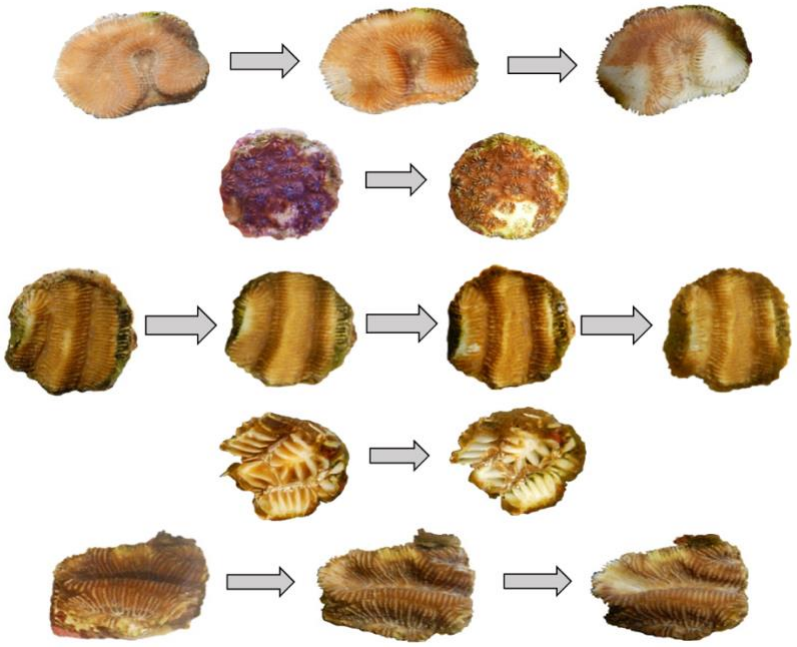
The role of algal symbionts (genus *Breviolum*) in the susceptibility of corals to Stony Coral Tissue Loss Disease in South Florida



Florida Department of Environmental Protection
Office of Resilience and Coastal Protection

The role of algal symbionts (genus *Breviolum*) in the susceptibility of corals to Stony Coral Tissue Loss Disease in South Florida

Final Report

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June 2021

Completed in Fulfillment of PO B65046 for

Florida Department of Environmental Protection
Office of Resilience and Coastal Protection
1277 N.E. 79th Street Causeway
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This report should be cited as follows:
Dennison CE, Karp RF, Weiler B, Goncalves A, del Campo J, Rosales S, Traylor-Knowles N, Baker AC. 2021. The role of algal symbionts (genus *Breviolum*) in the susceptibility of corals to Stony Coral Tissue Loss Disease in South Florida. Florida DEP. Miami FL. 1-23.

This report was prepared for the Florida Department of Environmental Protection, Office of Resilience and Coastal Protection by University of Miami. Funding was provided by the Florida Department of Environmental Protection Award No. B65046. The views, statements, findings, conclusions and recommendations expressed herein are those of the authors and do not necessarily reflect the views of the State of Florida or any of its sub-agencies.

Executive Summary

Since late summer 2014, Florida's Coral Reefs have experienced an unprecedented tissue loss disease known as stony coral tissue loss disease (SCTLD). Although it is still unknown why certain coral species are more susceptible to SCTLD than other as well as why some corals of the same species show differential susceptibility to this disease, several early observations correlate the associated symbiotic algae with the relative susceptibility of corals species (and individuals) to SCTLD. In this report we describe the physiological and molecular outcomes of a multifaceted experiment investigating the role of algal symbionts in five different Caribbean coral species whose susceptibility to SCTLD varies (*Colpophyllia natans, Diploria labyrinthiformis, Meandrina meandrites, Oribicella faveolata,* and *Psuedodiploria strigosa*). Through experimental bleaching and recovery, we manipulated a subset of cores from each colony away from their native symbionts towards *Durusdinium* and subsequently exposed cores containing *Breviolum* (and in some cases *Cladocopium*) or *Durusdinium* to SCTLD. Tissue biopsies were taken throughout the algal manipulations and disease exposure to monitor changes in coral-algal symbiosis and a subset of samples taken throughout the disease exposures were selected for downstream transcriptomics and microbial dynamics analyses (16S and 18S) Generally speak, we found that 87% of cores containing *Breviolum* presented with SCTLD-like lesions between 10 and 50 days after disease exposure, compared to 36% of unmanipulated *Cladocopium* cores (only found in *O. faveolata*) and only 19% of cores manipulated to contain *Durusdinium*. These results, suggest that in addition to a species-oriented disease susceptibility hierarchy, there is a hierarchy among different Symbiodiniaceae in their susceptibility to SCTLD, which we tentatively rank as *Breviolum* >> *Cladocopium* > *Durusdinium* >> *Symbiodinium*. Further analyses of host gene expression, symbiont gene expression, 16S microbial dynamics, 18S microbial dynamics, and histology associated with this project is still ongoing.

Acknowledgements

This project would not have been possible without the support from the Florida Department of Environmental Protection's Office of Resilience and Coastal Protection and the National Science Foundation. The Florida Keys National Marine Sanctuary (FKNMS) and the Florida Fish and Wildlife Conservation Commission (FWC) who permitted the collection of corals. The Coral Rescue Team at the Fish and Wildlife Institute for use of collection materials in particular Allan Anderson and Nathan Berkebile who helped with coral collections. Additionally, Katy Cummings (FDEP), Dr. Nicholas Parr (FDEP), Airielle Cathers (NOAA), Lonny Anderson (NOAA), Olivia Carmack (Smithsonian), Kelly Pitts (Smithsonian) and the M/V Makai, in particular Captain Joe Bailey and Captain Carl Leer, for assisting in coral collections. Alexandra Wen for countless hours assisting in the lab. All of whom helped advised on SCTLD transmissions, particularly Dr. Val Paul (Smithsonian) and Dr. Erinn Muller (Mote) and those who helped facilitate the collection of diseased colonies in particular Dr. Karen Neely and Dr. Andrew Bruckner. Finally, we would like to acknowledge the help of Victoria Barker, Kristi Kerrigan, Joanna Walczak, and Maurizio Martinelli for their assistance throughout this project.

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List of Acronyms

SCTLD: stony coral tissue loss disease
FRT: Florida Reef Tract
CNAT: *Colpophyllia natans*
DLAB: *Diploria labyrinthiformis*
MMEA: *Meandrina meandrites*
OFAV: *Oribicella faveolata*
PSTR: *Psuedodiploria strigosa*
NMDS: Non-metric Multi-dimensional Scaling
CoxPh: Cox Proportional Hazards
RR: Relative Risk
GLMM: Generalized Linear Mixed Model
PCA: Principal Component Analysis

1. BACKGROUND

1.1. Overview

Since the initial reports in 2014, stony coral tissue loss disease (SCTLD) has overwhelmed Florida's Coral Reef, formerly the Florida Reef Tract (FRT). Unlike other coral disease, SCTLD is unique in that no other coral disease is as persistent or widespread as SCTLD. This disease affects at least 21 scleractinian coral species, just under half of the reef-building species found in Florida. Additionally, both Endangered Species-Act listed, and the primary reef builders have shown the tell-tale SCTLD lesions that result in whole colony mortality. First reported off Virginia Key, Miami, FL, the disease is endemic to all of Florida's Coral Reef including the Dry Tortugas which until late May 2021 remained the only portion of Florida reefs untouched by this disease (Florida Coral Disease Response Research & Epidemiology Team, 2018; National Park Service, 2021). SCTLD has also been reported in the USVI, BVI, Mexican-Caribbean, The Bahamas, and some reefs in the Caribbean (Kramer et al., 2019). Although the pathogen(s) responsible for this unprecedented disease outbreak have remained elusive to scientists, early observations of this disease highlight several key areas of interest – in particular the small, symbiotic zooxanthellae that reside in the coral tissue.

Tissue pathology of SCTLD note that the first tissue layer affected is the gastrodermis, this is the cell layer that houses the algal symbionts. The cellular breakdown continues outwards until it presents as a surface lesion, this being the first visual sign of disease (Landsberg et al., 2020). Another key observation is the species susceptibility hierarchy which emphasizes that not all species are equally susceptible to SCTLD. The three stony coral species that are typically first affected by SCTLD (*Meandrina meandrites, Dichocoenia stokesii, and Colpophyllia natans*) all exclusively host *Breviolum*, with only very rare colonies of some species (e.g., *C. natans*) occasionally hosting different symbionts, usually in deep water. Moreover, all 8 of the stony coral species that have been classified as "highly susceptible" (*D. stokesii, M. meandrites, C. natans, Dendrogyra cylindrus, Diploria labyrinthiformis, Eusmilia fastigiata, Pseudodiploria clivosa,* and *P. strigosa*) also typically only host *Breviolum*, with only *Pseudodiploria* and *Diploria* sometimes hosting different symbionts (e.g., *Cladocopium* in deeper water). Additionally, most of the "intermediate susceptibility" species, such as *Orbicella annularis, O. faveolata, O. franks*i, and *Solenastrea bournon*i, also commonly host *Breviolum* in Florida, however they are also known to associate with a combination of other symbionts and modulate their symbiont communities in response to their environment and conditions. Moreover, almost all coral species that have low or no susceptibility to SCTLD (*Porites astreoides, P. porites, P. divaricata, P. furcata, Acropora palmata, A. cervicornis*) never usually host *Breviolum*. Exceptions to this pattern do occur, however: three coral species that are classified as "intermediate susceptibility" (*Montastraea cavernosa, Stephanocoenia intersepta*, and *Siderastrea siderea*) do not typically host *Breviolum*, and two taxa that are not susceptible to SCTLD (*Oculina spp.,* and *Cladocora arbuscul*a) do host *Breviolum* (although there is the possibility that these may be different *Breviolum* than those found in the other species). Regardless, while the pattern is not perfect, there is a high correlation between coral species tendency to host *Breviolum* and their susceptibility to SCTLD.

