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

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

[Final Report]

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Management Summary (300 words or less)

The goal of this project was to determine if there was any correlation between the presence of potential viral pathogens and SCTLD. Our research group approached this project from multiple directions, which included using TEM, RNA sequencing, and infection experiments with endosymbiont cultures. Using TEM, we analyzed corals collected from areas before the arrival of SCTLD (naïve to the disease) and coral species resistant to the disease (*Acropora cervicornis*). From our results, it appears that the previously described VLPs present in diseased and apparently healthy corals from endemic zone in Florida are not present (or present at the same abundance) in the naïve and resistant coral samples. While more replicates are needed, these seems to be a trend between SCTLD and VLPs based upon the TEM results. RNA sequencing was also applied to identify potential viral pathogens. The total viral community needs to be analyzed at greater depth; however, our major finding was that our naïve corals do not have significant levels of Alphaflexiviridae. Members of this viral family were implicated in other TEM studies and several genomes of Alphaflexiviridae were sequenced in USVI SCTLD lesions, but they were at most 57% similar at the amino acid level to our sequences from Florida corals. Thus, suggesting that the viruses from USVI are not the same as the ones from Florida. Lastly, infection experiments with endosymbiont cultures and SCTLD lesion material yielded no conclusive results, which suggests this process may be more complex than initially hypothesized.

Executive Summary (max 1 page)

Florida's coral reef is currently experiencing a multi-year disease-related mortality event that has resulted in massive die-offs in multiple coral species. The best available information indicates that the disease outbreak is continuing to spread throughout the Caribbean. The causative agent(s) of SCTLD has yet to be identified; however, previous work implicated a virus as a potential causative agent for SCTLD. To determine if there were potential viral pathogens associated with SCTLD, this project had two main goals: (1) to identify potential viral pathogens and (2) to determine if Symbiodiniaceae-associated viruses are pathogenic. These two goals were aimed to improve understanding of primary and secondary causes of SCTLD and ultimately prevent future outbreaks.

To accomplish the first goal, TEM along with RNA sequencing were performed to determine the presence of viral particles in Florida corals. These two methods were used in tandem to determine if there was a link between viral particles observed in diseased corals through TEM and their subsequent sequences through analyses of the same diseased coral samples. Samples of corals that are naïve to SCTLD (collected from areas before observable SCTLD outbreaks) and species resistant to SCTLD were also analyzed for presence of VLPs. Through TEM, there appears to be a difference in the abundance of VLPs described in Work et al. 2022 in diseased corals, corals from endemic zones, and corals naïve to SCTLD. Preliminary checks in the sequencing data were unable to find significant populations of Alphaflexiviridae sequences in the naïve *D. labyrinthiformis* sample. This suggests that there is a correlation between these sequences and disease, however, more samples are needed to confirm this.

To accomplish the second goal of determining if Symbiodiniaceae-associated viruses are pathogenic, three genera of Symbiodiniaceaewere exposed to fractions from healthy and diseased corals. These fractions included full mucus, bacteria isolated from the mucus, and the filtrate which should include any VLPs that were in the mucus. The density of these cultures was measured through fluorescence on a plate reader. There was no significant different in the cultures that were exposed to each disease fraction when compared to the subsequent controls. There was also no significant difference when they were exposed to each healthy fraction when compared to the subsequent controls. These results are consistent with other independent trials conducted by the Smithsonian Marine Station as well as another trial using these same three strains exposed to diseased coral. In those previous trials there was no evidence of negative effects from exposure to SCTLD samples.

Overall, there appears to be a correlation of VLPs observed in corals with TEM present in SCTLD endemic zones naïve zones. Our results do suggest that different viruses from the Alphaflexiviridae group could be associated with disease lesions, but more work needs to be done before any conclusions cane be made. A lack of a significant difference in the algal symbiont testing could be due to the cultures potentially not being representative of the endosymbiotic state. Physiological changes in the symbionts as they become free-living cells in culture when compared to a non-motile cell inside coral tissue could results in the differences that are seen in these trials. More investigations are recommended to further characterize the viral communities in Florida corals and causation between specific viruses and SCTLD needs to be established before making any solid conclusions. Other types of pathogens have not been ruled out as a primary cause of SCTLD, therefore, caution must still be exercised in focusing solely on a putative viral pathogen.

Acknowledgements
We would like to thank Dr. Alison Taylor and Elizabeth Elliot for their training and help with electron microscopy. Also, Dr. Thierry Work and his team in the Honolulu USGS facility for their assistance with processing and analyzing some of the TEM samples.

