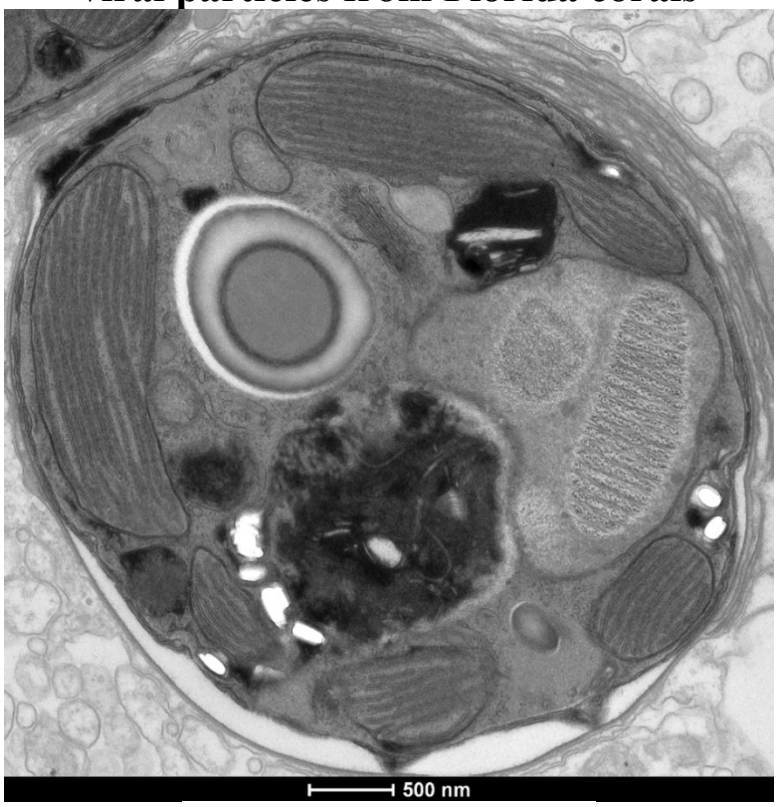
Identification and virulence testing of symbiont-associated viral particles from Florida corals



Identification and virulence testing of symbiont-associated viral particles from Florida corals

Final Report

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Management Summary (300 words or less)

The causative agent for SCTLD is still unidentified and recent studies have suggested the role of pathogenic viruses, which could be infecting the microalgal endosymbionts. Therefore, this project aimed to determine if pathogenic viruses are involved with SCTLD and if any specific viruses are involved. Utilizing transmission experiments with naïve coals, laboratory-infected corals and their associated controls were preserved for RNA sequencing and transmission electron microscopy (TEM). The use of RNA sequencing would identify constituents of the viral community (the virome) present on these corals, while TEM would allow for the observation of cellular changes and potential viral particles within coral cells. In addition, infection experiments with microalgae isolated from corals were conducted to see if SCTLD could be transmitted to them. Lastly, a time-course infection experiment was conducted to collect various sample types over time for SCTLD infections. For the transmission experiments, successful disease transmission was observed, and these samples were saved away for RNA sequencing and TEM. While there was an overall increase in virome diversity, there were interspecies differences in the responses to disease with no singular virus seemingly increasing in abundance during infection for all coral species. The TEM analysis did find evidence of virus-like particles (VLPs) in the coral endosymbionts; however, they were present in both healthy and diseased corals. Further, infection experiments with the microalgae suggested that the cultures used were not susceptible to SCTLD. However, over 200 samples were collected from the time-series experiments, which will provide a much clearer analysis of SCTLD for an upcoming project.

Executive Summary (max 1 page)

As stony coral tissue loss disease (SCTLD) continues to spread throughout the Caribbean and continues to kill off corals in Florida, the causative agent responsible for this malady is still unidentified. Because the causative agent is still unknown, the development of more targeted treatments and feasible diagnostic tools is severely hampered. Therefore, the objective of this project was to combine disease transmission experiments, RNA sequencing, and transmission electron microscopy to identify any potential etiological agents responsible for SCTLD.

Previous studies by other research groups have suggested that pathogenic viruses may play a role in SCTLD and could be infecting the microalgae endosymbionts. Therefore, our approach of using RNA sequencing instead of DNA sequencing (e.g., 16S rRNA sequencing, metagenomics) can capture the virome present in diseased samples. This approach was paired with the use of naïve corals that were collected from areas without SCTLD present (or before disease arrived), which would reduce background "noise" and help with identifying SCTLD-associated microbes. Additionally, samples used for RNA sequencing were also processed for transmission electron microscopy (TEM), which could reveal detailed changes to the corals at the cellular level as well as viral particles. Further, infection experiments with microalgae isolated from corals were conducted to see if SCTLD could be transmitted to them. Lastly, a time-course infection experiment was conducted to collect various sample types over time for SCTLD infections.

After RNA sequencing, virome profiling had revealed a shift in the coral virome in the presence of SCTLD, however analyses are still ongoing. At this time there is no singular putative causative viral agent associated with SCTLD. Interestingly, naïve and SCTLD diseased corals had signs of obvious filamentous viral-like particles (VLPs) across coral species. There was no significant difference in VLP abundance, membrane separation from symbiosomes, or the presence of crystal-like structures (potentially stress related) between healthy and diseased samples. Future measurements will include average size of symbionts, organization of symbionts within the tissue, and quantification of coral tissue. However, this led to the development of TEM processing protocols for corals that could produce consistent results and will be used for future studies. For the microalgae infections, there was no difference between cultures exposed to healthy samples versus disease samples. This was consistent with the results from independent studies conducted by two other research laboratories. Lastly, over 200 samples were collected from the time-series experiments. These samples will be used for a much larger and more detailed analysis of SCLTD progression via virome, microbiome, proteome, histological, TEM, and immunological analysis all done on shared samples.

Acknowledgements

We would like to thank the additional members of the Ushijima lab who helped with this project, Sukanya Dayal and Yesmarie De La Flor, the personnel at the Smithsonian Marine Station at Fort Pierce for all their help, especially Kelly Pitts, Jay Houk, Woody Lee, Mackenzie Scheuermann, and Kathryn Toth, Dr. Alison Taylor and Elizabeth Elliot at UNCW's Richard M. Dillaman Bioimaging Facility, and the personnel at the Keys Marine Lab as well as Dr. Karen Neely and her team who have helped with diseased coral collection.

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List of Acronyms

- Braken = Bayesian Re-estimation of Abundance with KRAKEN)
- CNAT = *Colpophyllia natans*
- DSTO = *Dichocenia stokesii*
- FL DEP = Florida Department of Environmental Protection
- FSW = filtered seawater
- HTS = high throughput sequencing
- MMEA = *Meandrina meandrites*
- MCAV = M*ontastraea cavernosa*
- OFAV = Orbicella faveolata
- RT-PCR = reverse transcription-polymerase chain reaction
- SMS = Smithsonian Marine Station
- SCTLD = Stony Coral Tissue Loss Disease
- SMS = Smithsonian Marine Station
- TEM = transmission electron microscopy
- UNCW = University of North Carolina Wilmington
- USDA ARS= United States Department of Agriculture Agricultural Research Service
- VLP = virus like particle

1. PROJECT DESCRIPTION

1.1. Background

Florida's coral reefs are currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of Florida's Coral Reef, and south to the Dry Tortugas. The best available information indicates that the disease outbreak is continuing to spread southwest and throughout the Caribbean.

Previous studies, including those by our research group, confirmed SCTLD to be an infectious disease (Aeby *et al.*, 2019; Muller *et al.*, 2020; Meiling *et al.*, 2021). However, the exact etiological agent has yet to be identified. Several bacterial groups are associated with disease (Meyer *et al.*, 2019; Rosales *et al.*, 2020; Becker *et al.*, 2021) and broad-spectrum antibiotics are mostly effective against disease lesions (Aeby *et al.*, 2019; Neely *et al.*, 2020; Shilling *et al.*, 2021; Walker *et al.*, 2021). It is unclear if bacteria are initiating disease or are playing more of an opportunistic role. For example, the bacterial pathogen *Vibrio coralliilyticus* may be causing coinfections with SCTLD that exacerbate lesion progression, but pure cultures of this microbe are unable to consistently initiate disease (Ushijima *et al.*, 2020). This was an important and concerning revelation that further complicates the situation, but it allowed researchers to rule out this pathogen as a primary agent, although it remains a threat to corals.

A recent study had identified viral-like particles (VLPs) within coral tissue that were associated with the microalgae symbionts, which appeared to resemble filamentous RNA viruses (Work *et al.*, 2021). The authors of that study suggested they may belong to the viral family Alphaflexiviridae based on morphology alone, and sequences of this family have been identified in USVI corals, albeit in both healthy and diseased specimens (Veglia *et al.*, 2022). Also, these VLPs were observed in both diseased and apparently healthy corals under TEM, so no connection between these VLPs and disease has yet to be established. There have been other described viruses associated with corals and their symbionts unrelated to SCTLD (Wilson *et al.*, 2001; Lohr *et al.*, 2007; Thurber *et al.*, 2009). Viruses typically outnumber bacteria in a 10:1 ratio in marine systems, so their mere presence does not provide strong evidence for their role as a primary pathogen.

The presence of these VLPs could be latent infections that manifest during host stress, which was observed in the previous viral studies (Wilson *et al.*, 2001; Lohr *et al.*, 2007; Thurber *et al.*, 2009). Alternatively, they could also be an opportunistic infection that occurs after a primary agent infects, or SCTLD may be a polymicrobial disease that requires infection with multiple pathogens. The latter two scenarios are observed with other coral diseases with the primary and secondary infections of *Montipora* white syndrome in Hawaii (Beurmann *et al.*, 2017) and the polymicrobial black band disease (Cooney *et al.*, 2002; Meyer *et al.*, 2016), respectively.

This project is building upon the current work of our research group, which is focusing on identifying any VLPs associated with diseased corals, determining if they are specific to SCTLD lesions, and establishing if there are any associations between VLPs and the start of SCTLD lesions. We already have several samples of SCTLD lesions as

well as healthy corals collected from the Key West Nursery in July 2017 and the Dry Tortugas National Park in January 2020 before the arrival of SCTLD. These samples of corals potentially naive to SCTLD will be advantageous to our study as it is unclear if apparently healthy corals in Florida's endemic zones are harboring latent infections, which could be problematic if we are dealing with a viral pathogen. These samples are currently being processed with their total RNA being sequenced (metatranscriptomics) to identify any RNA viruses present as well as being used for TEM to characterize any VLPs present, especially if there are any that resemble the ones described in Work et al. 2021. Funding from FL DEP provided support for more samples to be processed as well as the first steps of a time-course experiments so we can analyze SCTLD lesions at the early, ongoing, and late stages of infection. The results from this work significantly contributed to the knowledge pool of SCTLD and had direct implications for the management of this disease.

The objectives of this project were to 1) identify the filamentous viral-like particles observed to be associated with microalgae within Florida coral holobionts, and 2) determine if viral-like particles are associated with disease. The Tasks of this project included:

Task 1: Identify potential viral pathogens.

a) Extract total RNA from healthy and diseased corals for RNA sequencing to generate sequencing data.
b) Computational analysis of sequencing data to identify any potential viral sequences associated with healthy and diseased corals.
c) Use TEM to describe any viral-like particles associated with healthy and diseased coral holobionts.
d) Develop PCR assays for screening environmental or laboratory samples.

