr>
<tr>
<td>Otu014935</td>
<td><strong>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycidae(100);o__Synechococcales(100);f__Synechococcaceae(100);unclassified;unclassified;unclassified</strong></td>
<td>Dade vs Broward</td>
<td>12.8</td>
<td>16.9</td>
<td>22.6</td>
</tr>
<tr>
<td>Otu001669</td>
<td><strong>k__Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(100);o__Oceanospirillales(60);unclassified;unclassified;unclassified</strong></td>
<td>Dade vs Broward</td>
<td>3.5</td>
<td>2.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu003494</td>
<td><strong>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycidae(100);o__Synechococcales</strong></td>
<td>Dade vs Broward</td>
<td>2</td>
<td>2.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>OTU000275</td>
<td>k_Bacteria(100)</td>
<td>p_Planctomycetes(100)</td>
<td>c_Planctomycetales(100)</td>
<td>o_Pirellulales(99)</td>
<td>f_Pirellulaceae(99)</td>
</tr>
<tr>
<td>OTU000650</td>
<td>k_Bacteria(100)</td>
<td>p_Proteobacteria(100)</td>
<td>c_Gamma proteobacteria(100)</td>
<td>o_Oceanospirillales(80)</td>
<td>f_Endozoicomonaceae(78)</td>
</tr>
<tr>
<td>OTU000008</td>
<td>k_Bacteria(100)</td>
<td>p_Cyanobacteria(100)</td>
<td>c_Synechococcophyceae(100)</td>
<td>o_Synechococcales(100)</td>
<td>f_Synechococcaceae(100)</td>
</tr>
<tr>
<td>OTU005974</td>
<td>k_Bacteria(100)</td>
<td>p_Cyanobacteria(100)</td>
<td>c_Synechococcophyceae(100)</td>
<td>o_Synechococcales(100)</td>
<td>f_Synechococcaceae(100)</td>
</tr>
<tr>
<td>OTU000921</td>
<td>k_Bacteria(100)</td>
<td>p_Proteobacteria(100)</td>
<td>c_Gamma proteobacteria(74)</td>
<td>o_Cyanobacteria(100)</td>
<td>f_Synechococcophyceae(100)</td>
</tr>
<tr>
<td>OTU000053</td>
<td>k_Bacteria(100)</td>
<td>p_Cyanobacteria(100)</td>
<td>c_Synechococcophyceae(100)</td>
<td>o_Synechococcales(100)</td>
<td>f_Synechococcaceae(100)</td>
</tr>
<tr>
<td>OTU000399</td>
<td>k_Bacteria(100)</td>
<td>p_Cyanobacteria(100)</td>
<td>c_Synechococcophyceae(100)</td>
<td>o_Synechococcales(100)</td>
<td>f_Synechococcaceae(100)</td>
</tr>
<tr>
<td>OTU003548</td>
<td>k_Bacteria(100)</td>
<td>p_Proteobacteria(100)</td>
<td>c_Gamma proteobacteria(94)</td>
<td>o_Cyanobacteria(100)</td>
<td>f_Synechococcophyceae(100)</td>
</tr>
<tr>
<td>OTU000326</td>
<td>k_Bacteria(100)</td>
<td>p_Cyanobacteria(100)</td>
<td>c_Synechococcophyceae(100)</td>
<td>o_Synechococcales(100)</td>
<td>f_Synechococcaceae(100)</td>
</tr>
<tr>
<td>OTU</td>
<td>Taxonomy</td>
<td>Dade_vs_Broward</td>
<td>Dade_vs_Broward</td>
<td>Dade_vs_Broward</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------</td>
<td>----------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>OTU00381</td>
<td>k__Bacteria(100);p__Bacteroidetes(91);c__Flavobacteria(85);o__Flavobacteriales(85);f__Flavobacteriaceae(80);unclassified;unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU01421</td>
<td>k__Bacteria(99);p__Proteobacteria(89);unclassified;unclassified;unclassified;unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU00933</td>
<td>k__Bacteria(100);p__Bacteroidetes(100);c__Flavobacteria(88);o__Flavobacteriales(88);unclassified;unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU00334</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);unclassified;unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU01313</td>
<td>k__Bacteria(100);p__Proteobacteria(100);c__Alphaproteobacteria(100);o__Rickettsiales(100);f__Pelagibacteraceae(100);unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU00598</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);g__Prochlorococcus(100);unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU00839</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(98);o__Synechococcaceae(98);f__Prochlorococcus(79);unclassified;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU003905</td>
<td>k__Bacteria(100);p__Proteobacteria(89);unclassified;unclassified;unclassified;</td>
<td>26.6</td>
<td>34.7</td>
<td>58.2</td>
<td></td>
</tr>
<tr>
<td>OTU014935</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);unclassified;unclassified;</td>
<td>12.8</td>
<td>16.9</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>OTU001669</td>
<td>k__Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(100);o__Oceanospirillales(60);unclassified;unclassified;</td>
<td>2.8</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU</td>
<td>Taxonomy</td>
<td>Dade_vs_Panama</td>
<td>Pana_vs_Dade</td>
<td>Ratio</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------</td>
<td>--------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>OTU003494</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococccophycideae(100);o__Synechococccophycideae(100);f__Synechococccophycideae(100);g__Prochlorococcus(99);unclassified;</td>
<td>Dade_vs_Panama</td>
<td>2.6</td>
<td>2.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU000650</td>
<td>k__Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(100);o__Oceanospirillales(80);f__Endozoicomonaceae(78);unclassified;unclassified;unclassified;</td>
<td>Dade_vs_Panama</td>
<td>1.5</td>
<td>1.1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU00921</td>