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- VLP = virus-like particle
- AVLP = anisometric virus like particle
- DRTO = Dry Tortugas National Park
- TEM = transmission electron microscopy

1. DESCRIPTION

1.1. Project Summary and importance

The goal of this project was to investigate the potential virus-like particles (VLPs) described in Work et al. 2021 [1]. Currently, the cause of SCTLD is unknown, which poses a problem for management agencies trying to control the spread of this disease. Without a known pathogen, it is impossible to design feasible diagnostic tools, which is a major issue for coral restoration efforts. They cannot determine which areas are contaminated with SCTLD or which coral colonies are already infected if they do not present disease signs yet. Further, it is impossible to develop targeted treatment options if the pathogen is unknown, which leaves managers with a limited range of tools to control disease. This project was designed to approach this investigation from different directions, combining transmission electron microscopy (TEM), RNA sequencing, and infection experiments with endosymbionts. The work completed in this project includes: (1) a visual assessment of VLPs present or absent in corals never exposed to SCTLD (i.e., naïve corals), corals from regions with the disease, and species resistant to SCTLD through TEM; (2) identifying these potential sequences in both SCTLD corals and naïve corals through RNA sequencing; and (3) exposure of algal symbionts to potential viral agents.

2. METHODS

2.1. Transmission Electron Microscopy (TEM)

For TEM, coral samples were preserved for follow up analysis in a paraformaldehyde, glutaraldehyde, and Instant Ocean solution. Samples were analyzed by the Ushijima Lab at the UNCW Richard M. Dillaman Bioimaging Facility using the available FEI Tecnai Spirit BioTwin TEM.

For coral, fragments were decalcified with 10% calcium-free EDTA in DI water, with a pH of 7.0 adjusted with NaOH tablets, in a plastic container suspended off the bottom using cheesecloth to allow efficient decalcification. EDTA was changed every day and plastic containers were placed on a rotator at a speed of 80 rpm. Decalcification timing depended on the size of coral fragment. Throughout the decalcification, metal was not used to avoid ion interactions. After the coral had been fully decalcified, the tissue was then placed back into the fixative solution with the solution being 10 times the volume of the coral to ensure proper fixing. One mm^2 sections of the fixed coral was cut on an autoclaved glass petri dish using a sterile scalpel and placed into microcentrifuge tubes containing fixative solution. Tissue samples sit in the fixative solution for at least one day before processing.

To process the samples, the tissue was rinsed three times using a 0.1 M sodium cacodylate buffer with 0.35 M sucrose for 15 minutes each. After rinsing, tissue samples were post-fixed in a 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for two hours, similar to methods previously described [1]. After post-fixation, samples were rinsed two times for 15 minutes each in 0.1 M sodium cacodylate buffer. Samples were then dehydrated in a series of ethanol solutions (50%, 70%, 95%, 100% x2). After dehydration, samples were moved to BEEM capsules and embedded in 1:1 100% Spurr's resin with 100% ethanol for one hour. Tissue samples were then placed in 100% Spurr's resin overnight. The next morning, tissue samples were then added to new 100% Spurr's resin and left overnight in a 70°C oven. Paper with an ID was also embedded with the

tissue samples in the BEEM capsule. After embedding, tissue was cut out of the BEEM capsule, trimmed using a razor, and then cut into sections using a glass knife on a Lecia UC7 Ultramicrotome. Sections were placed on copper grids that were previously coated with formvar and stained with 2% uranyl acetate in 50% ethanol and subsequently stained with Reynold's lead citrate. Sections were imaged using the Tecnai Spirit BioTwin.

2.2. RNA Sequencing

All samples were fixed in RNAlater and stored at -80 °C. The samples for RNA sequencing were then shipped frozen to Dr. Michelle Heck (USDA ARS/Cornell) and her Ph.D. student Stephanie Preising (Cornell) for further processing. To identify viral sequences, total RNA was extracted from diseased, healthy, and diseased-adjacent corals using the Qiagen RNeasy extraction kit. Construction of cDNA libraries and sequencing was performed using Truseq Illumina library preparation kits and Illumina high throughput sequencing at Cornell University. Samples were sequenced for 150 bp length reads at approximately 50 million reads per sample on an Illumina NextSeq500. Poly-a enrichment was used to enrich for polyadenylated RNA, which should include members of Alphafelxiviridae. We have considered rRNA depletion as an alternative method to poly-a enrichment, given the recently released publication [2], and their identification of poly-adenylated Alphaflexiviridae viral sequences as a putative causative agent; however, we chose the poly-a enrichment method for our samples. We had sufficient high-quality RNA to run an rRNA depletion method as an alternative, if necessary, after the data analysis of the first runs.