Given this correlative evidence, the role of algal symbionts in the susceptibility of corals to SCTLD remains a priority for ongoing disease research. Through algal manipulation and SCTLD trials, we will have a better understanding of this disease. In particular, whether hosting symbionts (genus *Breviolum*) increases the susceptibility of corals to SCTLD in comparison to *Durusdinium*. Additionally, the biological samples taken before and during disease trials will assist in a more comprehensive grasp of holobiont responses to SCTLD. A better understanding of the role of algal symbionts in SCTLD susceptibility will improve our understanding of this disease to further direct research efforts.

1.2. Project Goals

This project tests the hypothesis that hosting algal symbionts in the genus *Breviolum*, either exclusively or predominantly, plays a role in determining why some coral species (and individuals) are more susceptible to stony coral tissue loss disease (SCTLD) than others. This is the first study to directly test in a controlled way whether different algal symbionts affect the susceptibility of corals to SCTLD. We tested this hypothesis by manipulating algal symbionts of five coral species (*Colpophyllia natans, Diploria labyrinthiformis, Meandrina meandrites, Oribicella faveolata,* and *Psuedodiploria strigosa*) through controlled bleaching and recovery in favor of *Durusdinium*. We then tested their susceptibility to SCTLD and compared it to unmanipulated controls containing native symbionts (typically *Breviolum* and *Cladocopium*). We also hypothesized that bleached corals might be less susceptible to SCTLD if they have fewer *Breviolum* symbionts and tested this by exposing a subset of bleached cores to SCTLD to investigate whether reductions in *Breviolum* density affect susceptibility (Fig. 1). Additionally, in order to extract as much information as possible from this unique experiment, we also used a variety of techniques to study the potential holobiomic and mechanistic responses underlying observed differences in susceptibility, including ITS-2 typing and qPCR and to identify and quantify different Symbiodiniaceae, 16S and 18S sequencing to study microbial dynamics, and TagSeq to investigate gene expression of both hosts (immune response) and algal symbionts as a result of disease exposure.

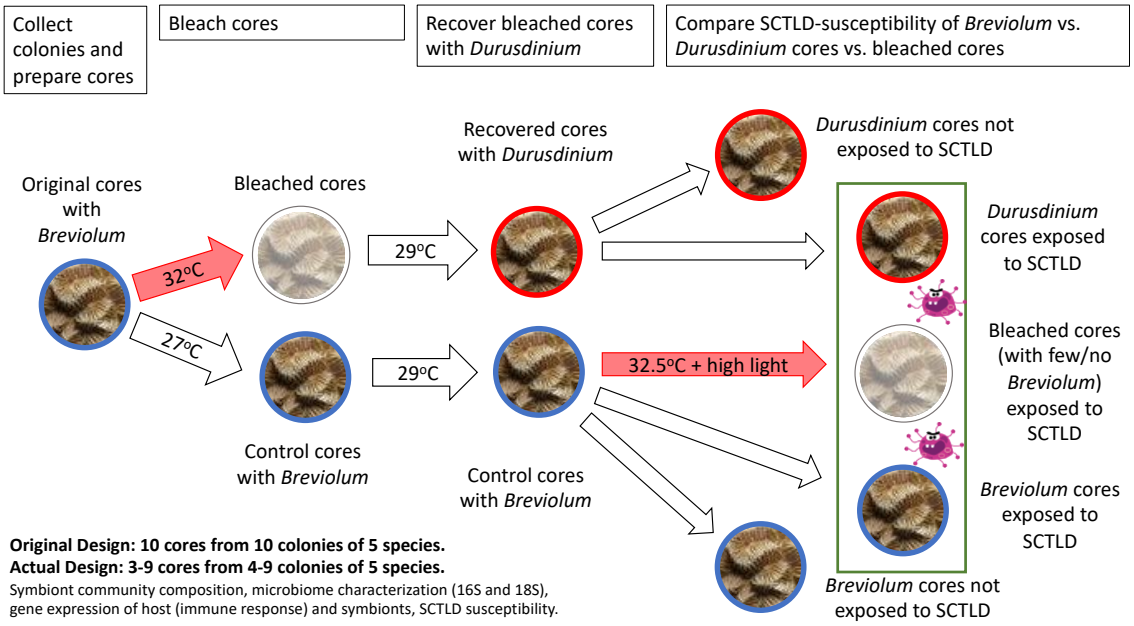

Figure 1: Experimental design for laboratory experiment.

2. METHODS

2.1. Coral Collections

Coral colonies were collected from the Dry Tortugas, FL on a three-day collection cruise on the M/V Makai in late January 2020. Upon surfacing from collection dives, photos and initial tissue biopsies were taken and preserved in 1% SDS in DNAB for initial symbiont identification (Fig. 3). Following collections, colonies were transported back to the University of Miami's Rosenstiel School of Marine and Atmospheric Science and acclimated to laboratory conditions. Given that the laboratory uses flow-through water taken from Bear Cut (within the SCTLD endemic zone), tanks were sanitized prior to coral arrival and water supply to the experimental tanks was UV-sterilized. After a week of acclimation to laboratory conditions, colonies were fragmented into 10-15 replicate, 2.5cm-diameter cores and maintained at ambient conditions for two months to promote recovery. Shortly after fragmenting, some species (predominantly *D. labyrinthiformis* and *O. faveolata*) experienced rapid tissue loss (RTL). This was likely due to the high density of freshly fragmented cores (N=678) in our tanks, and the low incoming water flow rate we used to ensure adequate UV sterilization of incoming water, resulting in excess mucus accumulation in the tank. To remedy this, a more powerful UV sterilizer was added to the system and cores were split between four different tanks to increase water volume. Following this unanticipated RTL, we were able to re-core some colonies (N=610) to maintain a high degree of colony replication and uniform genotypic representation across the experiment (Table 1).

Table 1: Distribution of genets (colonies) and cores throughout the experiment.

Site	Species		ORIGINAL TARGET	Initial collections	Initial Coring	After mortality and recore	Start of bleaching - cores reallocated to treatments	End of bleaching	End of recovery	Start of disease trial	Disease Trials Early Mortality
				28-Jan-20	21-Feb-20	27-Mar-20	20-Feb-20	30-Apr-20	30-Jul-20	23-Aug-20	28-Aug-20
Emerald Reef	DLAB	Genotypes				3	3	3	2	2	2
9/15/19		Cores				30	30	30	15	8	7
	OFAV	Genotypes				4	4	4	2	2	2
		Cores				38	38	38	20	15	4
Dry Tortugas	CNAT	Genotypes	10	13	13	13	13	13	13	13	10
01/28/2020-01/30/2020		Cores	100		140	150	150	150	80	55	28
	DLAB	Genotypes	10	13	13	10	10	10	8	8	6
		Cores	100		128	47	47	47	35	25	11
	MMEA	Genotypes	10	15	15	14	14	14	13	13	10
		Cores	100		144	88	88	88	77	50	25
	OFAV	Genotypes	10	14	14	13	13	13	11	10	8
		Cores	100		134	111	111	111	56	35	27
	PSTR	Genotypes	10	15	15	15	15	15	15	15	15
		Cores	100		132	146	146	146	123	81	47
	Total genotypes		50	70	70	72	72	72	64	63	53
	Total cores		500		678	610	610	610	406	269	149

2.2. Manipulation of Algal Symbionts (Task 1)

To manipulate the algal symbionts, a subset of cores was systematically bleached and recovered to promote recovery with *Durusdinium* (Fig. 2). Prior to the start of thermal manipulations, small tissue biopsies were taken using sterile razor blades and preserved in DNA/RNA Shield and stored in a -80C freezer for downstream analyses. Additionally, a MAXI Imaging Pulse Amplitude Modulated (IPAM) fluorometer (Walz, Effeltrich, Germany), was used to measure the photochemical efficiency of photosystem II (Fv/Fm), a proxy for Symbiodiniaceae community function, twice weekly in all cores. Experimental cores were then ramped to 32.5°C over the course of 10 days (0.5°C per day) and maintained at this temperature until Fv/Fm values declined by at least 50% of the initial value, or cores were visually bleached, whichever came first. Corals removed from the heat stress exposure in this way accrued between 5- and 11-degree heating weeks (DHWs) in total. When cores were removed from heat stress, they were allowed to recover in a new tank at 29°C for the next three months. To monitor recovery of algal symbionts, tissue samples were taken monthly and preserved in 1% SDS in DNAB for DNA extraction and algal identification and quantification. Additionally, Fv/Fm was monitored weekly during recovery as a non-invasive metric to monitor coral health and recovery. Throughout the course of this experiment, all cores (control and experimental)

were fed Reef Roids three times a week for 30 minutes.