Task 2: Determine if microalgal-associated viruses are pathogenic.

a) Test Koch's postulates with inoculation of microalgae or corals with disease samples.
b) Conduct a time-series experiment with SCTLD using complementary analysis (TEM, RNA sequencing, microbiome sequencing).

This work directly addressed **SCTLD Research Priority 1: Is SCTLD a biotic, abiotic, or combination disease?** Specifically, area 1a: Conduct a time-series study of SCTLD, from pre-exposure to tissue necrosis, using holistic and complementary methods of analysis; and 1c: Build on existing research to isolate and characterize the virus-like particles (VLPs) and/or related viruses observed in transmission electron microscopy (TEM) slides and develop probes to detect the relevant VLPs and/or viruses.

1.2. **Coral Reef Management Applications**

A major hurtle managers face with SCTLD is a lack of feasible diagnostic options as well as the disadvantage of an unknown etiological agent(s) when making management decisions. To address these issues, etiological agent(s) must be identified and a clear link to disease must be established. Determining if the cause of SCTLD is viral, bacterial, or polymicrobial in origin, would directly impact how diseased specimens are handled and

how biosafety is managed. For example, marine viruses may stay viable for longer periods of time than bacteria in environmental samples while any means of sterilization (e.g., filtration or disinfectants) would be different for bacterial versus viral pathogens. Another future outcome from this research is the development of feasible diagnostic tools. While SCTLD is prevalent in the Caribbean and most tissue loss cases are attributed to this disease, coral diseases cannot normally be diagnosed in the field (Work and Aeby, 2006). Further, we have no tools to screen local environments (i.e., sediment or water) for SCTLD pathogens for restoration efforts or study the spread of SCTLD through vectors like ballast water (Rosenau, 2021). So far it appears that through histology, SCTLD could be diagnosed to some degree (Landsberg *et al.*, 2020) however, this is an incredibly low-throughput and slow process that is not feasible for managing a currently spreading disease. Additionally, SCTLD could appear similar to other tissue loss diseases based on histology. The development of something like a PCR test specific for SCTLD agents would greatly speed up diagnostics. Our research lab (in collaboration with mAbDx Inc.) is already working with a rapid, antibody-based test for an opportunistic infection associated with SCTLD (Ushijima *et al.*, 2020). Therefore, this project has important implications for the development of tools and protocols for direct tracking and management of SCTLD.

The outcomes of this project will be incorporated into an on-going coral disease response effort which seeks to improve understanding about the scale and severity of the coral disease outbreak on Florida's Coral Reef, identify primary and secondary causes, identify management actions to remediate disease impacts, restore affected resources, and ultimately prevent future outbreaks. As such, collaboration amongst partners is encouraged when appropriate to avoid duplication of efforts and ensure alignment of needs. Coordination with other Principal Investigators is recommended and required, as appropriate.

2. METHODS

2.1. **Coral Collections and Transmission Experiments**

2.1.1. Coral Collections

All diseased corals used in this project were collected from the Florida Keys under permit number FKNMS-2022-049 issued to Dr. Blake Ushijima and permit number FKNMS-2019-160-A1 issued to Dr. Valerie Paul by The National Oceanic and Atmospheric Administration, Office of National Marine Sanctuaries. All corals were collected while on SCUBA using a hammer and chisel. All corals were transported to the Smithsonian Marine Station (SMS) to be held at the quarantine facility by collaborator Dr. Valerie Paul. At the SMS, diseased corals were maintained in oceanic seawater filtered down to 0.22 µm (FSW) and UV-sterilized to kill or deactivate any bacterial cells or viral particles, respectively. The seawater was kept in the dark and continuously recirculated through a UV-sterilizer, 20 µm-pore filter, and activated carbon filter. This treated seawater was also used for all experiments and was filtered down to 0.22 µm again prior to use. The corals were fragmented into manageable fragments (usually ~ 25 cm^2) using a masonry saw (Husqvarna MS 360) while smaller fragments would then be precision cut using a diamond band saw (Gryphon AquaSaw). Healthy and diseased

corals were not cut at the same time, using the same equipment, or without substantial disinfection between uses.

Naïve healthy corals mentioned in this report were collected during a collection cruise to Dry Tortugas National Park in January 2020 or were collected from the Key West nursery in the summer of 2017, both of which were before the arrival of SCTLD in those areas. These corals were kept at the SMS separate from other healthy corals in the treated seawater described above. The seawater in each holding tanks was constantly circulated through a UV-sterilizer and 20 μm-pore filter to maintain biosecurity.

2.1.2. Transmission Experiments

Various transmission experiments were set up to infect naïve coral fragments with "fresh" SCTLD using diseased fragments from the field. The transmission experiments used an experimental set up identical to previously described studies (Aeby *et al.*, 2019, 2021), with a few minor modifications. For these transmission experiments only contact transmission was used, i.e., the diseased fragment was physically put onto contact with the healthy corals. Control tanks use naïve fragments from the same colonies as the experimental corals except they were put into contact with naïve corals belonging to a different genotype. Water changes were conducted every other day and photographs were taken daily to visually monitor for disease transmission. Successful disease transmission was observed as tissue loss which may or may not include localized discoloration. If progressive disease lesions were observed, then the fragment was preserved in RNAlater and frozen at -80 °C.

2.2. **RNA Sequencing**

2.2.1. RNA Extraction

Coral fragments frozen in RNAlater and stored at -80 °C were thawed on ice. Approximately 30 mg of tissue was suspended in 600 μL of RLT buffer. The samples were cryogenically pulverized at 27 Hz for 3 minutes in a Retsch Mixer Mill. Following homogenization, the samples were centrifuged for 3 minutes at 15,000 x g and the supernatant was collected. One volume of 70% ethanol was added to the lysate and mixed gently. 700 μL of the sample was transferred to an RNeasy spin column and centrifuged for 30 seconds at 8000g. 300 μL of buffer RW1 was added to the column and spun followed by 500 μL of buffer RPE. The samples were eluted in 50 μL of RNase free water. DNase treatment followed immediately using the TURBO DNase kit following manufacturer's instructions. The samples were nano dropped and analyzed for size and quality using the AATI Agilent Fragment Analyzer at the Cornell Biotechnology facility.

2.2.2. Sequencing Prep:

The Samples were enriched for poly-adenylated (Poly-A+) transcripts and sequenced on the Illumina NextSeq 2K P2 2x100 paired-end reads at Cornell's Biotechnology center.

2.2.3. Data Analysis

The raw sequences were subjected to quality control parameters, low-quality reads were trimmed and the 3' adapter bases were removed from the reads. Using TRINITY, de novo transcriptomes were built, using the software packages Inchworm, Chrysalis, and

Butterfly. The transcriptomes were split into host and non-host transcriptomes. Host reads were assembled into candidate transcripts with Trinity, and super transcripts were generated. Non-host reads were aligned to viral sequences using the NCBI Viral Reference database and KrakenTools packages. Viral sequences were retrieved, and the package Braken was used to compute the abundance of viral species in the transcriptomes. KrakenTools KRONA plot script was used to generate visual plots of viral diversity within each sample. Geneious was used to map the read coverage of the putative viruses in each sample. Consensus viral sequences were verified with Geneious. All RNA sequencing data will be provided on a physical hard drive shipped to the DEP office.

2.2.4. RT-PCR

RNA was extracted from the diseased *C. natans* (CND30) corals using the Qiagen RNeasy extraction kit following the manufacturer's instructions. cDNA was synthesized using the iScript cDNA synthesis using OligoDT's and random hexamers according to the manufacturer's instructions. 500 ng of RNA was loaded into each RT reaction. The PCR was performed using Q5 Hot Start master mix and the following set of primers CHFV-1 Set 1, CHFV-1 Set 2, CHFV-2 Set 1, CHFV-2 Set 2 (Veglia *et al.*, 2022), Biden's mottle virus (BMV-1) Set 1, BMV set 2, Cl-Potyvirus universal primers, and Nlb-potyvirus universal primers. The gel was imaged on a 1.5% agarose gel. All the primers stated here were used due to previous hypothesis on Alphaflexiviridae being involved with SCTLD (Veglia *et al.*, 2022) or primers designed based on VLP morphology from (Work *et al.*, 2021).

2.3. **Transmission Electron Microscopy**

2.3.1. Sample Prep

Samples were fixed using a recipe originally from Thierry Work (Work *et al.*, 2021) to standardize fixation across experiments for comparative analyses. Samples were fixed in a combination of 2.5% glutaraldehyde and 2% paraformaldehyde in Instant Ocean (pH 8, 35ppt) and kept at 4°C. Samples were then decalcified in 10% EDTA (pH 7) shaking at room temperature until the skeleton dissolved. Coral tissue was then cut into 1 mm^3 chunks. Samples were rinsed 3 times for 15 minutes each with 0.35 M sucrose in a 0.1 M sodium cacodylate buffer solution. Samples were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at room temperature. Samples were rinsed twice for 15 minutes each with 0.1 M sodium cacodylate buffer. Samples were then dehydrated with a series of ethanol solutions (50, 70, 95, 100, 100%) for 15 minutes each. Samples were then added to a 1:1 mixture of Spurr's resin with 100% ethanol for 1 hour. Samples were then embedded in 100% Spurr's resin overnight. Fresh 100% Spurr's resin was added to samples and samples were put into a 70 °C vacuum oven overnight. Then 90 nm sections were cut with a diamond knife and placed onto a 0.25% formvar coated copper grid. Sections were stained with UranyLess for 5 minutes, rinsed with ultrapure water, stained with lead citrate for 5 minutes, rinsed with ultrapure water, and allowed to dry overnight. Sections were then imaged using a FEI Tecnai Spirit Bio Twin TEM at UNCW's Richard Dillaman Bioimaging Facility.

2.3.2. Sample Analysis

A protocol for quantitative measurements of TEM images was developed by UNCW. Each sample was imaged at the same magnification to obtain total area of gastrodermis, and number of symbionts present in the sample within an area on the grid. Each symbiont within this grid area on one section was imaged. Each symbiont was scored for size, presence of pyrenoid, presence of electron dense bodies, chloroplast gigantism, cavity inside membrane, size of cavity, separation from the symbiosome (the membrane-enclosed compartment within the host tissue housing the endosymbiont), visible thylakoid membranes, presence of VLPs, types of VLPs, number of starch granules, chloroplast breakdown, presence of crystal-like structures, and size of crystal-like structure area. Samples were measured using ImageJ.

2.4. **Microalgae Infection Experiments**

2.4.1. Microalgae Cultures

Cultures used in these experiments were obtained from Dr. Mary-Alice Coffroth's collections via the Smithsonian Marine Station. Cultures included *Symbiodinium* code Mf 10.02~ and *Breviolum* code Mf 1.05b.OI SCI 07-205. Cultures were kept in 1x f/2 (Guillard's f/2 nutrient solution) made with Instant Ocean (35 ppt, pH 8) under full spectrum lights at 35 µmol/m^2/sec in a 27°C incubator.

2.4.2. Infection Experiments

All seawater used in these experiments was collected offshore, filtered through a 0.22 µm filter and kept out of direct sunlight with constant recirculation through a UV-sterilizer, 20 µm pore filter, and an activated carbon filter. Prior to use, the seawater is filtered through a 0.22 µm filter.