Quality control, adapter trimming, host read removal, and rRNA depletion of the reads was performed using FASTQC. CLC Genomics Workbench 5.15, SPAdes, and Geneious 8.1.7. The contigs and read depth were evaluated after using Blastn against the entire virus database at NCBI. Cleaned contigs were mapped against viral reference genomes in a de novo assembly using Geneious and Snapgene software. The raw files were trimmed using FASTQC, and then from the trimmed sequenced reads, we removed coral and endosymbiont reads. The reference genomes were mapped to available genomes for stony corals using strict parameters to reduce the risk of removing unknown viral reads. The reads were assembled into a de novo assembly using the program TRINITY, resulting in assembled trinity.fasta files (i.e., the primary assemblies). Using TRINITY, we built transcripts from the primary transcripts. To build these super transcripts we collate the sample reads. We then sorted the super transcripts by sample to identify super transcripts present in the different sample types (e.g., naïve versus diseased corals). We used BLASTN and tBLASTx to identify viral sequences by aligning the assemblies to NCBI's viral genome database. By using tBLASTx we can identify viruses with more permissive parameters. We are running 'samtools idxstats' to quickly pull the number of reads mapped to each trinity-gene. We will compare viromes from each sample type to determine whether a potential causative viral presence is correlated to quantifiable differences in virome diversity. We will use constructed genomes to compare viral sequences across microalgae from healthy and diseased coral, look for polymorphisms, non-synonymous and synonymous mutations in the sequences. This will generate information on the virome leading to new directions in potential viral causative agents.

2.3. Infection Experiments

Algal cultures were obtained from the Smithsonian Marine Station and kept in continuous culture at UNCW. Cultures were originally from Mary-Alice Coffroth. The cultures include *Symbiodinium* sp. Mf 10.02~, *Durusdinium* sp. Mf 2.26-2, and *Breviolum* sp. Mf 1.05b.OI SCI 07-205. All three genera of symbionts were exposed to mucus and filtered bacteria from a diseased *Orbicella faveolata* colony and a healthy *O. faveolata* control colony from Broward County, FL. The diseased *O. faveolata* was originally from Broward County, FL, but was held in captivity for three months at UNCW before showing tissue loss disease signs. This coral was sampled during active tissue loss by collecting mucus and tissue along the lesion.

Algal samples were plated in triplicate in 96 well plates and fluorescence was measured using a plate reader at the emission 680 nm and excitation 465 nm. Baseline fluorescence was obtained before cultures were exposed in triplicate to mucus. Plates were then incubated at 28°C overnight. Fluorescence was then measured, 10 µL of RO water was added to each well to prevent evaporation, and plates were then incubated in a humidified light algal incubator at 27°C for the duration of the experiment. Plates were also measured on days two and six.

3. RESULTS

3.1. Task 1: Identify the filamentous viral particles observed to be associated with microalgae within Floridian corals.

3.1.1. Identification of any viral particles through TEM.

We focused on whether there are correlations between the presence of the VLPs described in Work et al. 2021 and diseased or healthy coral samples. Sequencing will identify any viral RNA; however, we must also establish if there is 1) a link between the VLPs observed and disease, and 2) if there is a link between the VLPs and specific viral sequences identified. From the initial study, the VLPs in question were observed in both diseased and apparently healthy coral samples, suggesting that the healthy corals may already be infected by this putative viral agent and do not display any gross disease signs. Further, Veglia et al. 2022 described the discovery of Alphaflexiveridae genomes (the viral family implicated in the previous TEM study) to be present in both SCTLD-affected and apparently healthy corals from the U.S. Virgin Islands. We do not know for sure if the VLPs are Alphaflexiveridae particles, but it does suggest that healthy corals from endemic zones may already be carrying a latent viral infection. Therefore, samples of corals that are naïve to SCTLD (collected from areas before observable SCTLD outbreaks) and species resistant to SCTLD were analyzed.

The major finding was that there appears to be a difference in the abundance of VLPs described in Work et al. 2022 in diseased corals, corals from endemic zones, and corals naïve to SCTLD. Apparently healthy *Siderastrea siderea* (n=2) and *Meandrina meandrites* (n=1) were sampled and prepared for TEM. These corals were collected from the January 2020 cruise to Dry Tortugas National Park (DRTO) and kept at the Smithsonian Marine Station in their own isolated tanks with seawater that had been filtered down to 0.22 µm and constantly flowed through a UV-sterilizer. These corals

were considered naïve to SCTLD since they were collected and quarantined before the detection of the disease at DRTO. The fragments were fixed in TEM fixative and shipped to Dr. Thierry Work at the USGS office in Honolulu, HI in Fall 2021. The major observation was the lack of any VLPs first described in Work et al. 2021 (**Figures 1 & 2**). There was no formal quantification done, but there was a clear distinction between these corals from DRTO and the healthy corals collected from Florida Keys described in the previous study.