<td>k__Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(74);unclassified;unclassified;unclassified;</td>
<td>Dade_vs_Panama</td>
<td>1</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU002499</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococccophycideae(100);o__Synechococccophycideae(100);f__Synechococccophycideae(100);g__Prochlorococcus(91);unclassified;</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU005974</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococccophycideae(100);o__Synechococccophycideae(100);f__Synechococccophycideae(100);g__Prochlorococcus(86);unclassified;</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU00933</td>
<td>k__Bacteria(100);p__Bacteroidetes(100);c__Flavobacteria(88);o__Flavobacterales(88);unclassified;unclassified;unclassified;</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU00598</td>
<td>k__Bacteria(100);p__Cyanobacteria(100);c__Synechococccophycideae(100);o__Synechococccophycideae(100);f__Synechococccophycideae(100);g__Prochlorococcus(100);unclassified;</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU00381</td>
<td>k__Bacteria(100);p__Bacteroidetes(91);c__Flavobacteria(85);o__Flavobacterales(85);f__Flavobacteriaceae(80);unclassified;unclassified;unclassified;</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU35636</td>
<td>k__Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(93);o__Oceanospirillales(68);unclassified;unclassified;</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU00217</td>
<td>k__Bacteria(100);p__Bacteroidetes(100);c__Flavobacteria(98);o__Flavobacterales(98);f__Cryomorphaceae(79);</td>
<td>Dade_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>OTU</td>
<td>Kingdom</td>
<td>Phylum</td>
<td>Class</td>
<td>Order</td>
<td>Family</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>--------</td>
</tr>
<tr>
<td>OTU00756</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU00148</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Oceanospirillales</td>
<td>Halomonadales</td>
</tr>
<tr>
<td>OTU00839</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Oceanospirillales</td>
<td>Halomonadales</td>
</tr>
<tr>
<td>OTU00195</td>
<td>Bacteria</td>
<td>Cyanobacteria</td>
<td>Synechococcales</td>
<td>Synechococcaceae</td>
<td>Prochlorococcus</td>
</tr>
<tr>
<td>OTU00326</td>
<td>Bacteria</td>
<td>Cyanobacteria</td>
<td>Synechococcales</td>
<td>Synechococcaceae</td>
<td>Prochlorococcus</td>
</tr>
<tr>
<td>OTU00348</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td>Gammaproteobacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU001421</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU001421</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU003905</td>
<td>Bacteria</td>
<td>Proteobacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU014935</td>
<td>Bacteria</td>
<td>Cyanobacteria</td>
<td>Synechococcales</td>
<td>Synechococcaceae</td>
<td></td>
</tr>
<tr>
<td>OTU003974</td>
<td>Bacteria</td>
<td>Cyanobacteria</td>
<td>Synechococcales</td>
<td>Synechococcaceae</td>
<td></td>
</tr>
<tr>
<td>OTU</td>
<td>Taxonomy</td>
<td>Broward vs_Panama</td>
<td>1</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-------------------</td>
<td>---</td>
<td>---</td>
<td>----</td>
</tr>
<tr>
<td>Otu003494</td>
<td>k_Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);g__Prochlorococcus(99);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>1.2</td>
<td>1.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu000275</td>
<td>k_Bacteria(100);p__Planctomycetes(100);c__Planctomycetia(100);o__Pirellulales(99);f__Pirellulaceae(99);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>1.2</td>
<td>1.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu000008</td>
<td>k_Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);g__Synechococcus(83);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>1</td>
<td>1.2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu002499</td>
<td>k_Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);g__Synechococcus(91);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu000053</td>
<td>k_Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);g__Synechococcus(91);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu000650</td>
<td>k_Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(100);o__Oceanospirillales(80);f__Endozoicomonaceae(78);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu000399</td>
<td>k_Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(100);o__Synechococcaceae(100);g__Synechococcus(85);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu00839</td>
<td>k_Bacteria(100);p__Cyanobacteria(100);c__Synechococcophycideae(98);o__Synechococcaceae(98);f__Synechococcaceae(98);g__Prochlorococcus(79);unclassified;</td>
<td>Broward_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Otu003548</td>
<td>k_Bacteria(100);p__Proteobacteria(100);c__Gammaproteobacteria(94);unclassified;unclassified;</td>
<td>Broward_vs_Panama</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>
Dual Site Analysis, Pairwise: (Dade County, Florida, USA-Broward County, Florida, USA; Dade County, Florida, USA-Bocas del Toro, Panama; Broward County, Florida, USA-Bocas del Toro, Panama).