In July 2022, a diseased *M. cavernosa* was left in seawater for one week to condition the seawater. The seawater was then added to cultures of *Symbiodinium* and *Breviolum* after being filtered through various filtration sizes. The samples included cultures by themselves with no diseased water added, cultures with the diseased water, cultures with diseased water that was filtered through of 0.45 µm filter, and cultures with diseased water that was filtered through a 5 µm filter. The cultures were exposed to the water for 12 days. Samples were saved in RNAlater and TEM fixative. These samples were not saved in paraformaldehyde, which was a protocol developed after this experiment. Raw data from these experiments were submitted to FL DEP with this report (titled **Microalgae Infection Experiments).**

In May 2023, diseased and healthy water was taken from the time series experiments. The healthy water had only healthy corals in it with no signs of SCTLD. The diseased water had a diseased coral in it that showed signs of transmission to the other corals in the tank. The water was from 4E and 4C (see time series section) and was filtered through a 5 µm syringe filter. Water from the healthy and diseased corals along with a filtered seawater control were added to cultures of *Symbiodinium* and *Breviolum* in quadruplicate in a 1:1 ratio of water to culture. Samples were saved in RNAlater, TEM fixative, and paraformaldehyde for fluorescence microscopy. Cultures were exposed for 8 days.

2.5. **Time Series Transmission Experiment**

2.5.1. Naïve and diseased corals

The time-series experiments used naïve *M. cavernosa* (*n*=4) and *O. faveolata* (*n*=1) that were collected from the Key West Nursery in July 2017 before the arrival of SCTLD. These corals were kept at the SMS separate from other corals in a 200-gal closed system using the sterilized seawater described above. Additionally, the system's water is constantly circulated through a UV-sterilizer and 20 µm-pore filter.

These naïve corals were fragmented using a masonry saw (Husqvarna MS 360) into approximately 4 x 4 cm fragments 10 days prior to the beginning of the experiment to allow time to heal. The five diseased *C. natans* colonies used in this experiment were collected from various reefs in the lower Florida Keys. Each diseased sample was trimmed using a masonry saw so that a disease lesion was present with approximately 8 x 8 cm of living tissue adjacent to the lesion. Each experimental replicate consisted of pre-exposure samples, and samples from both an experimental tank (with a diseased donor fragment) and a control tank (with a naïve donor fragment; outlined in **Figure 8.4.1**). A separate diseased *C. natans* colony was used per experimental replicate. Each experimental tank will consist of one diseased fragment (*Diseased Colony 1* in **Figure 8.4.1**) and four naïve fragments from the same colony (*Naïve Colony 2* fragments depicted in **Figure 8.4.1**). I.e., four naïve fragments are exposed to disease at the same time. The control tank consisted of four naïve fragments from the same naïve colony as in the experimental replicate (*Naïve Colony 2* in Figure 1) and one naïve fragment from a different colony (*Naïve Colony 3* in Figure 1).

2.5.2. Time Series Experimental Setup

After healing and before experimental set up, one fragment from each diseased colony and one fragment from each naïve colony was split into five fragments (approximately 1 x 1 cm) using a band saw for histology, TEM, metabolomics, multi-omics, and immunological assays (see below for further explanation of sample types). This served as *Timepoint 1* (pre-experiment) for this experiment. Control/healthy corals were cut before any experimental/diseased corals using a separate saw, which were thoroughly cleaned with 80% ethanol after each use. The healthy and diseased fragments were then arranged in an experiment and control tank as depicted in **Figure 8.4.1**. After 48 h of exposure, another experimental and control fragment was taken for *Timepoint 2*, and each fragment was split into five samples like the previous timepoint. There was a total of five timepoints during this experiment, however, *Timepoints 3-5* were taken depending on the development and progression of tissue loss lesions. The sampling record and progression of the disease lesions are depicted in **Figure 8.4.2**. The main signs noted was tissue loss and localized discoloration of the tissue, indicative of SCTLD (Aeby *et al.*, 2019, 2021). Representative photos of the disease lesions are depicted in **Figure 8.4.3.** and all photos taken were organized and submitted to FL DEP with this report **(**titled **Time Series File Folder)**.

The coral experimental set up used was similar to a previously described system for transmission experiments (Aeby *et al.*, 2021). Briefly, corals were kept in five L tanks filled with FSW (described above) under ambient sunlight outside under a 50% shade cloth in larger secondary tables filled with freshwater to control temperature. Chillers and heaters were used to adjust the temperature of the freshwater to keep the aquariums between 27 and 28 °C. Partial water changes were conducted every day for the first nine days and then every two days after that. All fragments were photographed every day to

track coral health and disease progression. Ammonia, nitrate, nitrite, and pH were periodically tracked using rapid test strips (API). All aquariums, water scoops, and grating used for this experiment were sterilized using a calcium hypochlorite solution (~10% final concentration), rinsed thoroughly with freshwater and left to dry for at least 48 h prior to use. Strict biosecurity measures were taken to ensure no cross-contamination and the instruments used for one tank were not used in another tank without sterilization.

2.5.3. Time Series Experiment Sample Types

Each of the five sample types collected from the fragments were for subsequent multi-omics extraction/analysis, metabolomics, histology, TEM, and immunological assays. Samples were saved in 20% Z-fix (made with FSW) for histology (at room temperature), a glutaraldehyde/paraformaldehyde mixture for TEM (at 4 °C), or flash frozen at -80 °C for metabolomics, meta-omics, and immunological analysis. This process will be repeated using apparently healthy corals from endemic zones for fiscal year 2023-2024 while all major analysis of these samples will be conducted in fiscal year 2024 – 2025.

2.5.4. Time Series Experiment Data analysis

Disease and disease progression on each fragment was determined visually and through the daily photographs taken of every fragment. Tissue loss and localized bleaching was determined by the disease signs described in previous experiments (Aeby *et al.*, 2019, 2021; Ushijima *et al.*, 2020). Additionally, all diseased fragments were screened for the toxic protein VcpA produced by the opportunistic pathogen *V. coralliilyticus* using the *VcpA RapidTest* immunoassay using the test and protocol previously described (Ushijima *et al.*, 2020). For disease progression on the donor fragments, tissue loss and was standardized as percent tissue loss over time and was measured on ImageJ. The procedure and calculations are described in a previous publication (Ushijima *et al.*, 2023).

3. RESULTS

3.1. **RNA Sequencing Results**

3.1.1. Viral Families detected in SCTLD corals and asymptomatic control corals

RNA was extracted by tissue scraping using liquid nitrogen, a bead mill homogenizer, and the Qiagen RNeasy extraction kit. The following coral samples were prepped for RNA-seq: Four experimental fragments and two control fragments of *Meandrina meandrites* (MMEA), two experimental fragments and one control fragment of M*ontastraea cavernosa* (MCAV), and three experimental fragments of and one control fragment of *Dichocenia stokesii* (DSTO). The samples were sent to the Cornell University sequencing facility for Illumina high throughput sequencing (HTS). HTS generated an average of 50-70 million trimmed high-quality reads from SCTLD symptomatic and asymptomatic controls of MMEA, MCAV, and DSTO. From these reads, approximately 0.05-0.01% of reads from each sample were classified as viral, which was expected as the majority of the reads matched to the host transcriptome. The viral diversity of corals is shown through the detection of different viral families. Every sample regardless of SCTLD presence or absence included reads mapping to viruses in the following families: baculoviruses, hantaviruses, *Peribunyaviridae*, and

Phycodnaviridae viruses (**Figure 8.1.1**). Reads aligning to *Peribunyaviridae* were detected in SCTLD-symptomatic DSTO, and not in the control asymptomatic sample. However, viruses in the order *Bunyavirales* were detected in both SCTLD and control samples, but with greater abundance in the SCTLD samples. Reads matching species in the *Tospoviridae* were detected in all samples sequenced, regardless of disease status. In the MMEA virome, reads aligning to the family *Phycodnaviridae* were present and abundant in the SCTLD samples, and not detected in the healthy samples. Linear mixed modeling analysis is in progress in consultation with a statistician to determine whether synergistic interactions in the virome correlate with disease status. There are several classes of viruses in the healthy and diseased fragments for all coral species, aminotype analysis will be performed to determine whether there is an association between a particular aminotype and SCTLD. Aminotyping is the prediction of differences in amino acid sequences for viral strains. It is useful in the analysis of virome data to infer relationships between certain viral sequences and disease or stress. Given that it may be challenging to identify a singular cause of SCTLD, aminotyping may give important information on any key viral drivers of the disease. A paper describing these datasets is in preparation for submission to a peer-reviewed journal. All raw data will be shared in public repositories such as NCBI, FigShare, and as supplementary documents published with the document.

3.1.2. Species Abundance in SCTLD corals and asymptomatic corals

The Braken (Bayesian Re-estimation of Abundance with KRAKEN) statistical method was used to compute the abundance of species in the samples. For MMEA samples, approximately 31% of the virus reads detected were classified in the order Bunyavirales. Among these reads, 27% aligned to Pepper chlorotic spot virus (PCSV), an ortho-tospovirus. Further analysis using Geneious detected similarities but not an exact match to (PCSV). This putative ortho-tospovirus is detected in the MMEA-diseased sample and further molecular characterization would be required to determine if a real ortho-tospovirus was present in the samples or if these reads originated from endogenous virus elements, known to be integrated into coral endosymbiont genomes.

3.1.3. RT-PCR

We were unable to amplify virus sequences from the CnD30 SCTLD sample. All primers set tested negative for CHFV-1, CHFV-2, BMV, and the potyvirus universal primer sets. Due to the negative RT-PCR results correlating with the absence of these sequences with the RNA sequencing data, we continued relying on the RNA sequencing data instead of PCR.

3.2. **TEM Results**

3.2.1. TEM Quantitative Measurements

To include more quantitative data with the TEM, post-imaging analysis was done. Images from eight corals were analyzed using ImageJ. These corals included the healthy control and transmitted diseased *M. meandrities* genotype 21, *D. stokesi* genotypes 2 and 3, and *M. cavernosa* genotype 3. To measure the symbionts, each symbiont within a grid section was measured for presence of VLPs, number of symbionts separated from

symbiosome, and the number of symbionts with crustal-like structures. Other measurements such as average size, chloroplast gigantism, and internal cavities will be measured for each symbiont. 77 symbionts from healthy *M. meandrities* 21 and 59 from diseased *M. meandrities* 21 were measured. 38 symbionts from healthy *D. stokesi* 2 and 45 from diseased *D. stokesi* 2 were measured. 49 symbionts *D. stokesi* 3 healthy and 37 from diseased *D. stokesi* 3 were measured. 19 symbionts from diseased *M. cavernosa* 3 and 18 from healthy *M. cavernosa* 3 were measured.

Obvious filamentous VLPs were present in 29.87% of symbionts in healthy *M. meandrities* 21 and 27.11% in the diseased sample. They were present in 39.47% of the symbionts in healthy *D. stokesi* 2 and 26.67% in the diseased sample. 28.57% of the symbionts in healthy *D. stokesi* 3 had signs of obvious filamentous VLPs while 45.96% in diseased *D. stokesi* 3 had signs. They were present in 33.33% of the healthy *M. cavernosa* 3 and 5.36% in the diseased (**Figure 8.2.1**).

Separation or swelling of the symbiosome was seen in 64.94% of symbionts in healthy *M. meandrities* 21 and 67.80% in the diseased sample. It was present in 15.79% of the symbionts in healthy *D. stokesi* 2 and 37.78% in the diseased sample. 25.64% of the symbionts in healthy *D. stokesi* 3 had signs separation or swelling of the symbiosome while 45.95% in diseased *D. stokesi* 3 had signs. It was present in 22.22% of the healthy *M. cavernosa* 3 and 100% in the diseased (**Figure 8.2.2**).