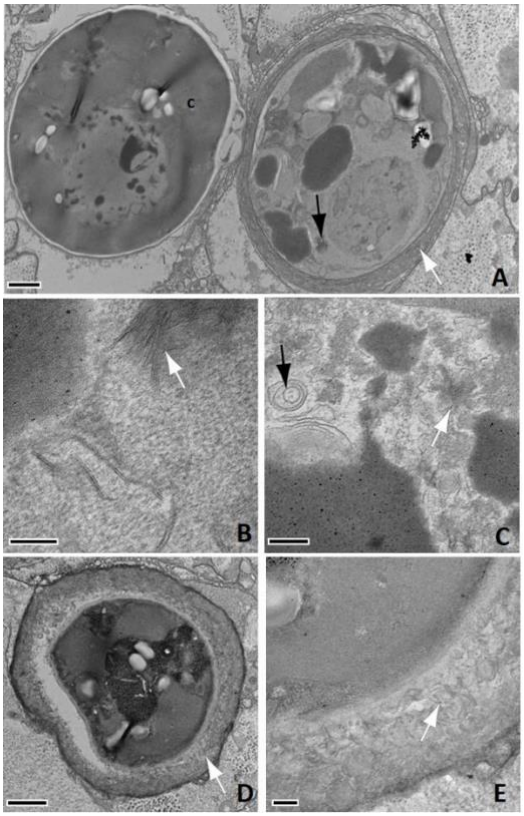

Figure 1. *Siderastrea siderea endosymbionts. A) Note normal symbiosome on the left and symbiosome populated by stacked membranes (white arrow) right. Endosymbiont on right also has intracytoplasmic accumulations of granular material and electron-dense bodies with rare AVLP (black arrow). Endosymbiont on left has indistinct thylakoid membranes in chloroplasts (c). Bar= 1 um. B) Detail of AVLP (arrow) associated with granular material and electron dense bodies. Bar = 100 nm. C) AVLP (white arrow) among granular material, electron-dense bodies, and membranes (black arrow); bar= 200 nm. D) Symbiont with enlarged chloroplasts with indistinct thylakoids and marked accumulations of membranes in symbiont (arrow). Bar= 1 µm. E) Detail of D; note scrolled appearance of membranes (arrow). Bar= 200 nm. Figure and photo credit Dr. Thierry Work (USGS).*

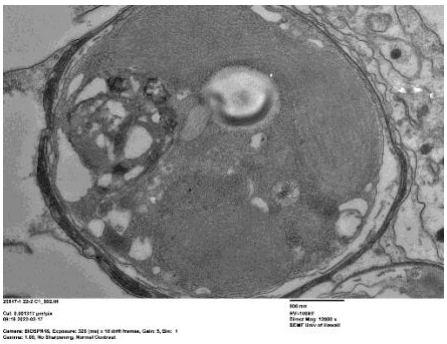

Figure 2: *Meandrina meandrites endosymbiont. A representative image of an endosymbiont from a colony collected from DRTO. Scale bar = 800 nm. No VLPs were found in these samples as previously described in Work et al. 2021. The endosymbionts in these M. meandrites appeared to have organized chloroplasts without obvious signs of any potential viral infections. Photo credit Dr. Thierry Work (USGS).*

TEM was also performed on a SCTLD-resistant species, *Acropora cervicornis*, to correlate the presence of the VLPs with SCTLD. The idea being that if the VLPs are associated with SCTLD, they should only be present in corals susceptible to the disease. The *A. cervicornis* used for this were kept at the UNCW coral spawning facility managed by Dr. Nikki Fogarty and were originally from the Florida Keys. From our initial findings, we are unable to detect any VLPs in the *A. cervicornis* samples while the chloroplast appears to have their expected shape and structure (**Figure 3**). This contrasts to the apparently healthy corals from endemic zones analyzed in previous studies where the gross tissue appears to be healthy, however, the cellular structures appear to be damaged and are filled with VLPs (excluding SCTLD-resistant *A. cervicornis*).