APPENDIX 9: R studio “picante” codes and ANOVA/Regression results for rarified OTU richness analyses of pair-wise location analyses. Parameters include: Collection Site, Collection Date, Temperature, Salinity, Calendar-Based Seasons, and Precipitation-Based Seasons.
```r
> rich.reg <- lm(richness.rar ~ metadata$Temp_C)
> summary(rich.reg)
Call: lm(formula = richness.rar ~ metadata$Temp_C)
Residuals:
     Min      1Q  Median      3Q     Max
-363.24  -90.84  -2.95  175.91  228.43
Coefficients:
                         Estimate Std. Error t value Pr(>|t|)
(Intercept)               3045.41     867.38   3.511 0.01279 *
metadata$Temp_C          -37.24      34.06  -1.093 0.3161
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
Residual standard error: 218.9 on 6 degrees of freedom
Multiple R-squared:  0.1662, Adjusted R-squared:  0.02719
F-statistic: 1.19 on 1 and 6 DF,  p-value: 0.3161

> rich.reg <- lm(richness.rar ~ metadata$Salinity_ppt)
> summary(rich.reg)
Call: lm(formula = richness.rar ~ metadata$Salinity_ppt)
Residuals:
     Min      1Q  Median      3Q     Max
-286.85  -160.83  -12.34  219.85  246.03
Coefficients:
                         Estimate Std. Error t value Pr(>|t|)
(Intercept)            -4974.13    6492.17  -0.766 0.4730
metadata$Salinity_ppt  -80.86     182.68  -0.443 0.6742
Residual standard error: 235.9 on 6 degrees of freedom
Multiple R-squared:  0.03162, Adjusted R-squared:  -0.1298
F-statistic: 0.1959 on 1 and 6 DF,  p-value: 0.6735

> rich.rar.aov <- aov(richness.rar ~ metadata$Seasons)
> summary(rich.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Seasons     3 162982   54327 1.1950 0.4182
Residuals           4 181812   45453

> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Seasons)
$metadata$Seasons
diff   lwr     upr   p adj
Spring-Fall 285.833  -1078.108  506.775 0.5274
Summer-Fall 450.500  -1513.448  654.448 0.4179
Winter-Fall 225.000  -1092.894  652.894 0.7310
Summer-Spring 164.666  -1166.821  1556.153 0.9038
Winter-Spring  60.833  -731.442  853.108 0.9880
Winter-Summer 225.500  -837.448  1288.448 0.8236

> rich.rar.aov <- aov(richness.rar ~ metadata$Season2)
> summary(rich.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Season2  1  60852  60852 1.2867 0.3000
Residuals         6 283941  47324

> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Season2)
$metadata$Season2
diff   lwr     upr   p adj
Rain-Dry 263.714 -832.768  1360.197 0.3001

> rich.rar.aov <- aov(richness.rar ~ metadata$Collection_Site)
> summary(rich.rar.aov)
Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Site  1 188439 188439 3.4041 0.1076
Residuals                 7 387519  55360

> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Collection_Site)
$metadata$Collection_Site
diff   lwr     upr   p adj
Dade.USA-Boca.Panama 291.200 -82.021  664.421 0.1076

> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Collection_Site)
$metadata$Collection_Site
diff   lwr     upr   p adj
Dade.USA-Boca.Panama 291.200 -82.021  664.421 0.1076
```