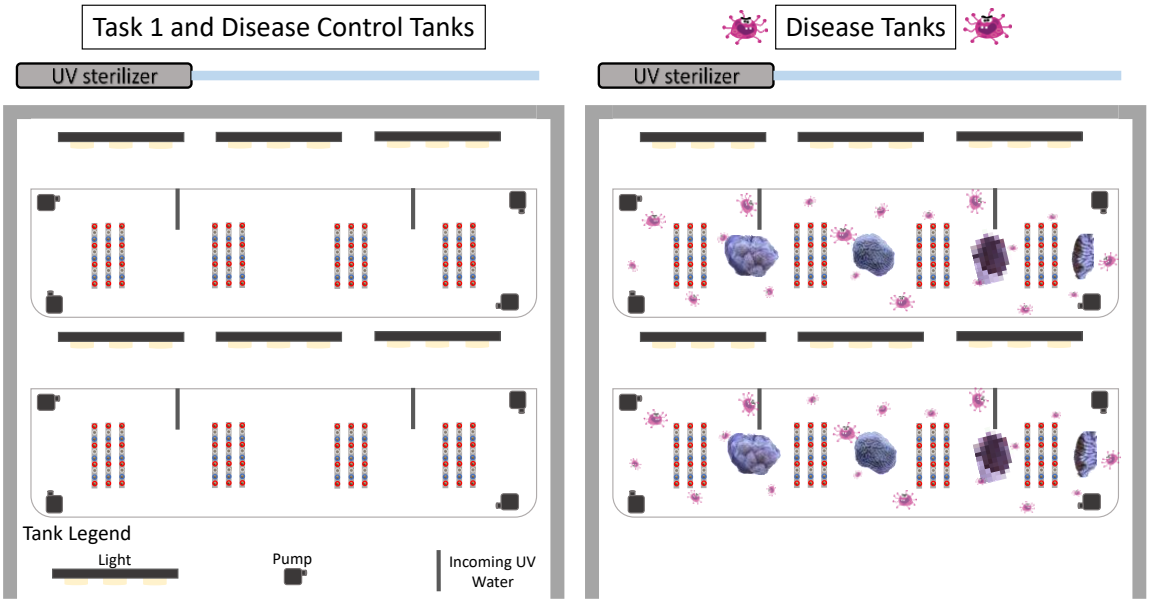

Figure 2: Tank setup for symbiont manipulations and disease exposures.

2.3. Symbiodiniaceae quantification and identification (qPCR; Task 1 and Task 2)

Throughout this experiment 1 polyp (~2mm) genetic biopsies were taken and preserved in 1% SDS in DNAB. Genomic DNA was extracted using a modified organic extraction protocol (Baker & Cunning, 2016) and the associated Symbiodiniaceae genera in each colony/core was characterized using real-time PCR (qPCR) assays at several time points during this experiment — initials, just before heat stress, at the start of recovery, every month for three months during recovery, and prior to disease exposure. TaqMan environmental master mix (Thermo Fisher Scientific) assays were used to amplify the actin gene in *Symbiodinium* (Winter, 2017), *Breviolum* (R. Cunning et al., 2015), *Cladocopium*, and *Durusdinium* (Ross Cunning & Baker, 2013), and qPCR data were used to identify associated algal symbionts and their relative abundance using the StepOne package for R (Cunning 2018) adapted for these data.

2.4. Disease Assays (Task 2)

Before beginning disease assays a final subset of cores were rapidly bleached (high light and high temperature) over the course of one week. Following recovery, cores were divided into six disease treatments based on species and associated algal symbionts: (1) manipulated + disease, (2) unmanipulated + disease, (3) manipulated, no disease, (4) unmanipulated, no disease (controls), (5) bleached + disease, and (6) bleached, no disease. Small tissue biopsies were taken from all cores, preserved in DNA/RNA Shield, and subsequently stored in a -80C freezer. To expose disease treatment cores to SCTLD, coral colonies showing active SCTLD lesions were collected from the Middle and Lower Keys (courtesy of Karen Neely) and transported to the University of Miami. Colonies

were then divided in half, based on lesion area, and divided between two tanks containing the experimental cores to be exposed. In this shared-water approach (i.e., non-contact assays) cores became infected with SCTLD over the course of 60 days (Fig. 2). Experimental tanks were outfitted with four pumps and one heater to ensure constant water circulation, and temperature and incoming water flow remained constant. Additionally, cores were moved within and between tanks daily to avoid within- and between-tank effects. Given the unpredictable nature of lesion formation, experimental cores were monitored daily with photos and small tissue biopsies were taken throughout the disease exposures and flash frozen until the end of disease trials. All cores (disease-exposed and disease-unexposed) were fed Reef Roids three times a week for 30 minutes.

2.5. Disease Progression and Survivorship

Core health and disease progression were monitored daily for signs of lesions and disease progression. Survival curves were generated using the Kaplan-Meier estimate (Kaplan and Meier 1958). To investigate the relationship between survival time (exposure days) and dominant Symbiodiniaceae, a Cox proportional-hazards model (Cox, 1972) was used. Additionally, for species with mixed algal communities (*C. natans, D. labyrinthiformis, O. faveolata,* and *P. strigosa*) a mixed-effects model was employed to investigate the effects of the dominant algal symbiont and colony (i.e., genotype and species) on the rate of infection. In addition, given that the majority of the cores in the experiment did not host a single algal genus, but rather a mix of multiple genera, a Cox proportional-hazards model was used to calculate relative risk over time to assess the contribution of non-dominant Symbiodiniaceae, the relative abundance of *Breviolum* vs. *Durusdinium*, which were the two genera found in all four species used in the mix effects model. Statistical analyses were performed with the survival 3.2-7 (Therneau 2015) and survminer 0.4.8 (Kassambara et al. 2019) packages for R.

2.6. Microbial and transcriptomic dynamics (Task 3 and Task 4)

In addition to samples used for symbiont characterization, one-polyp tissue samples were also preserved in DNA/RNA Shield and co-extracted for DNA and RNA to investigate eukaryotic (18S) and prokaryotic (16S) microbial dynamics (DNA) and host gene expression (RNA). Following the conclusion of the disease assay, a subset of samples was chosen for extractions and analysis. Samples of interest were identified based on the following criteria:

1) Dominant Symbiodiniaceae genus;
2) Background Symbiodiniaceae genera;
3) Fate following disease exposure;
4) Availability of time series samples before, during, and at the end of lesion progression;
5) Cores of interest based on survival time and lesion progression rate.

Flash-frozen samples were transferred to Zymo Bead Bashing tubes with DNA/RNA Shield. Prior to extraction, samples were homogenized using a MiniPrep for 7

minutes at 1-minute intervals. The samples were then processed using the ZymoBIOMICS Magbead DNA/RNA kit (Zymo Research) and extracted on the Kingfisher™ Flex Purification System (Thermofisher). Further processing is explained in the sections below.

2.7. Transcriptomics and host gene expression (Task 3)

Raw RNA sequence data was returned from the sequencing core facility on May 20, 2021, and uploaded to the Pegasus supercomputer cluster at the University of Miami. A total of 350 zipped raw data files, approximately 100-400 MB each, were obtained. These files will be quality controlled using FastQC for high throughout sequencing pipelines. This will provide data on essential sequence quality parameters (e.g., per-base sequence quality, per-sequence quality scores, per-base sequence content, per base GC content, per base N content, sequence length distribution, sequence duplication levels, and Kmer content). Poor quality reads which fall below the quality threshold will be removed. Adapter sequences will then be trimmed from the read ends using BBDuk. After an additional FastQC quality control check will then be performed to ascertain that read quality was not compromised during the BBDuk trimming process.

To align sequences against reference genomes we will use Spliced Transcripts Alignment to a Reference (STAR). Expression of transcripts will be quantified using Salmon, which accounts for common biases and enhances expression quantification accuracy. Expression quantification data will be analyzed in R Studio to determine how genes are differentially expressed between the six different treatment groups, with particular focus on comparisons within genotypes and within species. A Principal Component Analysis (PCA) will be performed to visualize this expected differential host expression of genes.

2.8. Prokaryotic Microbial Dynamics (Task 4)

Extracted DNA was amplified with 16S rRNA gene primers using Earth Microbiome Project (EMP) protocols. PCR products were cleaned with AMPure XP beads (Beckman Coulter, Brea, CA) and subsequently amplified with barcodes before being normalized to 4nM. Following normalization, 5uL of each sample was pooled and sent to the Center for Genome Technology at the University of Miami Miller School of Medicine for sequencing.

In total, 377 samples were sequenced for microbiome analysis, which included four negative controls. Samples were split across two Illumina MiSeq runs, and raw sequence reads from the two runs were returned from the facility on May 18 and May 21, 2021. Here we report preliminary analyses from the first batch of samples. In the first run, there was a total yield of 25,761,119 sequences and they ranged per sample from three reads (in a negative control) to 250,557. From the 188 samples that were sequenced in the first run, five were below the sequencing read threshold (<13 reads) and were not analyzed further.

The data were analyzed using the Qiime2-2018.11 software package (Bolyen et al. 2019) and processed using the Cutadapt plugin (Martin 2011) to remove primers, and the DADA2 plugin (Callahan et al. 2016) to assign Amplicon Sequence Variants (ASVs).

This resulted in a total of 15,001 ASVs which ranged in frequency per sample, between 1 – 749,258 (median=24). Both negative controls from the first sequencing run had zero ASVs assigned. ASVs were taxonomically classified with a fitted classifier using the function feature-classifier classify-sklearn and a trained Silva-132-99-105-806 database (Bokulich et al. 2018). Chloroplast and mitochondria ASVs were removed from the analysis which left 13,976 ASVs. The data was then transferred to R version 4.0.5.

2.9. Eukaryotic Microbial Dynamics (Task 4)

The SCTLD pathobiome-extracted DNA was amplified for microeukaryotes using the specific non-metazoan primer pair 616F (forward: 5'-TTAAARVGYTCGTAGTYG-3') and UNonMet_DB (reverse: 5'-CTTTAARTTTCASYCTTGCG-3') targeting a 531 bp amplicon from the 18S rRNA gene. Next generation sequencing library preparation followed the Earth Microbiome Project 18S protocol. Briefly, the PCRs were conducted using Platinum Hot Start 2X Master Mix (Invitrogen, Carlsbad, CA) and 10 µM of the forward (Illumina 5' adapter, primer pad, primer linker, and forward 616F primer) and reverse primers (reverse complementary of 3' Illumina adapter, golay barcode, primer pad, primer linker, and reverse UNonMet_DB primer) (Integrated DNA Technologies, Coralville, IA) for a final volume of 25 µL and 0.2 µM concentration. Amplification was completed using the following PCR profile: 94 °C for 3 min; 35 cycles each at 94 °C for 45 s, 57 °C for 60 s, 72 °C for 90 s; a final extension at 72 °C for 10 min. The resultant PCR products were verified at ~600 bp by gel electrophoresis at 80 V for 60 min using a 100bp ladder (Promega, Madison, WI) and cleaned of adapter dimers using a 1:1 Agencourt AMPure bead (Beckman Coulter, Indianapolis, IN) to input DNA ratio. Cleaned PCR products were quantified using the Qubit 4.0 fluorometer dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA), normalized to 4 nM by dilution using molecular grade water, and pooled for Illumina MiSeq 2x300 bp sequencing at the Center for Genome Technology at the University of Miami Miller School of Medicine. Sequence reads were completed at the facility on May 26, 2021, but the sequencing run failed and will be troubleshooting why this occurred.