Crystal-like structures were seen in 12.99% of symbionts in healthy *M. meandrities* 21 and 84.75% in the diseased sample. They were present in 0% of the symbionts in healthy *D. stokesi* 2 and 0% in the diseased sample. 0% of the symbionts in healthy *D. stokesi* 3 had crystal-like structures while 32.43% in diseased *D. stokesi* 3 had signs. They were present in 5.55% of the healthy *M. cavernosa* 3 and 36.84% in the diseased (**Figure 8.2.3**). All raw data and TEM images associated with this task was submitted to FL DEP with this report (titled **TEM images and files).**

3.3. **Microalgae Infection Experiments**

3.3.1. Trial 1 results (Summer 2022)

For the *Breviolum* cultures, the cell counts appeared to increase over time for every treatment. The culture exposed to 0.45 µm diseased water had the highest cell counts on day 8. The cultures exposed to unfiltered diseased water had the lowest cell counts by day 8, but still had an increase in cell counts over time. For the *Symbiodinium* cultures, the cell counts appeared to increase over time for every treatment expect the culture exposed to unfiltered disease material. These results are consistent with the previous four trials run by the Smithsonian Marine Station and UNCW. All raw data files and representative images were submitted to FL DEP with this report (titled **Microalgae Infection Files).**

3.3.2. Trial 2 results (Summer 2023)

The second trial of microalgae exposures utilized the tank water from some of the time-series transmission experiments described here. Specifically, these trials used water from time-series tanks 4C (control tank with just naïve, healthy corals) and 4E (which contained an infectious *C. natans* with tissue loss). The diseased fragments in tank 4E transmitted disease to various *M. cavernosa* fragments via tank water (i.e., the infected fragments were not in direct contact with the *C. natans*), so the potential infectious agents should have been present. However, there were no signs of significant microalgae

mortalities over a period of eight days for any of the species used (**Figure 8.3.2**). For the *Breviolum* sp. there was a trend of cell concentrations to be increasing over time in the treatment with diseased tank water and the FSW control, but none of the changes over time in any of the three treatments were significant (n=4, 2-way ANOVA, p=0.0957). For the *Symbiodinium* sp. there were no noticeable trends or significant changes to the cell concentrations for any of the treatments (n=4, 2-way ANOVA, p=0.1307). These results were consistent with the initial trial in Summer 2022 as well as the independent trials run by the Smithsonian Marine Station.

3.4. **Time-Series Experiment**

A transmission experiment using naïve *M. cavernosa* (n=4) and *O. faveolata* (n=1) was completed using diseased *C. natans* and a diseased *M. cavernosa*. This main experiment is still on-going, so all the tables and figures are for the results at the time this report was written. An overview of the sampling timepoints and status of each coral fragment is outlined in a Gantt chart (**Figure 8.4.2.**) as well as daily photographs provided to FL DEP (titled **Time Series Files)** included with this report. The progression of tissue loss was measured for all donor fragments and is depicted in **Figure 8.4.4** with raw data provided in the **Time Series Files** folder included with this report. Some level of disease progression was observed on all diseased donor *C. natans* fragments (n=5) in the experimental tanks except for donor CnD-48 in tank 4E where the lesion appeared to have stopped progressing after minimal tissue loss. Tank 4E is also the only tank where there was no noticeable disease progression (tissue loss or localized discoloration) on any of the naïve *O. faveolata* fragments. However, the *O. faveolata* fragments in tank 4E did appear to be releasing a noticeable amount of mucus into the tank, which was not observed with the corresponding *O. faveolate* fragments in control tank 4C (from the same *O. faveolata* colony).

Some of the diseased donor fragments were replaced with new diseased fragments to ensure disease transmission. The donor diseased *C. natans* fragment (CnD-44) in experimental Tank 1E was replaced on day 19 with a diseased *M. cavernosa* (McD-106) due to the original donor having 100% mortality on day 11 and the remaining recipient fragment not being becoming initially infected. The diseased donor fragment in tank 4E (CnD-47) had acute tissue loss and had 100% tissue lost by day 5 (**Figure 8.4.2. and Figure 8.4.4.**). It was replaced with diseased *C. natans* (CnD-49), which had 100% tissue loss by day 7. A third diseased *C. natans* from diseased colony CnD-48 was added (day 8), but had 100% tissue loss within 48 h. All the diseased corals were screened using the VcpA immunoassay, but all fragments were VcpA negative.

For every experimental fragment that was sampled, the corresponding control fragments were also sampled. We were able to divide each fragment into the five sample types planned for this experiment (histology, TEM, metabolomics, multi-omics, and immunology). The full catalog of samples was provided to FL DEP (titled **Time Series Files)** included with this report.

4. DISCUSSION

4.1. **Investigating SCTLD Using RNA Sequencing**

RNA sequencing has revealed a plethora of putative viruses in the microbiome of SCTLD and asymptomatic (healthy) Florida corals. While there appears to be a shift of the virome when SCTLD experimental and asymptomatic controls are compared, there is not yet enough evidence to pinpoint a single causative virus. Interestingly, virome signatures associated with disease progression are likely to be coral-species specific. For example, there is an increase in the presence of viral reads aligning to the family Peribunyaviridae in the DSTO and MMEA SCTLD experimental fragments compared to their controls, but a decrease of these reads in the MCAV fragments compared to the control fragment. Notably, there are *Phycodnaviridae* sequences detected in all of the samples sequenced, but very few in the MMEA healthy samples. Viruses in the *Phycodnaviridae* family are icosahedral double-stranded DNA viruses and primarily infect algae. Further analyses, such as linear mixed modeling, principal components analysis and aminotyping analysis are needed to conclude if a single virus can be attributed as the causative agents. These analyses, plus additional molecular biology experiments may help to determine whether the viral sequences detected belong to actively replicating viruses.

In this report, the TEM evidence of Florida corals affected with SCTLD and even the healthy corals suggest a viral infection in the endosymbionts. However, it is unclear if infection of these endosymbionts occurs before, after, or during infection of the coral host by an unknown agent(s). Our current analysis pairing RNA-sequencing data with TEM imaging of the uncharacterized VLPs suggests several viruses in the coral virome, however, currently there is no indication of one putative causative viral agent.
While we continue to analyze our RNA-sequencing data, we aim to develop RT-PCR/PCR assays to screen coral and microalgae samples. From the first batch of samples submitted for RNA-seq we used an aliquot of high-quality RNA (500ng) to create a cDNA library using BioRad iScript kit. We did a preliminary PCR with the following primer sets: CHFV-1 and CHFV-2 aligning to the putative *Alphaflexiviridae* sequences identified in (Veglia *et al.*, 2022), BMV primer sets aligning to the *Biden's mottle virus* identified in a previously published zooxanthellae RNA-seq run, and universal primers aligning to viruses in the family *Potyviridae*. In this PCR we were unable to detect any of the viruses. However, the sequences aligning to the *Alphaflexivirdae* viruses detected in our first RNA-seq run have very low identity to CHFV-1 and CHFV-2, and may explain the lack of detection in our RT-PCR. We aim to develop primers to the sequences detected in our RNA-seq run.

4.2. **Investigating of SCTLD Using TEM**

There were visible differences between the healthy controls and the diseased corals within their symbionts, however, these results were just trends and none of the differences were statistically significant. Diseased corals had signs of necrosis or apoptosis, separation or welling of the symbiosome, and chloroplast breakdown. Separation from the symbiosome or swelling of the symbiosome was previously seen in SCTLD samples (Landsberg et al., 2020). The swelling or separation from the symbiosome could be a result of the breakdown of the algal-host symbiotic relationship and could be due to stress.

Crystal-like structures were seen in previous SCLTD samples, deemed crystalline inclusion bodies (Landsberg *et al*., 2020); however, our structures are seen within the

symbiont instead of within the tissue as previously described. It is hypothesized that these crystal structures could be uric acid, which are present when there is high nitrogen to phosphorus ratios (Rosset *et al.*, 2017). There are signs of apoptosis by presentation of possible apoptotic bodies in the *M. cavernosa* 3 samples which could indicate that the disease was at a different progression stage or presented differently in this coral. Further quantification will be needed to fully assess the health state differences and to determine the cellular pathology of SCTLD.

4.3. **Microalgae Infection Experiments**

Various studies have proposed that SCTLD involves a breakdown of the symbiosis between the endosymbiotic microalgae and coral host (Landsberg *et al.*, 2020; Work *et al.*, 2021; Beavers *et al.*, 2023), therefore, it was reasonable to hypothesize that cultures of these microalgae are susceptible to SCTLD. However, the various attempts by labs at the Smithsonian Marine Station and UNCW have found that the *Breviolum*, *Durusdinium*, and *Symbiodinium* cultures we shared are not susceptible to SCTLD. This was further supported by recent communications with Rebecca Gibbel and Marilyn Brandt (University of the Virgin Islands). Gibbel exposed cultures of *Fugacium*, *Breviolum*, *Cladocopium*, and *Durusdinium* to samples from healthy and diseased corals with no significant effects on algal cell concentrations regardless of treatment (Gibble & Brandt, *per. comm*.). These results collectively suggest that these microalgae cultures from Mary-Alice Coffroth are not suitable for infection with SCTLD. We recommend that if this experiment is repeated then different algal cultures/strains should be used. However, if new cultures are isolated from corals then there should be more attention should be put on the purification and identification procedures following the new phylogeny (LaJeunesse *et al.*, 2018) to ensure they are coral endosymbionts and not potential non-symbiotic contaminants.

There are multiple hypotheses as to why these three laboratories have been independently unable to infect these microalgae with SCTLD. First, there are previous studies that demonstrate that viral infections of microalgae only cause cell lysis under stressed conditions (e.g., thermal stress) (Wilson *et al.*, 2001; Lohr *et al.*, 2007). This is reasonable because various latent viral infections will not cause host cell lysis or other cytopathies unless under stressed conditions. However, it has not been demonstrated that SCTLD is initially instigated by a viral pathogen but while coral-to-coral disease transmission can occur under controlled, non-stressful conditions, which is contradictory to the hypothesis that a stress-induced viral infection is taking place (Aeby *et al.*, 2019, 2021; Meiling *et al.*, 2021). If conditions like elevated water temperatures or increased UV radiation are needed for microalgae infection, then one should expect that to be needed for coral infections.

Second, it is still unclear if the cytopathies observed with the microalgae (Landsberg *et al.*, 2020; Work *et al.*, 2021) are the result of a pathogen directly infecting the algal cells, a response from the host, or a secondary infection of the algae. While it is tempting to speculate that an unknown pathogen is infecting these endosymbionts directly; it is plausible that these microalgae, which must maintain some type of immune tolerance with the host (Yuyama *et al.*, 2018), may be adversely affected by the host immune system that is trying to respond to a systemic infection. Therefore, it is possible that

cultures of microalgae outside of their host (the coral) may not respond to exposure to SCTLD samples due to a lack of the host responses or signals.

Third, these microalgae cultures may not be representative of the endosymbionts in corals. Although some species of coral endosymbionts culturable in the laboratory, there are significant physiological changes that occur when shifting from an intracellular relationship to free-living in culture (Maruyama and Weis, 2021). Related to this, is that these microalgae strains have been in culture for the past few decades. It is currently unknown what mutations these cultures have accumulated over the years or if they affect susceptibility to SCTLD.