113
> rich.rar.aov <- aov(richness.rar ~ metadata$Collection_Date)
> summary(rich.rar.aov)

    Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Date  6 321896   53649   2.343 0.462
Residuals                 1  22898 22898

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 '.' 0.1 ' ' 1

> TukeyHSD(rich.rar.aov)

Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = richness.rar ~ metadata$Collection_Date)

$metadata$Collection_Date

       diff lwr upr p adj
Dade.USA - Broward.USA  41 8 371.7428 0.8160514

> rich.reg <- lm(richness.rar ~ metadata$Temp_C)
> summary(rich.reg)

Call: lm(formula = richness.rar ~ metadata$Temp_C)
Residuals:
     Min      1Q  Median      3Q     Max
-388.20  -179.39    28.45  158.47  302.80
Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  2660.14     947.79   2.807   0.0263 *
metadata$Temp.C   57.23      35.65   1.605   0.1524

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 '.' 0.1 ' ' 1

Residual standard error: 245.2 on 7 degrees of freedom
Multiple R-squared:  0.2691,   Adjusted R-squared:  0.1647
F-statistic: 2.577 on 1 and 7 DF,  p-value: 0.1524

> rich.reg <- lm(richness.rar ~ metadata$Salinity_ppt)
> summary(rich.reg)

Call: lm(formula = richness.rar ~ metadata$Salinity_ppt)
Residuals:
     Min      1Q  Median      3Q     Max
-371.10  -116.10    5.90   54.77  319.90
Coefficients:
              Estimate Std. Error t value Pr(>|t|)
(Intercept)   -1796.69    1526.08  -1.177   0.278
metadata$Salinity.ppt  87.50      45.35   1.930   0.095