3. RESULTS & DISCUSSION

3.1. Manipulation of coral symbioses in the laboratory (Task 1)

Following coral collections, initial symbiont communities in *C. natans, M. meandrites,* and *P. strigosa* were dominated by Symbiodiniaceae in the genus *Breviolum*, while *D. labyrinthiformis* was dominated by *Breviolum* and/or *Durusdinium* and *O. faveolata* was typically dominated by *Cladocopium* (Fig. 3).

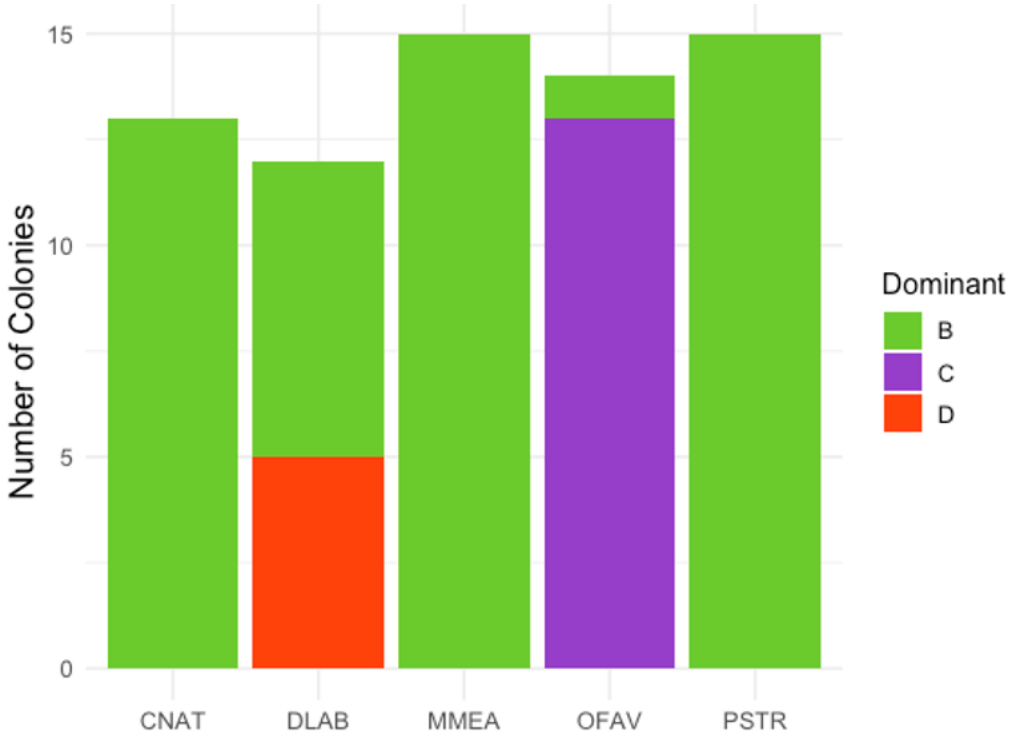

Figure 3: Dominant Symbiodiniaceae genera in colonies collected from the Dry Tortugas in January 2020 (pre-exposure).

Following experimental bleaching, the relative abundance of *Symbiodinium*, *Breviolum*, *Cladocopium*, and *Durusdinium* in each core was assessed after 1 and 2 months of recovery. The proportion of each symbiont genus was quantified and a 2-dimensional NMDS plot and an analysis of similarities (ANOSIM) performed to test whether pre-bleaching and post-bleaching timepoints were significantly different, as well as whether there were differences in response between species (Fig. 4). For each species, the ANOSIM statistic was significant ($p<0.01$) with 4 of the 5 species (*C. natans*, *D. labyrinthiformis*, *O. faveolata*, and *P. strigosa*) shifting their communities in favor of *Durusdinium*.

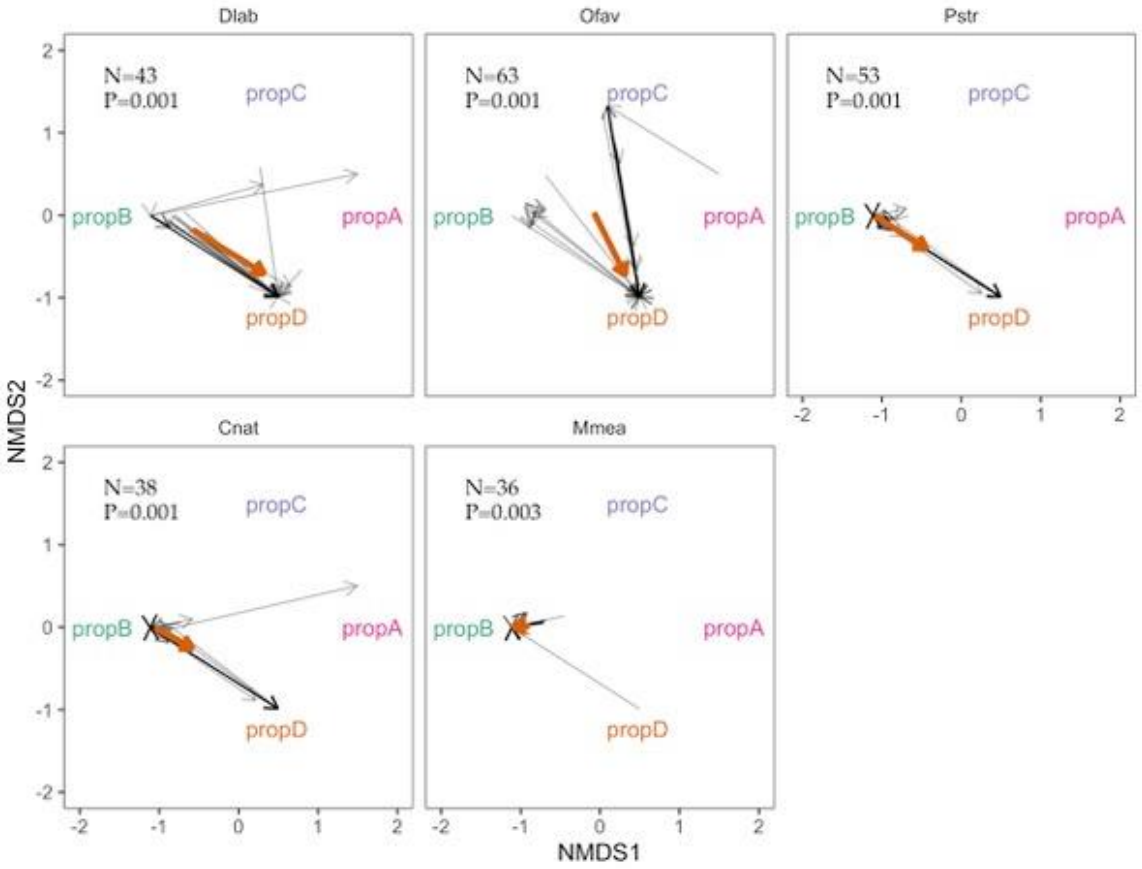

Figure 4: A 2-dimensional NMDS plot of symbiont communities for the 5 species used in the disease pre-bleaching and post-bleaching. Light black vectors show the direction and magnitude of symbiont shuffling in individual cores, with the tail being the initial core symbiont community and the head of the arrow showing the final symbiont community. The light red arrow shows the mean trajectory for each coral species.

The probability of each species associating with *Durusdinium* as a result of bleaching was then quantified using a 3-dimensional NMDS analysis and the Bray-Curtis dissimilarity metric. This method measured how closely related the initial and final communities were to being fully *Durusdinium*-dominant (with a value of 1 being *Durusdinium* dominant and 0 having a symbiont community made up of non-*Durusdinium*). Since the symbiont distributions are quasi-binomially distributed, the propensity to host *Durusdinium* (propensity D) can be viewed as the probability that a coral core from a given species shifts its symbiont community to being *Durusdinium*-dominant. The propensity to associate with *Durusdinium* following bleaching and recovery varied by coral species (p=1.17x10-8) with *D. labyrinthiformis* and *O. faveolata* being most likely to associate with *Durusdinium* as a result of bleaching and recovery (Fig. 4), but these species also tended to host more *Durusdinium* to begin with. The other two species, *C. natans* and *P. strigosa* may have had to rely on the uptake of *Durusdinium* from the surrounding water column instead of internal communities of *Durusdinium. M. meandrites* was the only species that did not change its symbionts in favor of *Durusdinium*. Finally, the propensity of *Durusdinium* correlated significantly

with degree heating weeks (DHWs) (p=4.08x10-13) and the more time the coral core spent at temperature (32.5oC) the more likely it was to shift its community in favor of *Durusdinium* (Fig. 5).