4.4. **Implications of a Time-Series Experiment**

The samples generated from the time-series experiment are the first set of corals for a larger overarching project to apply various analyses to the same set of samples. This first trial uses naïve corals that have not been contaminated by microbes associated with SCTLD while the upcoming trial, part of the proposal submitted for next fiscal year (Fiscal Year 2023-2024), will use apparently healthy corals collected from endemic zones. Analysis of these two trials will provide a powerful comparison and could reveal a difference between naïve corals and diseased colonies as well as exposed corals from Florida waters. The analysis of all these samples will take place in the following fiscal year (Fiscal Year 2024-2025).

The use of naïve corals is essential for various reasons. First, there is some evidence that suggests SCTLD infections at the cellular level occur before the manifestation of the gross disease signs – tissue loss or localized discoloration (Landsberg *et al.*, 2020). However, the incubation period (the point of infection and the gross signs of disease) is unknown. Further, if SCTLD is a polymicrobial disease requiring various pathogenic microbes, contamination with some of the pathogen consortium could be occurring without corals displaying any signs of disease. Second, the potential involvement of viral pathogens (regardless of they are primary, secondary, or opportunistic pathogens) (Work *et al.*, 2021; Veglia *et al.*, 2022) along with evidence suggesting a depression of key components of the host immune system following SCTLD infection (Beavers *et al.*, 2023) could indicate that the initial infection does not cause gross disease signs. A similar situation occurs with the human pathogen human immunodeficiency virus (HIV) that will cause significant immunodepression resulting in acquired immunodeficiency syndrome (AIDS) after a lengthy incubation period and patients are highly susceptible to a range of bacterial and fungal opportunistic infections (reviewed in Elfaki, 2014). An environmental example is with the Pacific oyster (*Crassostrea gigas*), which have lines bread to survive infection by the virus OsHV-1 that can decimate populations. However, it was discovered that while some oysters can survive OsHV-1 infection, the infection depresses their immunes system to the point that they become more susceptible to infection by the bacterial pathogen *Vibrio aestuarianus* (Azéma *et al.*, 2016). Taking these considerations into account, it may not be possible to fully trust “healthy” corals from endemic zones as true controls for comparative experiments.

5. MANAGEMENT RECOMMENDATIONS

- With no current access to serological tests or PCR for viruses associated with SCTLD, early detection via scouting is strongly recommended.
- New research on development of early disease biomarkers is a priority for early detection and development of field-deployable diagnostic tests. This would benefit greatly from the analysis of the time-series samples.
- Standardizing fixatives and sample preparation methods is recommended for TEM analyses to compare across studies. Quantitative measurements should be utilized for TEM.
- Based on various independent trials conducted by three separate research laboratories, some of the microalgae cultures distributed by Dr. Mary-Alice Coffroth do not seem to be susceptible to SCTLD exposure. It is possible that new microalgae cultures more representative of what is present in Florida corals are needed to conduct laboratory experiments.
- Specific consideration should be taken with the source of apparently healthy corals used in experiments as well as seawater sources and biosecurity implemented in experiments and restoration efforts. Filtration of seawater down to 0.22 μM to remove bacterial pathogens and re-circulation (not just a single pass) through at least UV-sterilizers are recommended to reduce potential viral pathogens.
- Caution should be taken when drawing conclusions based on the identification of viral reads in the RNAseq datasets. Many viruses integrate into the host genome and the reads may not be derived from a real virus infection of zooxanthellae. Moreover, contaminant viruses may be present in the virome due to contaminating sources, such as coral dietary components used in the laboratory
- Due to the seemingly complex nature of SCTLD, a more holistic analysis of a controlled set of samples is suggested. This could be carried out with the samples saved away during the time-series experiment.

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7. TABLES

Table 7.1: Viruses in RNA-seq data: Viruses reads identified at the species level from the high throughput sequencing. Selected samples are displayed representing each species and disease status.

Virus Name	MCAV Control	MMEA control	DSTO control	MCAV experimental	MMEA experimental	DSTO experimental
Choristoneura fumiferana granulovirus	1876	10668	1919	990	10950	3523
Oxbow orthohantavirus	2499	3922	1204	746	1522	1175
Pepper chlorotic spot orthotospovirus	1143	1563	332	83	692	955
Shamonda orthobunyavirus	183	347	192	43	1270	560
BeAn 58058 virus	700	817	164	115	777	687
Proteus virus Isfahan	231	203	13	5	432	175
Chrysochromulina ericina virus	19		16	2	11	24
Propionibacterium phage PFR2	16	15	8	4	265	13
Bacillus virus Bcp1					244	
Choristoneura rosaceana entomopoxvirus		159	38		98	116
Pandoravirus neocaledonia		5	77		13	150
Apocheima cinerarium nucleopolyhedrovirus		103			50	
Pseudomonas phage Lu11		45	24		18	34
Human endogenous retrovirus K	79	65	15	13	69	62
Melanoplus sanguinipes entomopoxvirus	34	4	3	3	6	5
Escherichia virus P1	3	7			32	13
Escherichia virus DE3	5	30		3	64	
White clover mosaic virus	2	12			2	
Brome mosaic virus	2	7	4	12	3	4
Simbu orthobunyavirus	34	31			41	37
Ribgrass mosaic virus		2				

Vibrio phage VH7D		23			3	
Escherichia virus Lidtsur	11	28	10	4	10	11
Phaeocystis globosa virus		35	3	1	21	1
Red clover vein mosaic virus		3			1	
Murid betaherpesvirus 2		1	24			7
Spodoptera litura granulovirus	16		2		8	11
Klebsiella phage ST16-OXA48phi5.4						
Peanut stunt virus					2	5
Moumouvirus	9	6		2	8	6
Synechococcus phage ACG-2014g			13			17
Staphylococcus phage SPbeta-like	15	21	5		22	10
Escherichia virus RCS47	2	10	2			1
Beauveria bassiana victorivirus 1		16			2	
Enterobacter phage phiT5282H					1	
Alternanthera mosaic virus	1				1	
Peridroma alphabaculovirus				21	2	
Sucra jujuba nucleopolyhedrovirus					19	2
Acinetobacter phage AM101	11			19		
Salinibacter virus M8CR30-2			2			
Rhizoctonia solani RNA virus HN008					17	
Synechococcus phage S-WAM1		1	14		3	17
Pandoravirus macleodensis	2	10	3	1	7	2
Cafeteria roenbergensis virus	10			5	15	2

Escherichia virus phiX174	1			1	15	1
Torque teno mini virus 3	1		1	1	1	15
Serratia phage Muldoon		1		1	1	2
Caulobacter virus CcrPW					11	
Squirrelpox virus		6	2		3	3

Table 7.2: The most abundant virus families in the RNA-seq data from selected samples representing each species, and disease status.

	MCAV Control	MMEA control	DSTO control	MCAV experimental	MMEA experimental	DSTO experimental
Betabaculovirus	1876	10668	1919	990	10950	3523
Hantaviridae	2499	3922	1204	746	1522	1175
Tospoviridae	1143	1563	332	83	692	955
Peribunyaviridae	183	347	192	43	1270	560
Chordpoxviridae	700	817	164	115	777	687
Siphoviridae	231	203	13	5	432	175
Phycodnaviridae	19		16	2	11	24
Siphoviridae	16	15	8	4	265	13
Herelleviridae					244	
Poxviridae		159	38		98	116
Pandoraviridae		5	77		13	150
Baculoviruses		103			50	
Myoviridae		45	24		18	34
Retroviridae	79	65	15	13	69	62
Poxiviridae	34	4	3	3	6	5
Myoviridae	3	7			32	13
Siphoviridae	5	30		3	64	
Alphaflexiviridae	2	12			2	
Bromoviridae	2	7	4	12	3	4
Peribunyaviridae	34	31			41	37
Virgaviridae		2				

MCAV= *Montastaea cavernosa*; MMEA = *Meandrina meandrites*; DSTO = *Dichocoenia stokesii*.

Table 7.3: Tanks and coral colony ID organization for the time-series experiment.

Tank	Tank Type	Donor Coral Species	Donor Coral Colony ID	Donor Coral Health State	Recipient Coral Species	Recipient Coral Colony ID	Recipient Coral Health State
1C	Control	OFAV	OfH-106 yellow	Healthy - Naive	MCAV	McH-102(1) blue	Healthy - Naive
2C	Control	MCAV	McH-102(1) blue	Healthy - Naive	MCAV	McH-104 orange	Healthy - Naive
3C	Control	MCAV	McH-104 orange	Healthy - Naive	MCAV	McH-101 white	Healthy - Naive
4C	Control	MCAV	McH-101 white	Healthy - Naive	MCAV	McH-103(4) red	Healthy - Naive
5C	Control	MCAV	McH-103(4) red	Healthy - Naive	OFAV	OfH-106 yellow	Healthy - Naive
1E	Experimental	CNAT	CnD-44 Replaced with McD-106 on 6/5/2023 (day 19)	Diseased - Field Diseased - Field	MCAV	McH-102(1) blue	Healthy - Naive
2E	Experimental	CNAT	CnD-45	Diseased - Field	MCAV	McH-104 orange	Healthy - Naive
3E	Experimental	CNAT	CnD-46	Diseased - Field	MCAV	McH-101 white	Healthy - Naive
4E	Experimental	CNAT	CnD-47 Replaced with CnD-49 on 5/22/2023 (day 5) Replaced with CnD-48 on 5/24/2023 (day 7)	Diseased - Field Diseased - Field Diseased - Field	MCAV	McH-103(4) red	Healthy - Naive
5E	Experimental	CNAT	CnD-48	Diseased - Field	OFAV	OfH-106 yellow	Healthy - Naive

MCAV= *Montastaea cavernosa*; *OFAV = Orbicella faveolata*; CNAT = *Colpophyllia natans*.

8. FIGURES

8.1. **Figures for RNA Sequencing Results**

Figure 8.1.1: The presence of viral families per species. MMEA, DSTO, and MCAV: The presence or absence of viral families across MMEA, DSTO, MCAV experimental fragments (SCTLD) and the control fragments (asymptomatic).