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 '.' 0.1 ' ' 1

Residual standard error: 231.8 on 7 degrees of freedom
Multiple R-squared:  0.3472,   Adjusted R-squared:  0.254
F-statistic: 3.723 on 1 and 7 DF,  p-value: 0.09498
### Analysis of Richness by Season

```r
> rich.rar.aov <- aov(richness.rar ~ metadata$Seasons)
> summary(rich.rar.aov)

Df  Sum Sq  Mean Sq  F value  Pr(>F)
metadata$Seasons  3  217639  72546 1.012 0.46
Residuals        5   358319  71664

> TukeyHSD(rich.rar.aov)

Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Seasons)

metadata$Seasons
                                 diff     lwr     upr     p adj
Spring-Fall                      -100.0 -1309.7943 1109.7943 0.9889864
Summer-Fall                     -401.2 -1483.2729  680.8729 0.5658499
Winter-Fall                     -240.0 -1636.9501  1156.9501 0.9167488
Summer-Spring                   -301.2 -1127.6469  525.2469 0.5774197
Winter-Spring                   -140.0 -1349.7943 1069.7943 0.9712924
Winter-Summer                   161.2 -920.8729 1243.2729 0.9427157
```

### Analysis of Richness by Collection Site

```r
> rich.rar.aov <- aov(richness.rar ~ metadata$Collection_Site)
> summary(rich.rar.aov)

Df  Sum Sq  Mean Sq  F value  Pr(>F)
metadata$Collection_Site        1  223238  223238 3.531 0.102
Residuals                      7  442544  63221

> TukeyHSD(rich.rar.aov)

Tukey multiple comparisons of means
95% family-wise confidence level
Fit: aov(formula = richness.rar ~ metadata$Collection_Site)

metadata$Collection_Site
                              diff     lwr     upr     p adj
Broward.USA-Boca.Panama        316.95 -81.88878 715.7888 0.1022974
```

### Analysis of Richness by Collection Date

```r
> rich.rar.aov <- aov(richness.rar ~ metadata$Collection_Date)
> summary(rich.rar.aov)

Df  Sum Sq  Mean Sq
metadata$Collection_Date       8  665782  83223
```
> rich.reg<-lm(richness.rar~metadata$Temp_C)
> summary(rich.reg)
Call:
  lm(formula = richness.rar ~ metadata$Temp_C)
Residuals:
     Min       1Q   Median       3Q      Max
-448.29  -75.29    90.54   159.66   242.71
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 3521.59     1255.51 2.805   0.0263 *
metadata$Temp_C   -85.34      45.19  -1.889  0.1009
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 251 on 7 degrees of freedom
Multiple R-squared:  0.3376,    Adjusted R-squared:  0.2429
F-statistic: 3.567 on 1 and 7 DF,  p-value: 0.1009
> rich.reg<-lm(richness.rar~metadata$Salinity_ppt)
> summary(rich.reg)
Call:
  lm(formula = richness.rar ~ metadata$Salinity_ppt)
Residuals:
     Min       1Q   Median       3Q      Max
-388.22  -161.40    -15.22   200.16   302.78
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  -1598.87     1663.03  -0.961    0.368
metadata$Salinity_ppt    81.87      49.36   1.659    0.141
Residual standard error: 261.3 on 7 degrees of freedom
Multiple R-squared:  0.2821,    Adjusted R-squared:  0.1796
F-statistic: 2.751 on 1 and 7 DF,  p-value: 0.1412
> rich.rar.aov<-aov(richness.rar~metadata$Seasons)
> summary(rich.rar.aov)
             Df Sum Sq Mean Sq F value Pr(>F)
metadata$Seasons  3 295463  98488  1.33  0.363
Residuals         5 370319  74064
> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = richness.rar ~ metadata$Seasons)
$ metadata$Seasons
            diff      lwr     upr     p adj
Spring-Fall -226.0000 -1646.150 1194.150 0.9317756
Summer-Fall -434.6667 -1519.324  649.991 0.5109515
Winter-Fall   3.0000  -1415.150 1425.150 0.9999991
Summer-Spring -208.6667 -1293.324    875.991 0.8892134
Winter-Spring 231.0000  -1189.150 1651.150 0.9277514
Winter-Summer 439.6667  -644.991 1524.324 0.5028020

116
> rich.rar.aov<-aov(richness.rar~metadata$Season2)
> summary(rich.rar.aov)

                 Df Sum Sq Mean Sq  F value  Pr(>F)
metadata$Season2  1 260642 260642 4.503 0.0715 .
Residuals        7 405140  57877
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> TukeyHSD(rich.rar.aov)
Tukey multiple comparisons of means
95% family-wise confidence level

    Fit: aov(formula = richness.rar ~ metadata$Season2)

$metadata$Season2

 diff  lwr  upr    p adj
Rain-Dry -361 -763.2543 41.25429 0.071498

APPENDIX 10:
R studio “picante” codes and results for Inverse Simpson/Regression for rarified OTU Alpha diversity analyses of pair-wise location analyses. Parameters include: Collection Site, Collection Date, Temperature, Salinity, Calendar-Based Seasons, and Precipitation-Based Seasons.