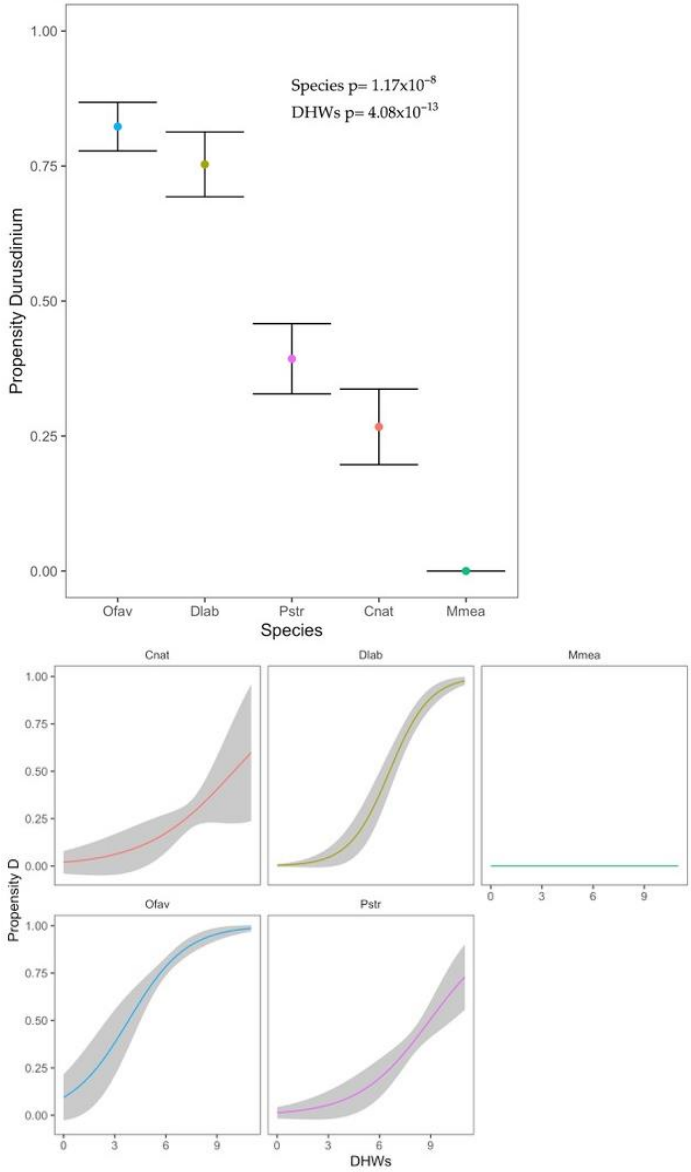

Figure 5: Propensity (probability) of recovery with *Durusdinium*. Top panel: Propensity (probability) to recover with *Durusdinium* as a result of coral bleaching, with 1 being a fully *Durusdinium*-dominated community. Bottom panel: The probability to recover with *Durusdinium* as a function of accumulated heat stress (DHWs).

Finally, a subset of the initial samples was processed for ITS2 amplicon sequencing (Illumina MiSeq) using Sym_Var primers (Hume et al, 2018) to identify any strain-level differences in Symbiodiniaceae communities within coral species, between coral species, and between collection sites. The majority of *Breviolum* were ITS-type *Breviolum minutum* (*Breviolum* B1) but additional types were also detected. Future

analysis will assess for species-level (within-genus) differences in Symbiodiniaceae to affect disease susceptibility (Fig. 6).

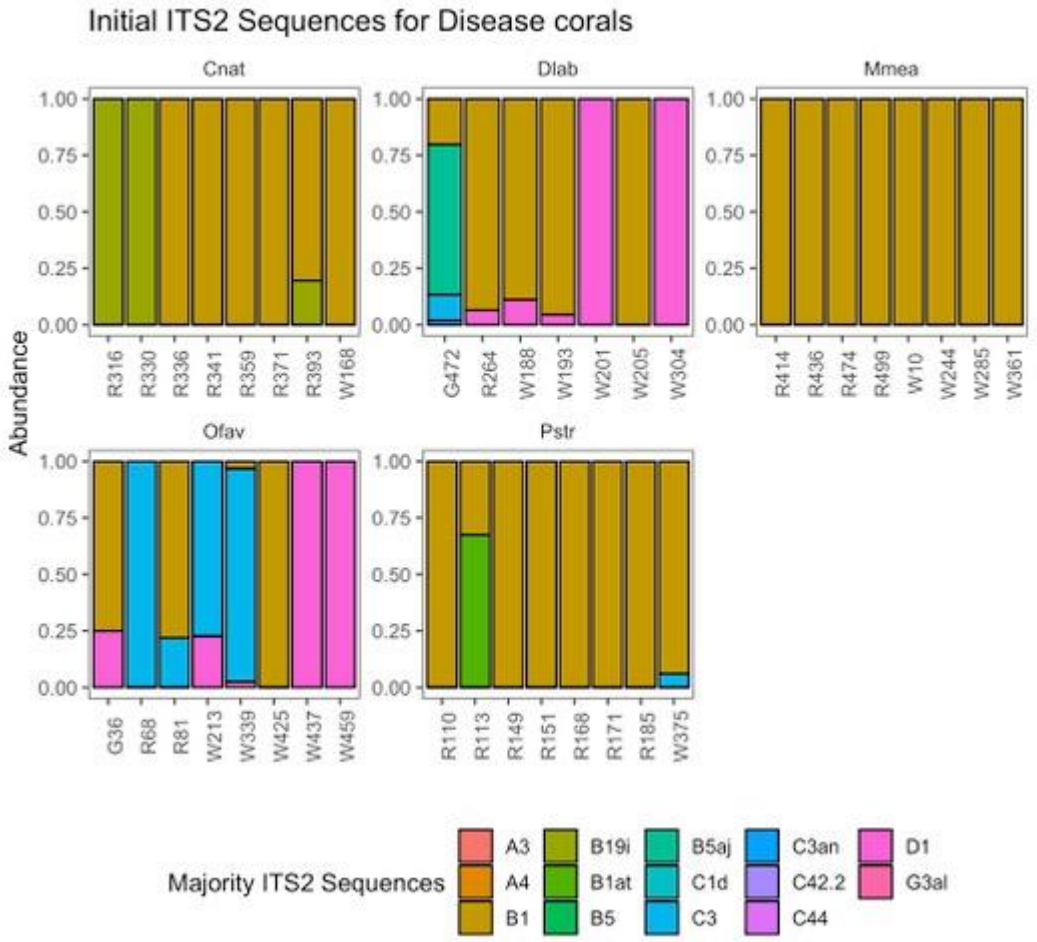

Figure 6: ITS2 sequences for initial symbiont communities (pre-bleaching) of corals used in the disease assays.

3.2. Assessing SCTLD susceptibility of corals hosting *Breviolum* vs. *Durusdinium* vs. bleached (Task 2)

We found that cores in disease treatments could develop SCTLD-like lesions via non-contact (waterborne) exposures. Non-contact assays minimize the chances of lesions due to inter-colony aggression which in turn could exacerbate SCTLD. These findings confirm growing evidence that SCTLD can be transmitted through the water with major implications for disease transmission (Muller et al. 2018).

This experiment also supported our hypothesis that algal symbionts play a role in SCTLD-susceptibility. Survivor curves generated using a Kaplan-Meier estimate (Fig. 7) indicate the rate of SCTLD infection varies as a function of algal symbionts.

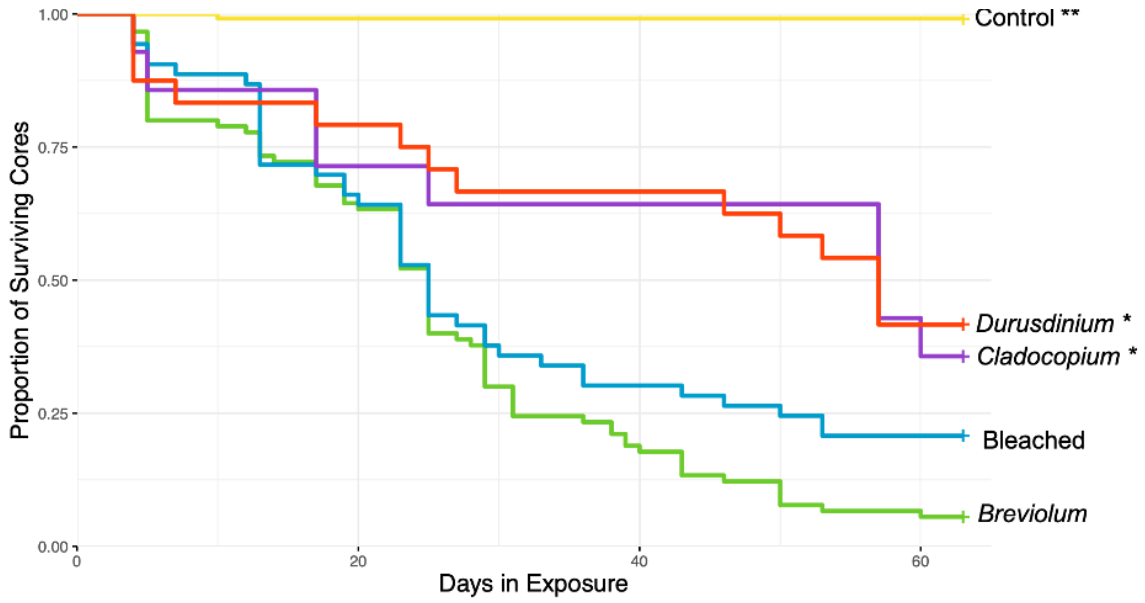

Figure 7: Kaplan-Meier survival curves for disease exposures.

The Cox proportional—hazards (CoxPh) model also showed that cores predominantly associated with *Durusdinium* or *Cladocopium* were significantly less likely to present with SCTLD-like lesions compared to cores that were bleached or which contained *Breviolum* (Fig. 8). However, it is important to note that *Durusdinium* and *Cladocopium* were not immune. In fact, as the number of days in disease exposure increased the survival rates also declined in these cores, suggesting that differences are relative, not absolute.