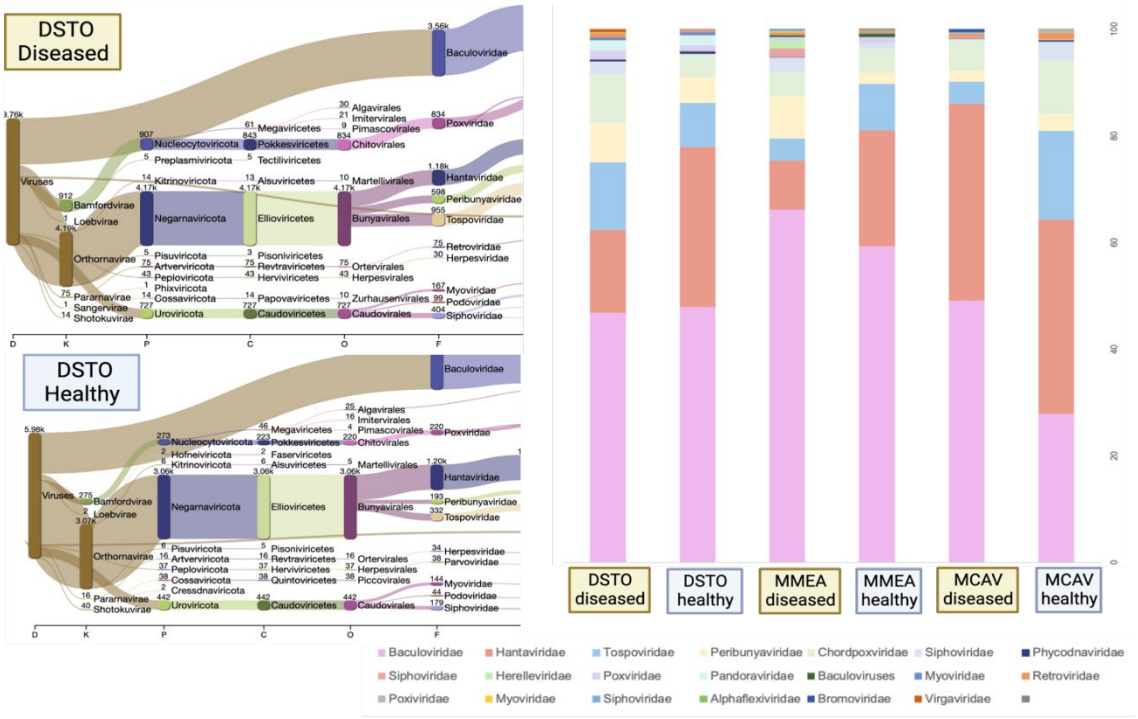

Figure 8.1.2: The distribution of viral reads per family. MCAV= *Montastaea cavernosa*; MMEA = *Meandrina meandrites*; DSTO = *Dichocoenia stokesii*.

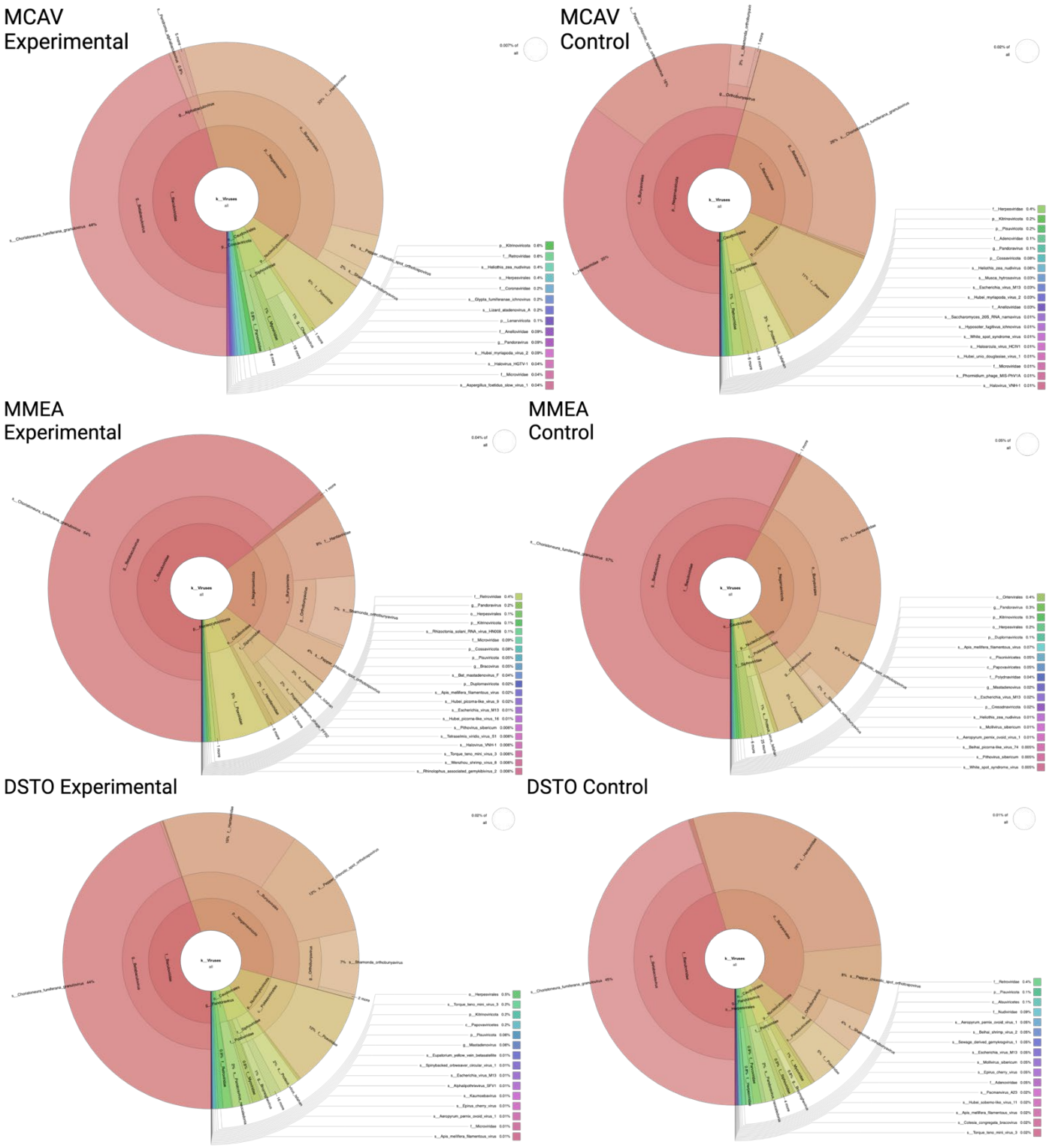

Figure 8.1.3: Viral species abundance of SCTLD Corals and the asymptomatic control corals. KRONA plots showing the species abundance of the SCTLD corals MMEA, MCAV, and DSTO, compared to the asymptomatic healthy controls.

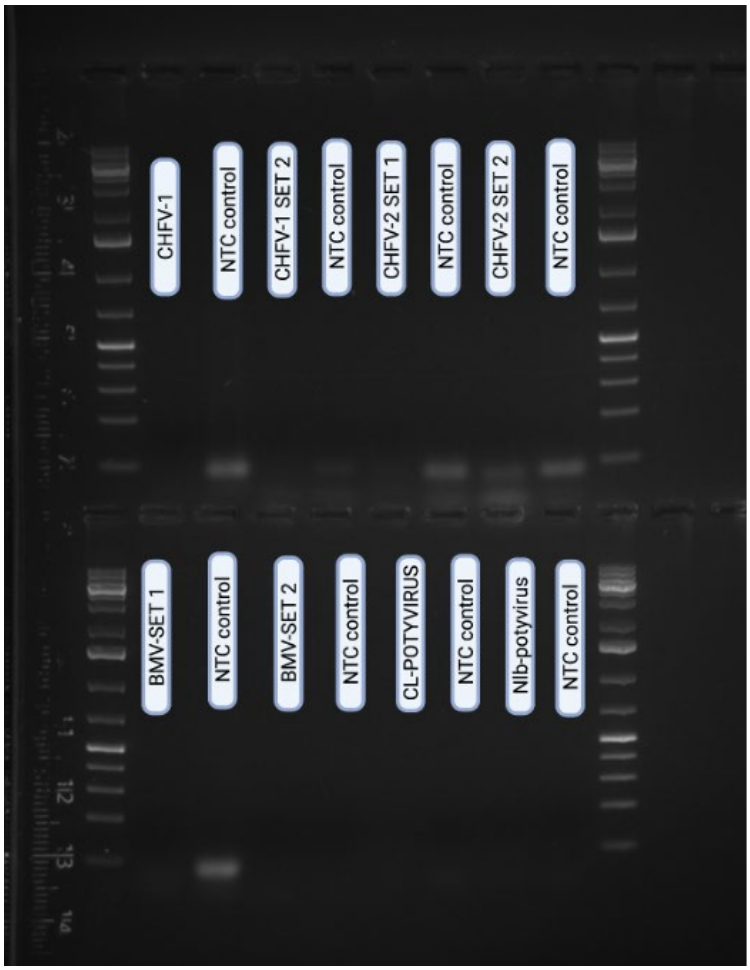

Figure 8.1.4: RT-PCR to detect the presence of BMV, Potyviruses, CHFV-1 and CHFV-2. RNA was extracted from the CND30 SCTLD and an RT-PCR was performed testing the following set of primers, CHFV-1 Set 1, CHFV-1 Set 2, CHFV-2 Set 1, CHFV-2 Set 2, Biden's mottle virus (BMV-1) Set 1, BMV set 2, Cl-Potyvirus universal primers, and Nlb-potyvirus universal primers. No bands were detected for any of the primer sets.

8.2. **Figures for TEM**

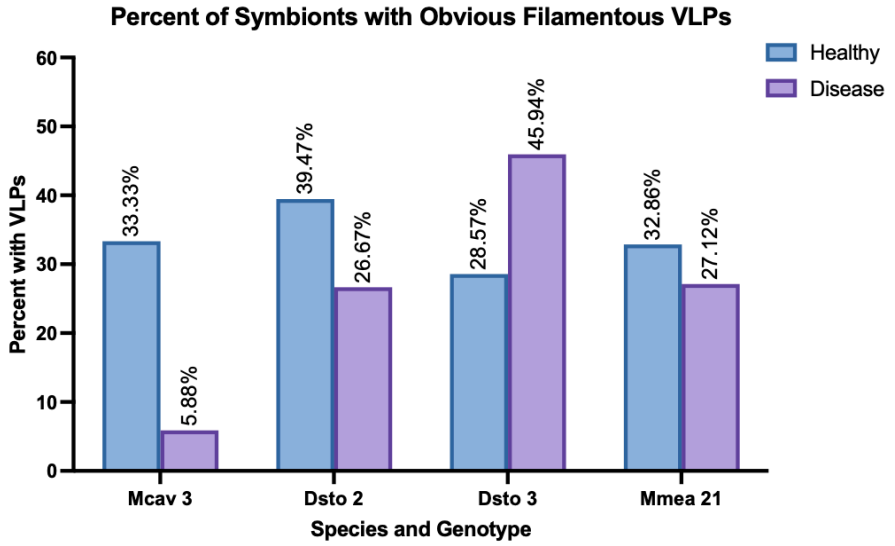

Figure 8.2.1: Percent of symbionts per coral with obvious filamentous VLPs. The percent of symbionts per coral that had signs of obvious VLPs. Blue is the healthy control and purple in the diseased sample. Each coral is separated by genotype.

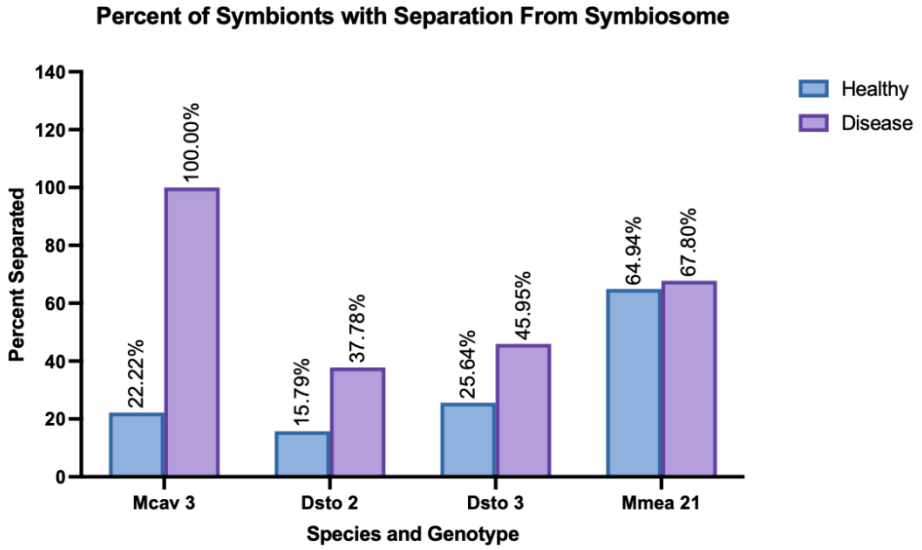

Figure 8.2.2: Percent of symbionts per coral with separation from symbiosome. The percent of symbionts per coral that had separation or swelling of the symbiosome. Blue is the healthy control and purple in the diseased sample. Each coral is separated by genotype.