> diversity.rar<-diversity(rar.comm, index="invsimpson")
> div.rar.aov<-aov(diversity.rar~metadata$Collection_Site)
> summary(div.rar.aov)

                 Df Sum Sq Mean Sq  F value Pr(>F)
metadata$Collection_Site 1 0.0   0.0 0.000 0 0.999
Residuals               6 28.230 4.705

> div.rar.aov<-aov(diversity.rar~metadata$Collection_Date)
> summary(div.rar.aov)

                 Df Sum Sq Mean Sq F value Pr(>F)
metadata$Collection_Date 6 28.230 4.705 1.007 0.643
Residuals               1  4.673 4.673

> div.reg<-lm(diversity.rar~metadata$Temp_C)
> summary(div.reg)

Call: lm(formula = diversity.rar ~ metadata$Temp_C)
Residuals:
     Min       1Q    Median       3Q      Max
-3.99033 -0.64668 -0.19485  1.15550  3.26033
Coefficients:
              Estimate Std. Error t value Pr(>|t|)
(Intercept)   12.27930   8.98743  1.3667   0.2206
metadata$Temp_C -0.22214   0.35287 -0.6289   0.5519

Residual standard error: 2.268 on 6 degrees of freedom
Multiple R-squared:  0.06191, Adjusted R-squared: -0.0944
F-statistic: 0.396 on 1 and 6 DF,  p-value: 0.5524

> div.reg<-lm(diversity.rar~metadata$Salinity_ppt)
> summary(div.reg)

Call: lm(formula = diversity.rar ~ metadata$Salinity_ppt)
Residuals:
     Min       1Q    Median       3Q      Max
-3.76543 -0.38214  0.21759  0.87444  3.15214
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  58.46882   60.87704  0.9607   0.3704
metadata$Salinity_ppt -1.45855   1.71344 -0.8512   0.4273

Residual standard error: 2.213 on 6 degrees of freedom
Multiple R-squared:  0.1078, Adjusted R-squared: -0.04994
F-statistic: 0.7247 on 1 and 6 DF,  p-value: 0.4273

> div.rar.aov<-aov(diversity.rar~metadata$Seasons)
> summary(div.rar.aov)

                 Df Sum Sq Mean Sq F value Pr(>F)
metadata$Seasons 3  6.608  2.203  0.597  0.469
Residuals        4 26.296  6.574

> div.rar.aov<-aov(diversity.rar~metadata$Season2)
> summary(div.rar.aov)