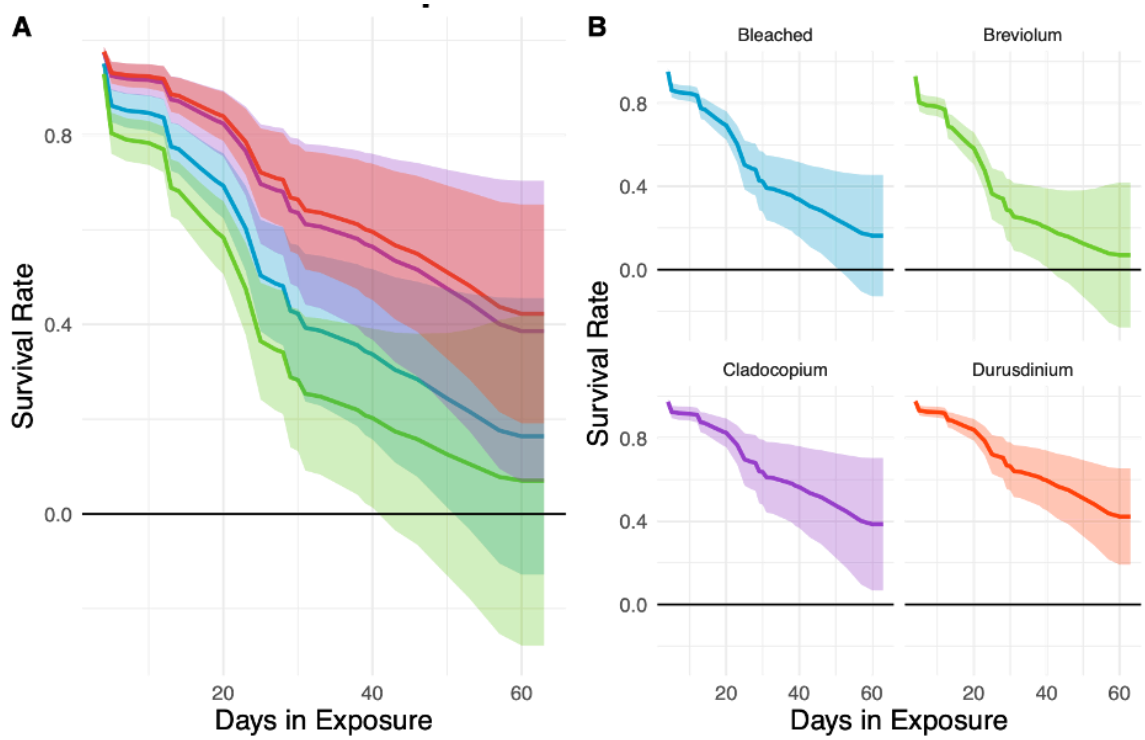

Figure 8: Cox Proportional Hazard (CoxPh) model of survival rates based on associated Symbiodiniaceae.

The generalized linear mixed effect model further supports the hypothesis that the probability of SCTLD incidence primarily depends on algal associations, with coral species and/or genotype not playing statistically significant roles (Fig. 9). As with the CoxPh model, *Durusdinium* and *Cladocopium* are statistically less susceptible to SCTLD compared to cores hosting *Breviolum* or bleached cores which contain few symbionts (Fig. 9). However, *O. faveolata* was the only species in this experiment that contained cores dominated by both *Durusdinium* and *Cladocopium*, so this finding should be tested in other species as well. Moreover, preliminary ITS-2 typing from Task 1 indicates that the *Cladocopium* in *O. faveolata* is *Cladocopium* C3, which behaves more like *Durusdinium* during thermal stress than *Cladocopium*. As such, this finding may be specific to this *Cladocopium*.

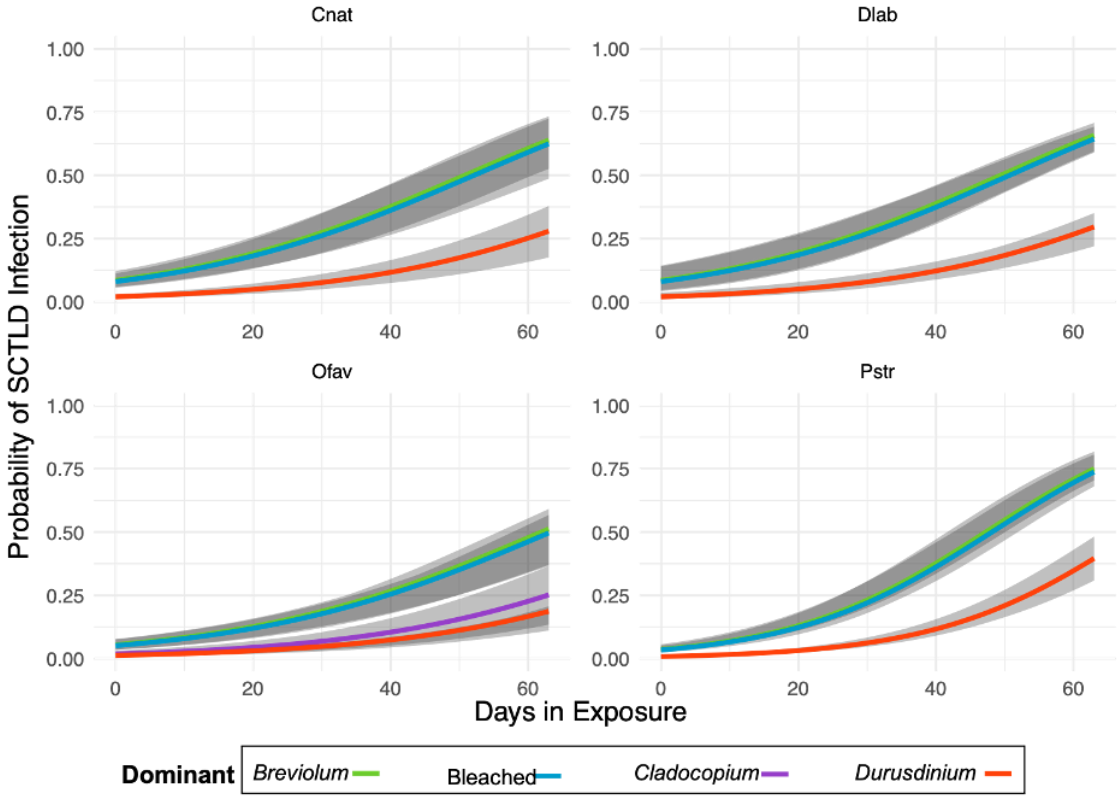

Figure 9: Generalized linear mixed effects model (GLMM) which includes the random effect of genotype and inherently species.

Following the results of the survival curves generated using the Kaplan-Meier estimate, CoxPh Model, and the GLMM, we suggest there is a hierarchy in SCTLD-susceptibility as a function of the Symbiodiniaceae hosted. We tentatively rank Symbiodiniaceae susceptibility as follows: *Breviolum* >> *Cladocopium* > *Durusdinium* >> *Symbiodinium*. Although no cores in this experiment were dominated by *Symbiodinium*, we include it in this ranking based on field observations suggesting corals

that associate with *Symbiodinium* (e.g., *Acropora cervicornis* and *A. palmata*) do not appear to be susceptible to SCTLD.

We also developed a model to account for the effect of different proportions of *Breviolum* vs. *Durusdinium* on SCTLD susceptibility. Briefly, we used a CoxPh to calculate the relative risk (RR) over time. We found that, for *Durusdinium*, the RR falls below one at ~20%, indicating that a colony containing at least 20% *Durusdinium* is less at risk of becoming infected with SCTLD, whereas for *Breviolum* the RR exceeds 1 at ~75%, indicating that a colony containing 75% *Breviolum* becomes more at risk of infection (Fig. 10). Overall, associating exclusively with *Breviolum* carries a risk two and a half times greater than that of *Durusdinium*.

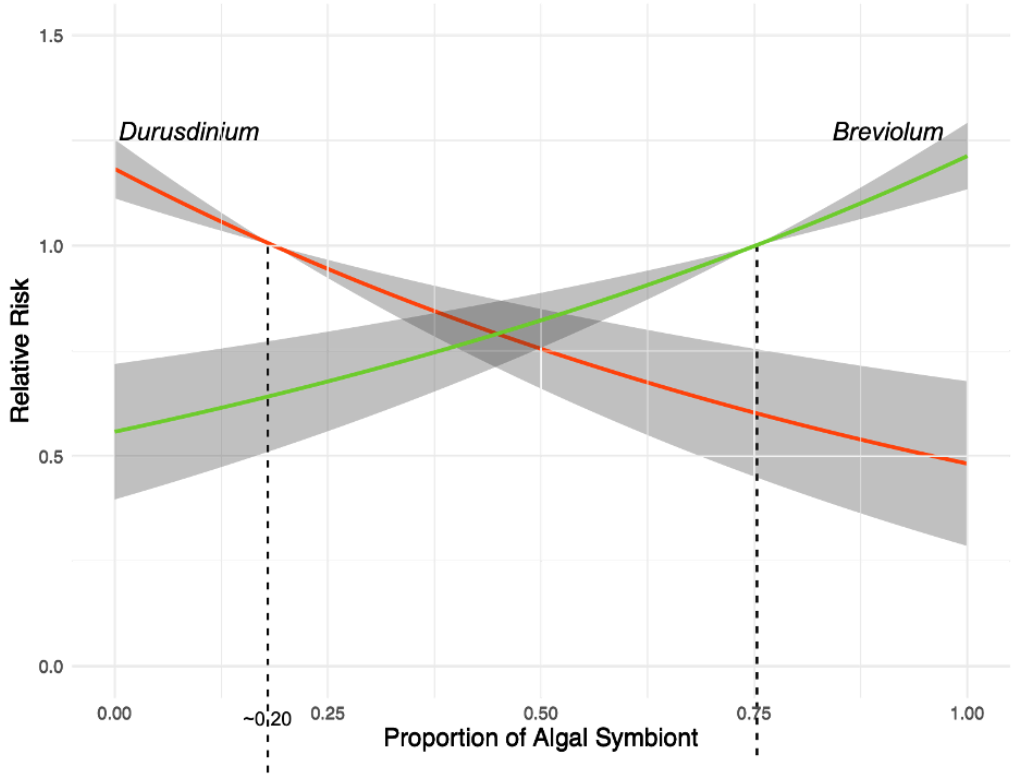

Figure 10: CoxPh model of the relative risk over time of different proportions of *Durusdinium* and *Breviolum.*

3.3. Compare differences in host gene expression (immune response) in *Breviolum* vs. *Durusdinium* vs. bleached corals in response to SCTLD exposure (Task 3)

After delays due to COVID-19, TagSeq sequences used for gene expression analysis were returned on May 20, 2021. A total of 288 samples were sent for sequencing and preliminary data of the raw reads indicates that each sample generated between 5 and 7 million reads. Our next steps will be to run a basic quality control (QC) of the raw

sequences using FastQC to generate basic statistics for each sequence. Samples will then be trimmed and put through another round of QC. We will then use STAR Align to align sequences against their reference genomes prior to gene expression analysis. The overall pipeline is illustrated in Fig. 11.