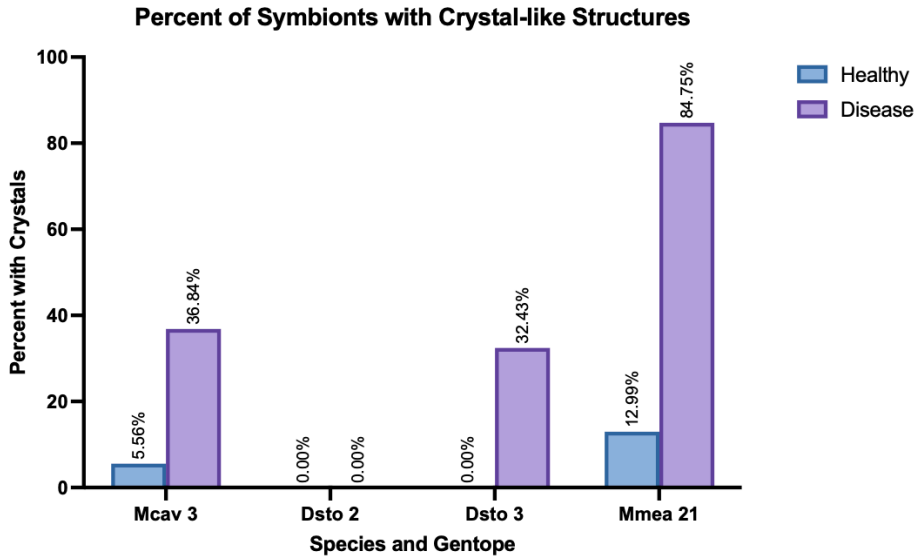

Figure 8.2.3: Percent of symbionts per coral with crystal-like structures. The percent of symbionts per coral that had crystal-like structures within the symbiont. Blue is the healthy control and purple in the diseased sample. Each coral is separated by genotype.

8.3. **Figures for Microalgae Infection Experiments**

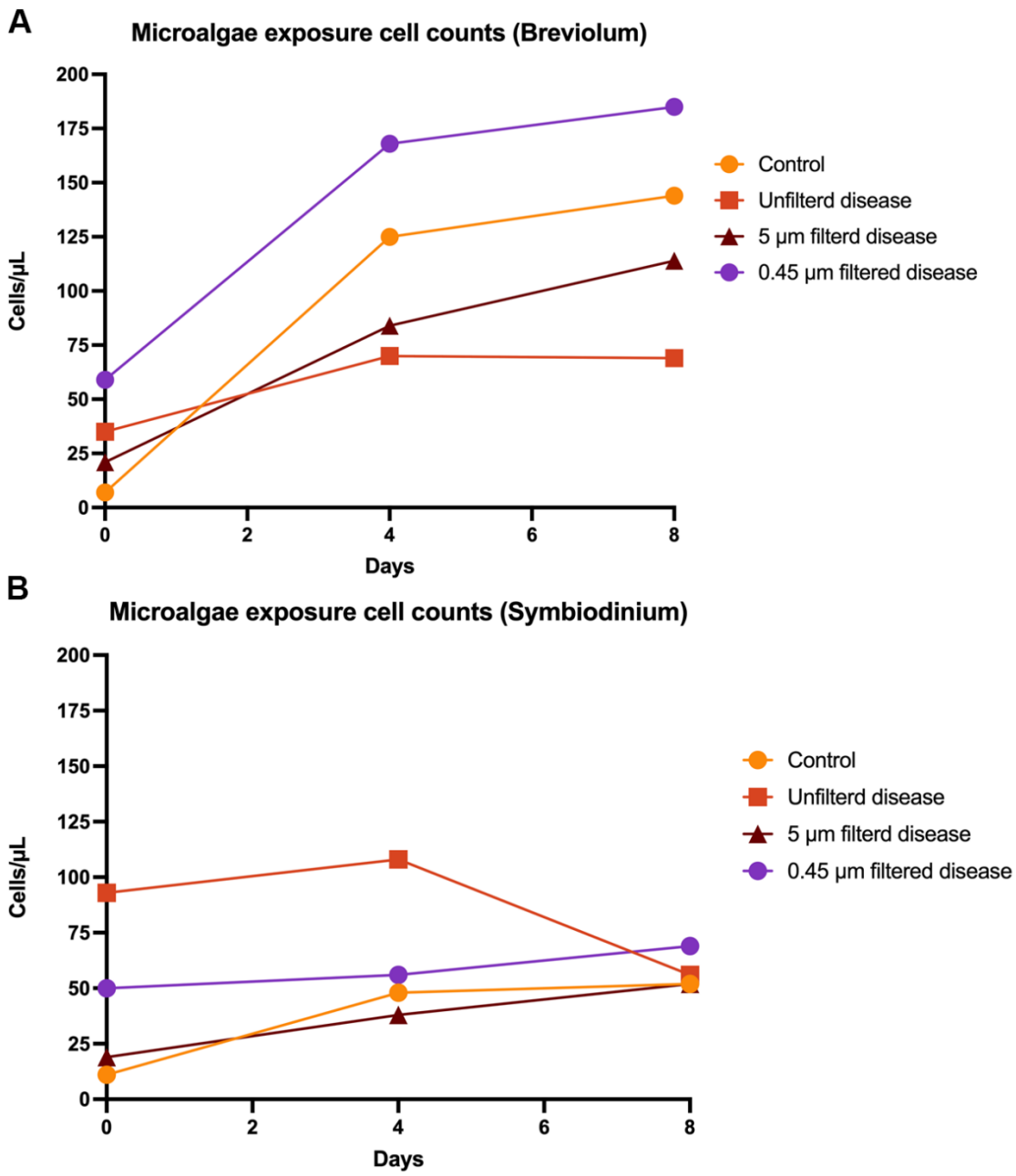

Figure 8.3.1: Trial 1 results of microalgae exposure experiment. Cultures of A) *Breviolum* sp. and B) *Symbiodinium* sp. exposed to the tank water from healthy corals or diseased corals that has been used directly, filtered down to 5 µM, or 0.45 µM.

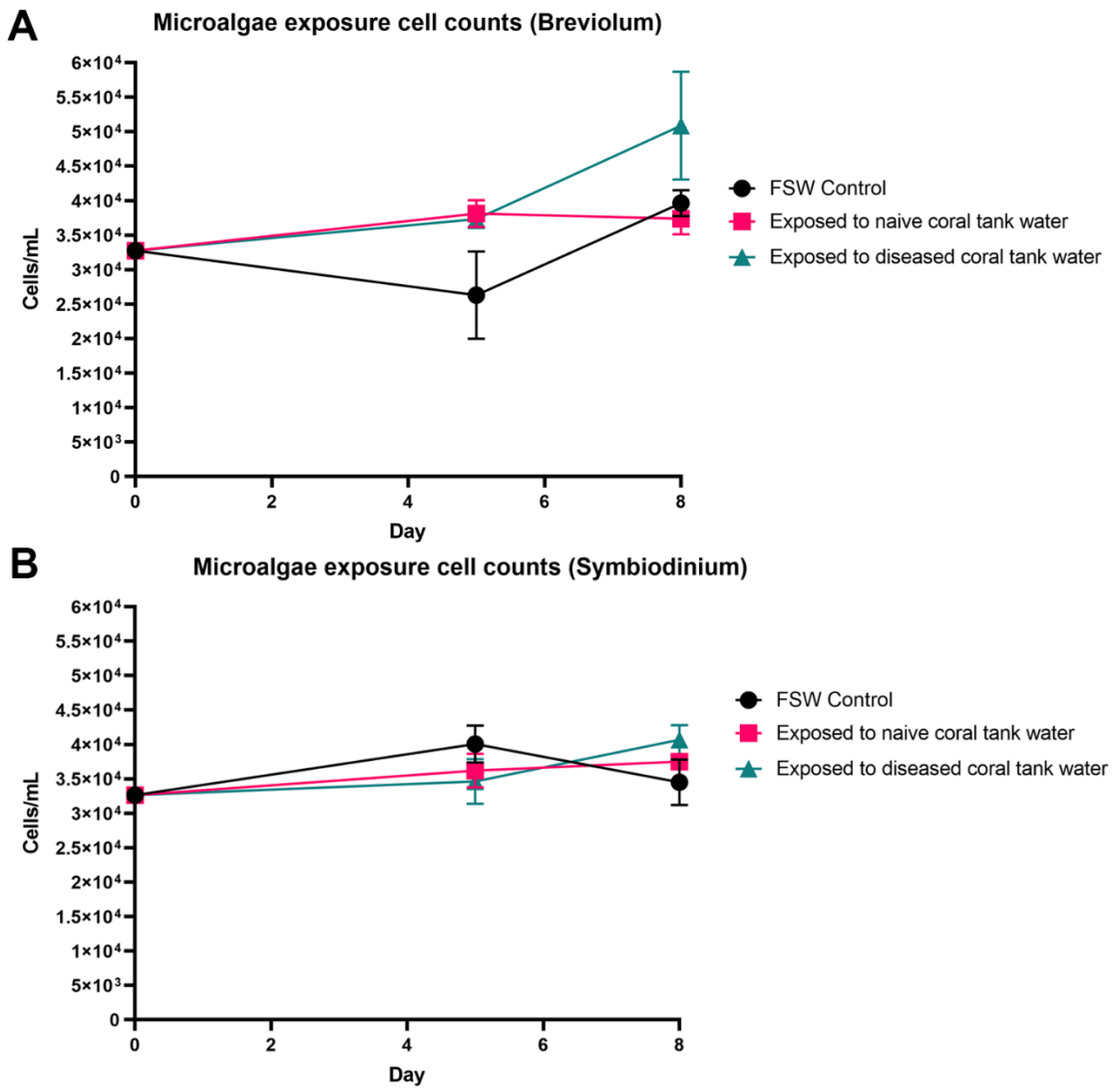

Figure 8.3.2: Trial 2 results of microalgae exposure experiment. Cultures of A) *Breviolum* sp. and B) *Symbiodinium* sp. exposed to the tank water with naïve healthy corals, diseased corals, or FSW. The tank water used for this experiment was from the time-series experiment tanks 4C (control) and 4E (experimental). Tank 4E had confirmed disease transmission and acute tissue loss progression on all infected fragments. Cell counts for Breviolum sp. (n=4, 2-way ANOVA, p=0.0957) and Symbiodinium sp. (n=4, 2-way ANOVA, p=0.1307) were not statistically different across treatments. Error bars on the data points represent standard error of the mean.