                 Df Sum Sq Mean Sq  F value Pr(>F)
metadata$Season2 1  2.978  2.978  0.597  0.469
Residuals       6 29.926  4.988
```r
> diversity.rar <- diversity(rar.com, index="invsimpson")
> div.rar.aov <- aov(diversity.rar ~ metadata$Collection_Site)
> summary(div.rar.aov)

    Df  Sum Sq Mean Sq F value  Pr(>F)
metadata$Collection_Site 1   21.51  21.509   4.257 0.078 .
Residuals                7   35.36   5.052
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> div.rar.aov <- aov(diversity.rar ~ metadata$Collection_Date)
> summary(div.rar.aov)

    Df  Sum Sq Mean Sq F value  Pr(>F)
metadata$Collection_Date 7    53.05  7.578   1.982 0.500
Residuals                1    3.83  3.827

> div.reg <- lm(diversity.rar ~ metadata$Temp_C)
> summary(div.reg)

Call:
  lm(formula = diversity.rar ~ metadata$Temp_C)
Residuals:
          Min       1Q   Median       3Q      Max
-3.6586 -1.7415  0.1696  1.3835  3.7853
Coefficients:                       Estimate Std. Error t value Pr(>|t|)
(Intercept)                19.60031    9.48806   2.066   0.0777 .
metadata$Temp_C           -0.55714     0.35675  -1.561   0.1625
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 2.455 on 7 degrees of freedom
Multiple R-squared:  0.2582 ,  Adjusted R-squared:  0.1523
F-statistic: 2.437 on 1 and 7 DF,  p-value: 0.1625

> div.reg <- lm(diversity.rar ~ metadata$Salinity_ppt)
> summary(div.reg)

Call:
  lm(formula = diversity.rar ~ metadata$Salinity_ppt)
Residuals:
          Min       1Q   Median       3Q      Max
-3.0996 -1.5240 -0.5844  1.6010  3.5087
Coefficients:                       Estimate Std. Error t value Pr(>|t|)
(Intercept)                -24.91820    15.01421  -1.660   0.1409
metadata$Salinity_ppt     -0.88553     0.44607  -1.985   0.0876 .
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 2.28 on 7 degrees of freedom
Multiple R-squared:  0.3601 ,  Adjusted R-squared:  0.2687
F-statistic: 3.939 on 1 and 7 DF,  p-value: 0.08756

> div.rar.aov <- aov(diversity.rar ~ metadata$Seasons)
> summary(div.rar.aov)

    Df  Sum Sq Mean Sq F value  Pr(>F)
metadata$Seasons             3  39.993  13.331   3.947 0.0868 .
Residuals                5  16.889   3.378
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> div.rar.aov <- aov(diversity.rar ~ metadata$Season2)
> summary(div.rar.aov)

    Df  Sum Sq Mean Sq F value  Pr(>F)
metadata$Season2             1  21.511  21.511   4.257 0.078 .
Residuals                7  35.361   5.052
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

---

118
> diversity.rar <- diversity(rar.comm, index="invsimpson")
> div.rar.aov <- aov(diversity.rar ~ metadata$Collection_Site)
> summary(div.rar.aov)

    Df Sum Sq Mean Sq F value  Pr(>F)
metadata$Collection_Site  1  22.96  22.958   7.396 0.0298 *
Residuals                 7  21.73   3.104
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> div.rar.aov <- aov(diversity.rar ~ metadata$Collection_Date)
> summary(div.rar.aov)

             Df Sum Sq Mean Sq
metadata$Collection_Date  8 44.69   5.586

> div.reg <- lm(diversity.rar ~ metadata$Temp_C)
> summary(div.reg)

Call: lm(formula = diversity.rar ~ metadata$Temp_C)

Residuals:
  Min      1Q  Median      3Q     Max
-2.5433 -1.2922  0.7583  1.1286  1.9204

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   28.9549    8.7528   3.308   0.0130 *
metadata$Temp_C -0.8681     0.3150  -2.756   0.0283 *

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.75 on 7 degrees of freedom
Multiple R-squared:  0.5203,   Adjusted R-squared:  0.4518
F-statistic: 7.593 on 1 and 7 DF,  p-value: 0.02828

> div.reg <- lm(diversity.rar ~ metadata$Salinity_ppt)
> summary(div.reg)

Call: lm(formula = diversity.rar ~ metadata$Salinity_ppt)

Residuals:
  Min      1Q  Median      3Q     Max
-1.9604 -1.6489 -0.7093  1.6861  2.2654

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) -22.5165    12.2879  -1.832   0.1096
metadata$Salinity_ppt   0.8146     0.3064   2.647   0.0607 .

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.931 on 7 degrees of freedom
Multiple R-squared:  0.4161,   Adjusted R-squared:  0.3327
F-statistic: 4.988 on 1 and 7 DF,  p-value: 0.06067

> div.rar.aov <- aov(diversity.rar ~ metadata$Seasons)
> summary(div.rar.aov)

          Df Sum Sq Mean Sq F value  Pr(>F)
metadata$Seasons  3 29.640  9.879   3.282  0.117
Residuals         5 15.051  3.010

> div.rar.aov <- aov(diversity.rar ~ metadata$Season2)
> summary(div.rar.aov)

          Df Sum Sq Mean Sq F value  Pr(>F)
metadata$Season2  1 24.680 24.680   8.637 0.0218 *
Residuals         7 20.000  2.858
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
**APPENDIX 11:**
R studio “picante” codes and Regression result for rarified OTU richness analysis of Broward single site location of temperature.