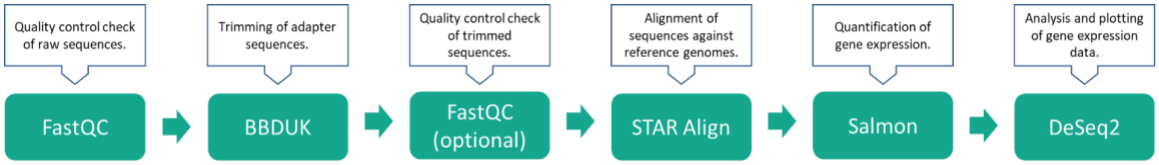

Figure 11: Host gene expression analysis pipeline.

A total of 354 samples were sent for sequencing and a total of 275 were successfully sequenced (Table 2). *O. faveolata* was chosen for initial analysis as the genome/transcriptome is readily available. Preliminary principal component analysis (PCA) was performed comparing manipulated cores, those from Task 1 with manipulated algal symbionts (i.e., bleached and recovered), and non-manipulated cores, or those that were not exposed to heat stress and recovered in Task 1 (Fig. 12a). This was further broken up into looking at dominant algal genera (Fig. 12b). Although no clear patterns exist, these are preliminary results, and we will continue to investigate host gene expression (and Symbiodiniaceae gene expression) particularly picking apart differences in healthy vs. disease cores and looking at differential gene expression throughout disease exposures across individual cores (i.e., time series).

Table 2: Distribution of samples and reads across species sequenced for gene expression analyses.

Species	Number of samples	Number of reads
C. natans	51	5-7 million
D. labyrinthiformis	30	5-7 million
M. meandrites	34	5-7 million
O. faveolata	77	5-7 million
P. strigosa	83	5-7 million

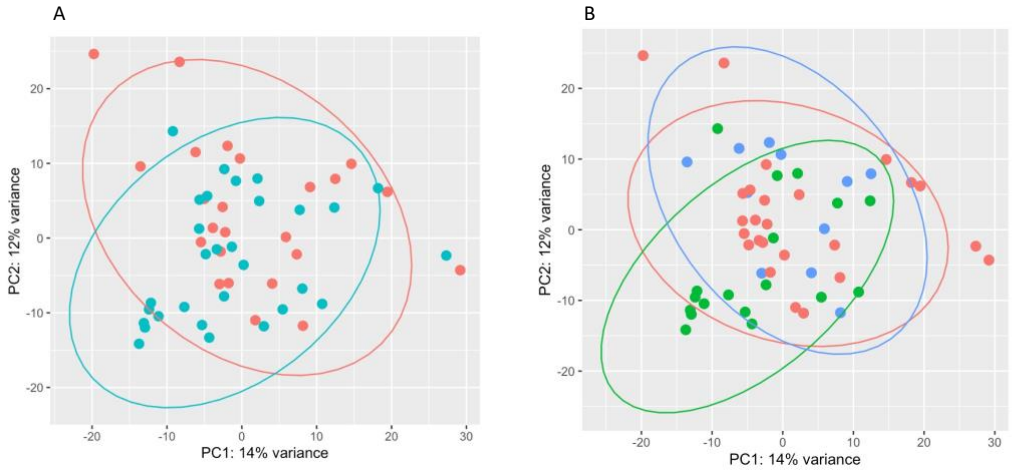

Figure 12: Preliminary principal component analysis (PCA) of manipulated and non-manipulated cores

3.4. Changes in coral microbiome as a result of SCTLD exposure and their dependence on algal symbiont community (Task 4)

3.4.1. Prokaryotic microbial dynamics

Sequence reads were filtered to remove low frequency amplicon sequence variants (ASVs) (<20% of the data), which resulted in 3,317 ASVs. These ASVs were then used for subsequent alpha- and beta-diversity analysis. For alpha-diversity, a Shannon Diversity Index showed that symbiont manipulations slightly increased microbial diversity in *Breviolum* and in *Durusdinium* but declined sharply in *Cladocopium* (Fig. 13; top panel). Once exposed to SCTLD there was a slight increase in microbial alpha diversity in corals hosting *Durusdinium* in both manipulated and non-manipulated corals, and in corals hosting *Cladocopium* that were manipulated (Fig. 13, bottom panel). In beta-diversity analysis (using the VEGAN package), there was a distinct microbial shift between samples that were exposed to a SCTLD disease and those that were exposed to a healthy coral (PERMANOVA pvalue<0.001; R2=0.04; Fig. 13). There was also a difference in microbial dispersion and samples exposed to SCTLD had a more dispersed community than samples exposed to the healthy coral (ANOVA padj<0.001). There was also a difference in microbial composition among corals with the three dominant symbiont communities (PERMANOVA pvalue<0.05; R2=0.02), but a pairwise comparison of the three symbionts showed that only *Durusdinium* vs *Breviolum* were different (padj<0.05, Fig. 14).

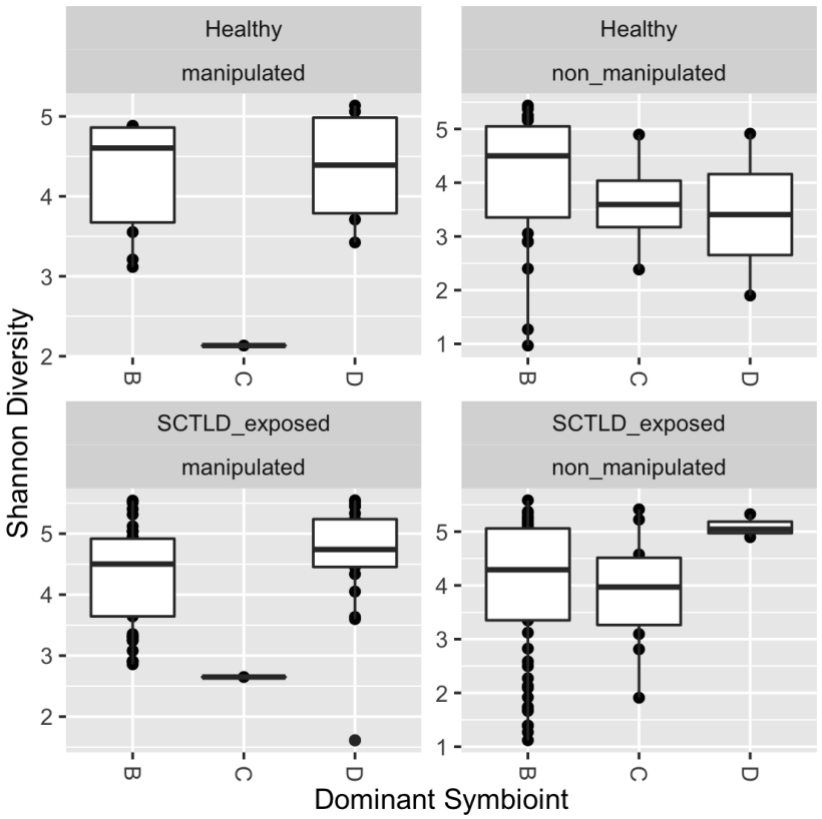

Figure 13: Differences in alpha-diversity among treatments and the dominant symbionts *Breviolum* (B), *Cladocopium* (C), and *Durusdinium* (D). Shannon diversity is parsed by corals which had manipulated or non-manipulated symbionts, and corals that were exposed to either a healthy or SCTLD-infected coral.

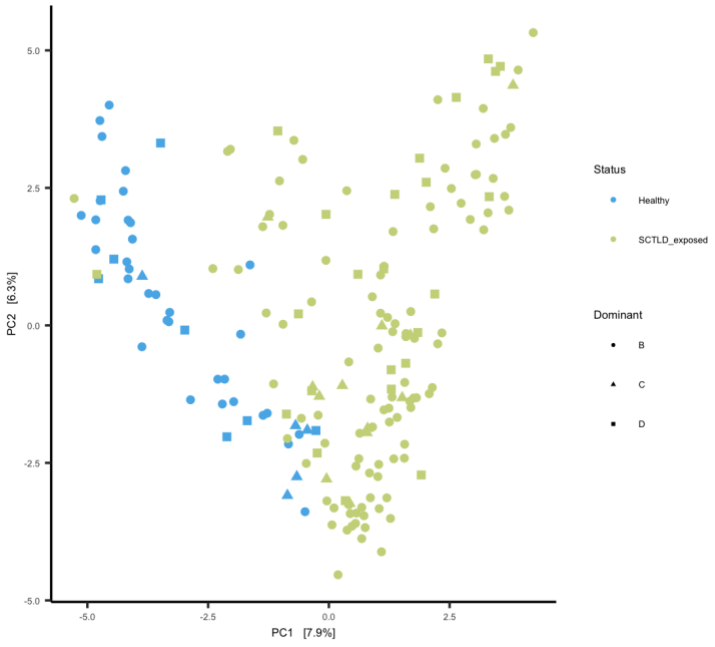

Figure 14: Distinct beta-diversity patterns to SCTLD exposure. Principal component analysis (PCA) with a Euclidean distance. The figure is colored by corals that were exposed to either a healthy or to a SCTLD-infected coral and shapes are based on the dominant symbionts in this study *Breviolum* (B), *Cladocopium* (C), and *Durusdinium* (D).