8.4. **Figures for Time-Series Experiment**

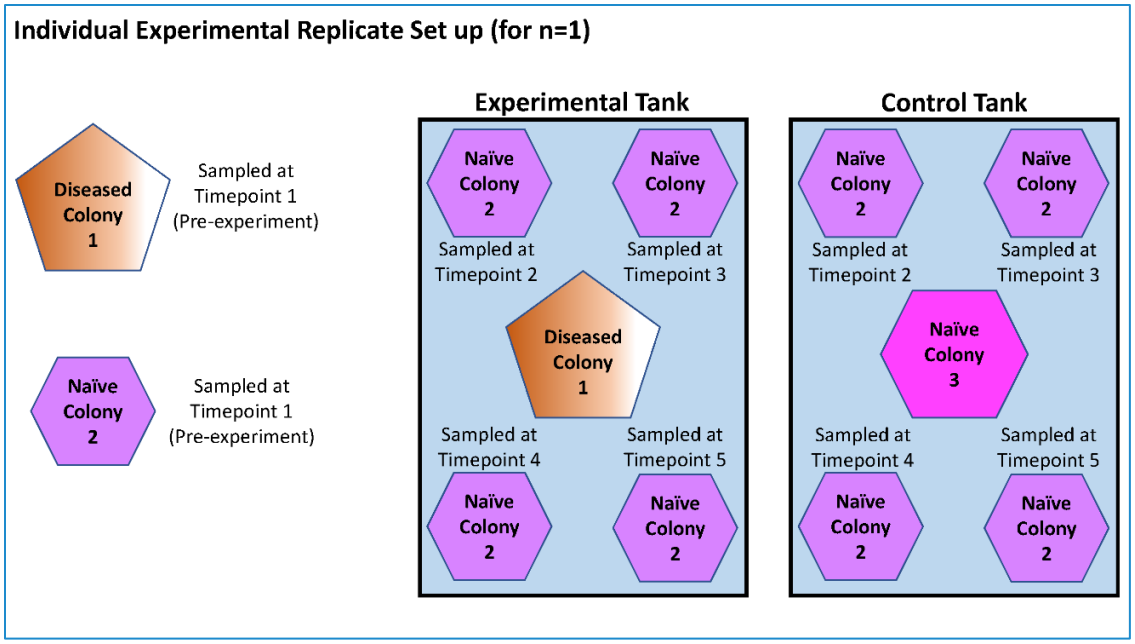

Figure 8.4.1. Schematic of an individual experimental replicate for the proposed time-series experimental set up. Each experimental replicate will consist of an experimental tank, where fragments of naïve, apparently healthy corals will be exposed to a fragment with SCTLD, and a control tank, where fragments of naïve healthy corals from the same colony will be exposed to a naïve, apparently healthy coral from a different colony. With the controls included, there will be a total of 10 different samples per replicate per sample type.

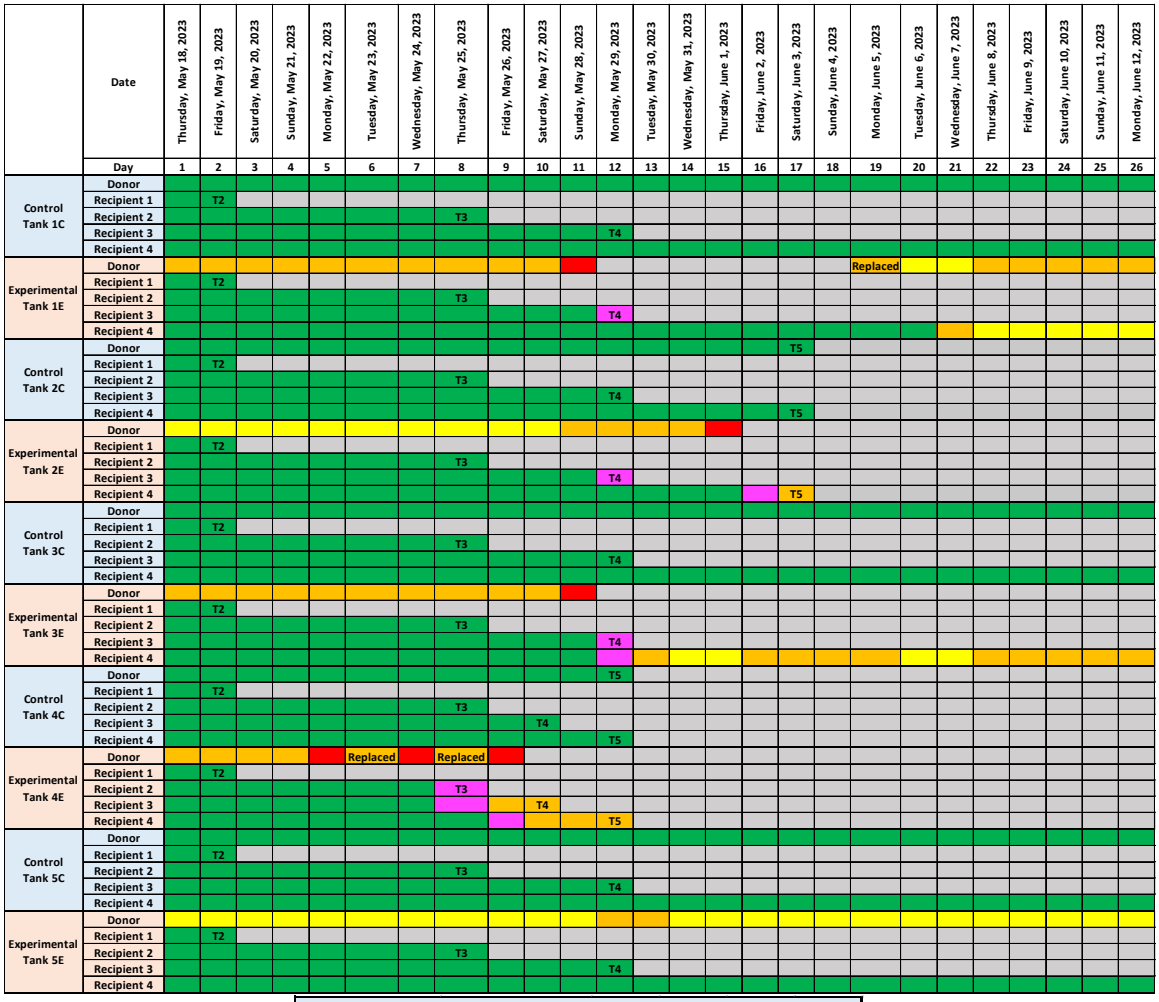

Figure 8.4.2. Gantt chart summarizing the time-series experiment (experiment still ongoing). The chart summarizes the progression of disease of each fragment (via the colors in the key below), what timepoint they were sampled for (i.e., T1, T2, T3, etc.) and if the diseased donor fragment was replaced ("Replaced"). Each column represents a different day, and each row is a different fragment.

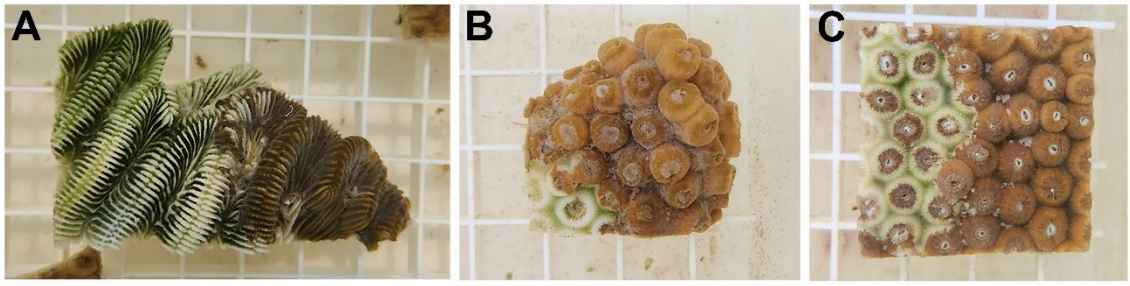

Figure 8.4.3. Representative photos of the disease signs observed during the time-series experiment. A) Diseased *C. natans* CnD-49; B) Infected *M. cavernosa* McH-103(4) approximately 36 h after initial disease signs was observed; C) Infected *M. cavernosa* McH-104 approximately 48 h after initial disease signs was observed. All fragments depicted here are developed tissue loss lesions that progressed across the fragment, indicative of SCTLD. The grating squares are 1.5 cm x 1.5 cm.

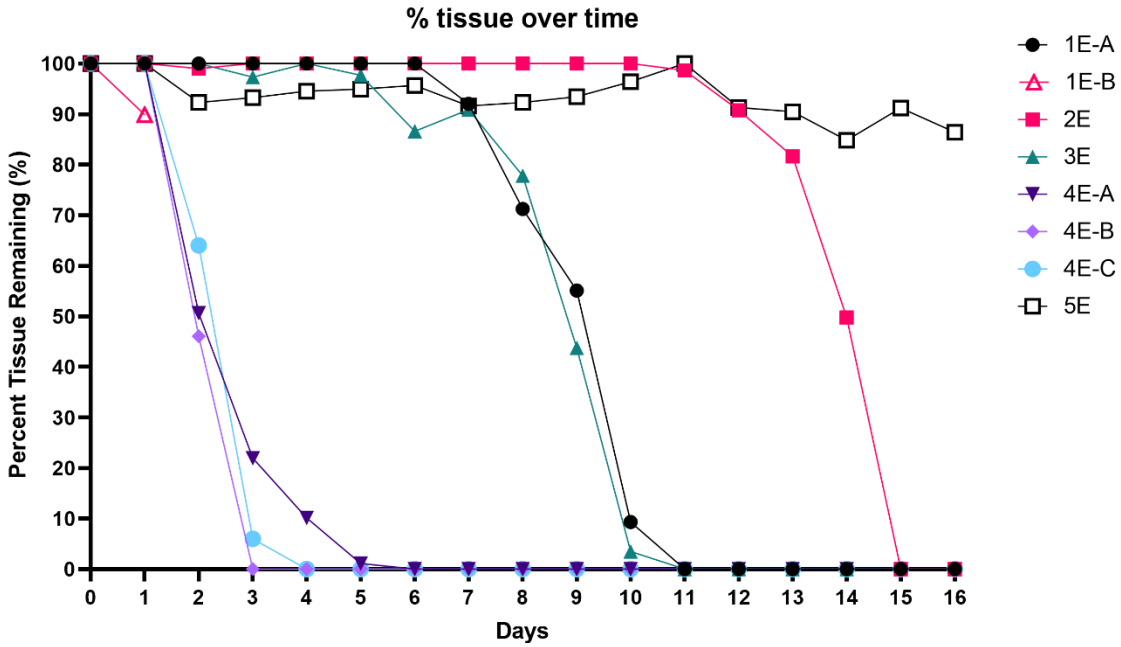

Figure 8.4.4. Percent tissue remining over time for diseased donor fragments used for the time-series experiments (experiment still ongoing). The total percent tissue over time calculated by measuring total living tissue using ImageJ (y-axis) that is plotted over how long each fragment was in a tank. The diseased donor fragments are labeled by what tank they were in, and letters indicate the different donors that were replaced. For 1E-A = frag CnD-44 in tank 1E, that was replaced with 1E-B = frag McD-106. For 4E-A = frag CnD-47 in tank 4E, that was replaced with 4E-B = frag CnD-49, that was then replaced with 4E-C = CnD-48.