```r
> rich.reg <- lm(richness.rar ~ metadata$Temp_C)
> summary(rich.reg)
Call:
  lm(formula = richness.rar ~ metadata$Temp_C)
Residuals:
  65.70        -31.64         -82.70         48.64
Coefficients:             Estimate  Std. Error   t value  Pr(>|t|)
(Intercept)           5093.11      466.77     10.911     0.00829 **
metadata$Temp_C      -103.82       17.38      -5.975     0.02689 *
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 85.22 on 2 degrees of freedom
Multiple R-squared:  0.9469,     Adjusted R-squared:  0.9204
F-statistic: 35.7 on 1 and 2 DF,  p-value: 0.02689
```

**APPENDIX 12:**
R studio “picante” codes and Regression result for rarified OTU Alpha diversity analysis of Broward single site location of temperature.

```r
> div.reg <- lm(diversity.rar ~ metadata$Temp_C)
> summary(div.reg)
Call:
  lm(formula = diversity.rar ~ metadata$Temp_C)
Residuals:
  0.15415       -0.06054       -0.25369       0.16009
Coefficients:             Estimate  Std. Error   t value  Pr(>|t|)
(Intercept)             22.35104     1.32718    16.840     0.00351 **
metadata$Temp_C         -0.58603     0.04941     -11.860     0.00703 **
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Residual standard error: 0.2423 on 2 degrees of freedom
Multiple R-squared:  0.9869,     Adjusted R-squared:  0.979
F-statistic: 140.7 on 1 and 2 DF,  p-value: 0.007033
```
11.0 SUPPLIMENTARIES

SUPPLEMENTARY 1: *A. compressa* sponge spicule images used to verify taxonomy of samples used for study, characterized as oxea diactinal monaxial as seen 1000x magnification with compound microscope. (IMAGES 1-8).

**IMAGE 1:** BCN50: 3/1/2011. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.

**IMAGE 2:** BCN50: 5/10/2011. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.
**IMAGE 3:** BCN50: 9/1/2011. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.

**IMAGE 4:** BCN50: 9/10/2011. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.
**IMAGE 5:** DCN31: 3/17/2011. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.

**IMAGE 7:** DCN32: 12/6/2010. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.

**IMAGE 8:** DCN32: 5/9/2011. Individual siliceous spicules in *A. compressa*, 1000x magnification with compound microscope.
SUPPLEMENTARY 2: R studio “picante” results for Simper Similarities percentages, pair-wise comparisons of the three collection locations of host sponge *A. compressa* microbiomes identified by OTUs (attached CD).


Frade, P. R., K. Roll, K. Bergauer, G.J. Herndl. (2016). “Archael and Bacterial Communities Associated with the Surface Mucus of Caribbean Corals Differ in Their Degree of Host Specificity and Community Turnover Over Reefs”. PLoS One 11(1)


Inside R Forum: http://www.inside-r.org/packages/cran/vegan/docs/fisher.alpha


Sipkema, D. L. de Laeger. Unpublished data. NCBI.


Weigel, B. L. and P. M. Erwin. (2015). “Intraspecific Variation in Microbial Symbiont Communities of the Sun Sponge, Hymeniacidon heliophila, from Intertidal and Subtidal Habitats”. Applied and Environmental Microbiology 86(6).