Controlled bleaching and recovery were generally accompanied by changes in the relative abundance of dominant bacterial taxa (Fig. 15). An exception was *Meandrina meandrites*, which remained dominated by the bacterial taxa Rhodospirillales, despite severe bleaching. However, *M. meandrites* did not change its symbionts in response to bleaching and recovery, suggesting that these bacterial shifts occur as a result of the shifts in symbiont community that occurred following recovery, not the environmental stress that caused the initial bleaching. Previous studies have found that bacterial taxa Rhodobacterales are specifically associated with SCTLD and in this experiment we also found that Rhodobacterales increase in relative abundance in samples exposed to SCTLD. Moreover, across all timepoints, corals with *Durusdinium* that did not get SCTLD also did not experience increases in their relative abundance of Rhodobacterales (Fig. 12). Overall, the microbiome does appear to be affected by the treatments and we will continue to explore these data for additional findings.

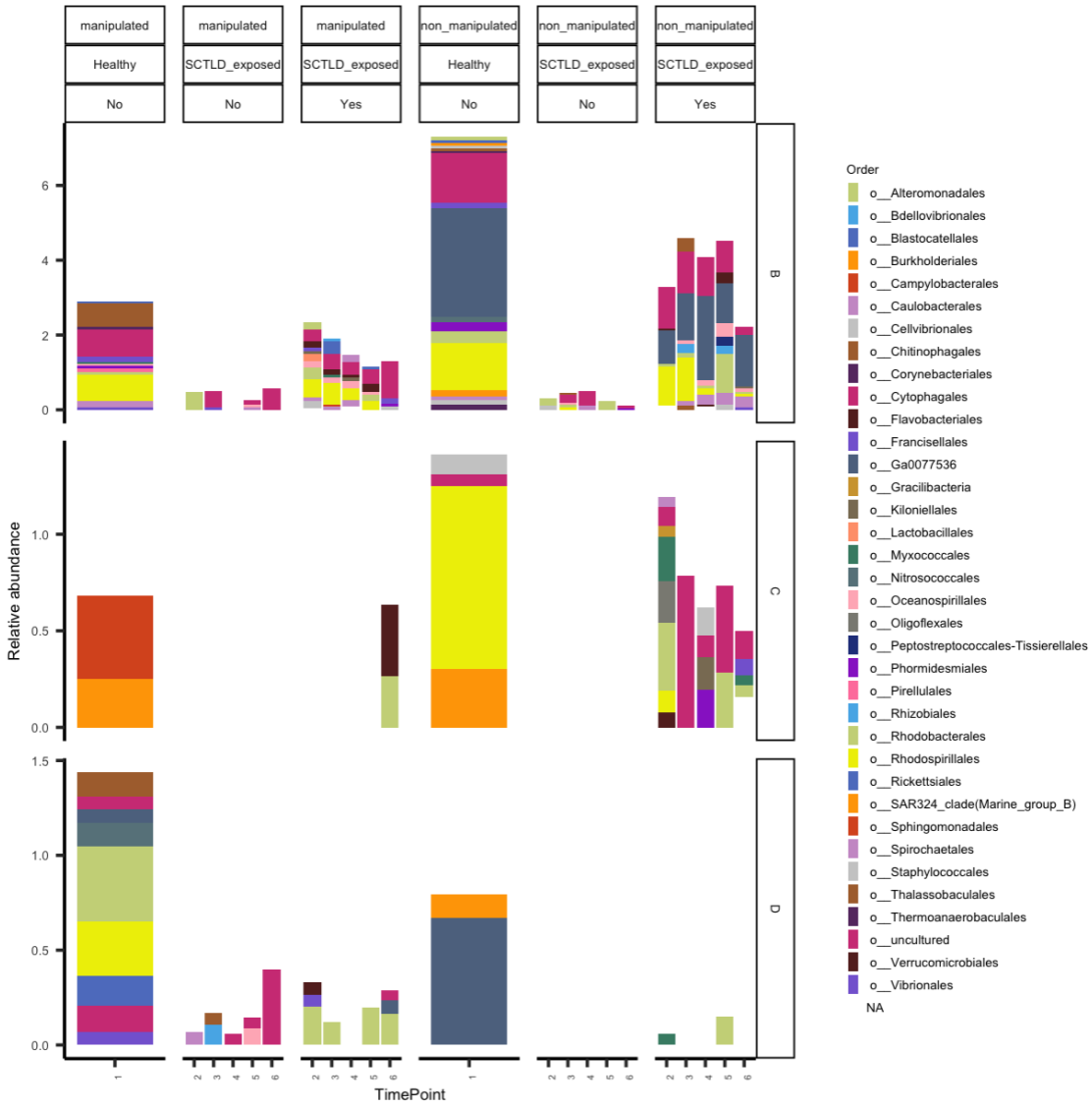

Figure 15: Differences in relative abundances of the most abundant microbial genera (>0.05%) per time point. Each stacked color bar represents a different bacteria order. On the horizontal axis the figure is grouped by coral which had manipulated or non-manipulated symbionts, corals that were exposed to either a healthy or to SCTLD coral, and by corals that became diseased ("Yes", or "No"). On the vertical axis, the figure is parsed by three symbiont genera examined in this study: *Breviolum* (B*), Cladocopium* (C), and *Durusdinium* (D).

3.4.2. Eukaryotic microbial dynamics

Unfortunately, the 18S sequencing runs failed for this portion of the experiment. We are currently working with the sequencing facility to identify where the errors occurred.

4. PRELIMINARY CONCLUSIONS

We found strong evidence that different algal symbionts play a role in a coral's susceptibility to SCTLD. Based on data on coral health paired with qPCR data to identify and quantify different algal symbionts, we conclude that corals that associate exclusively or predominantly with the genus *Breviolum* are more susceptible to SCTLD than those associating with other algal genera. Although this is the first time this has been reported, these results are supported by initial field observations regarding a disease susceptibility hierarchy which tend to support this conclusion. Further investigation of these and other data suggest that there is susceptibility hierarchy in which corals hosting *Breviolum* appear to be more susceptible to SCTLD compared to corals hosting *Cladocopium* and *Durusdinium*, which are in turn more susceptible than corals hosting *Symbiodinium*, which do not appear to be susceptible (based on the observation that corals which exclusively host Symbiodinium, such as Acropora spp., are not susceptible to SCTLD). *Cladocopium* and *Durusdinium* are not immune to SCTLD, but even small differences in susceptibility might translate to observable differences in the incidence of SCTLD on reefs. We also note that the "intermediately susceptible" coral species tend to have flexible relationships and can associate with multiple symbionts. Through this lens, the differential susceptibility of this group can be better explained based on the algal symbionts they associate with.

Background symbionts also appear to play a role in SCTLD susceptibility. Even hosting background amounts of *Breviolum*, (~25%) increases the risk of SCTLD infection. This could explain why large colonies of *O. faveolata*, which tend to have a mosaic pattern of symbionts across individual colonies, can show patchy distributions of SCTLD incidence within colonies. Additionally, these results, in addition to our understanding of how environmental factors influence symbiont assemblages, may help better explain why corals living less than two miles distant from the first reports of SCTLD in turbid chronically stressful environments that tend to favor *Durusdinium* may have survived the disease outbreak.

Although much of the results of Task 1 and Task 2 are finalized, further investigations are being explored. One of the overall takeaways from this experiment is that even though corals associated with *Breviolum* appear to be more than two times as susceptible to SCTLD than those associating with *Durusdinium* or *Cladocopium*, *Durusdinium* and *Cladocopium*-dominated individuals are not immune to SCTLD. One major point of discussion is the interpretation of the *ex-situ* lab results vs. *in-situ* field observations. Corals that were exposed to SCTLD remained in a "disease bath" for ~60 days. During this time there was a constant and concentrated influx of SCTLD (either from source colonies or disease progression from cores in the experiment). So, even though *Cladocopium* and *Durusdinium*-dominated hosts may not be immune in a lab trial, when translated to the field it is unlikely waterborne disease concentrations reached equivalent levels on reefs.

These findings also offer some implications for field observations, like the spatio-temporal symbiont mosaics in "intermediately" susceptible coral species, like *O. faveolata*. Algal community structure in *O. faveolata* tend to be fairly mosaic with portions of colonies associating with different algal symbionts based on micro-habitats (Kemp et al., 2015). In reference to these findings, disease dynamics in such colonies

may present haphazardly with some sections of a colony succumbing to SCTLD while others remain resilient. Since these algal communities are rather fluid, this begins to explain why repeated disease outbreaks on an individual are possible.

With respect to the preliminary results from 16S microbial sequencing, we found significant microbial shifts between healthy and lesion samples ($p<0.001$), and when dominant algal taxa are factored into these results, there are significant differences in the microbial composition among corals associated with *Breviolum* and *Durusdinium*. Furthermore, *M. meandrites* which exclusively associated with *Breviolum* B1, and did not modify their associations following heat stress, remained dominated by Rhodospirillales which have been associated with SCTLD lesions. *Durusdinium* cores that did not get SCTLD did not increase the relative abundance of this group. Further investigation and analysis are necessary to validate and better interpret these results.

A final methodological conclusion from this study is the use of non-contact assays for large-scale SCTLD assays. Previous experiments have relied on the use of contact assays to transmit SCTLD. In contrast, our experiment used a "disease bath" approach to create experimental tanks that introduced healthy cores to SCTLD using an approach that more closely mimics the waterborne pathways by which SCTLD is transmitted in the field. We recommend its use in future studies.

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