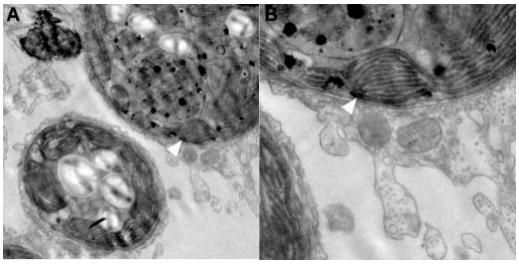

Figure 3: *Acropora cervicornis endosymbionts. A) two endosymbionts from a healthy A. cervicornis fragment (the whole fragment was sectioned after decalcification. B) the same endosymbionts but zoomed in. The white arrow points to a representative, healthy chloroplast.*

Additional TEM samples have been preserved including corresponding fragments used for RNA sequencing (see below). A majority of these samples have completed the decalcification process and have been stored in fixative for follow-up studies. However, additional samples are still required to increase the sample size for this process, but the focus should be on naïve corals never exposed to SCTLD.

3.1.2. Samples sent in for RNA sequencing and generation of sequencing data.

The RNA for the first sequencing run was extracted from the following corals collected from the Florida Keys and Dry Tortugas National Park (DRTO) (Table 1). The date of collection of *D. labyrinthiformis* precedes the dates of SCLTD detection in the

area and serves as a sample to compare viral transcripts with those from diseased *C. natans* corals. The samples were sequenced at the Transcriptional, Regulation, and Expression (TReX) facility at Cornell University.

Table 1. *The first batch of samples sent for RNA sequencing.*

Sample name	Sample State	RNA data: ng/uL and RQN*	Submission for RNA-seq date	Sequencing
CnD-30	Diseased *C. natans* from the FL Keys	35.0ng/uL | 8.4	2/09/22	Illumina HTS (50 million reads minimum)
CnD-313	Diseased *C. natans* from the FL Keys	100 ng/uL | 8.0	2/09/22	Illumina HTS (50 million reads minimum)
CnD-27	Diseased *C. natans* from the FL Keys	22 ng/uL | 8.6	2/09/22	Illumina HTS (50 million reads minimum)
DLAB	Naïve *D. labyrinthiformis* from DRTO	10 ng/uL | 9.0	2/09/22	Illumina HTS (50 million reads minimum)

*RQN stands for RNA-quality number and is from 1-10, only samples that score above a 7.5 are sequenced since they contain little to no degradation. This quality control step was performed for all samples being used for RNA seq and RT-PCR experiments.

The second sequencing run was submitted for RNA-sequencing in late March. Our collaborators at the Smithsonian Marine Station conducted in-house transmission experiments with SCTLD-affected corals using the following species: *Dichocoenia stokesii, Meandrina meandrites, and Montastraea cavernosa* (Table 2). Experimental and control fragments were sent to Cornell University and RNA was extracted from the coral and the Symbiodiniaceae endosymbiont in tandem. The samples were submitted for RNA quality control and immediate sequencing at the TReX facility on the Cornell campus.

Table 2. *The second batch of samples sent for RNA sequencing.*

Sample	Description*	ng/uL of RNA	Sequencing
1-DSTO	***Dichocoenia stokesii*** (Before transmission experiment began)	99.5	Illumina HTS (50 million reads minimum)
2-DSTO	***Dichocoenia stokesii*** (Before transmission experiment began)	104.0	Illumina HTS (50 million reads minimum)
3-DSTO	***Dichocoenia stokesii*** (control fragment)	226.7	Illumina HTS (50 million reads minimum)
4-DSTO	***Dichocoenia stokesii*** (control fragment)	85.5	Illumina HTS (50 million reads minimum)
5-DSTO	***Dichocoenia stokesii*** (Experimental fragment)	97.0	Illumina HTS (50 million reads minimum)
6-DSTO	***Dichocoenia stokesii*** (Experimental fragment)	138.8	Illumina HTS (50 million reads minimum)
7-DSTO	***Dichocoenia stokesii*** (Before transmission experiment began)	90.0	Illumina HTS (50 million reads minimum)

8-DSTO	***Dichocoenia stokesii*** (Before transmission experiment began)	109.3	Illumina HTS (50 million reads minimum)
9-DSTO	***Dichocoenia stokesii*** (Experimental fragment)	592.7	Illumina HTS (50 million reads minimum)
10-DSTO	***Dichocoenia stokesii*** (Experimental fragment)	163.6	Illumina HTS (50 million reads minimum)
11-MMEA	***Meandrina meandrites*** *(Experimental fragment)*	105	Illumina HTS (50 million reads minimum)
12-MMEA	***Meandrina meandrites*** *(Experimental fragment)*	145	Illumina HTS (50 million reads minimum)
13-MMEA	***Meandrina meandrites*** *(experimental fragment)*	122	Illumina HTS (50 million reads minimum)
14-MMEA	***Meandrina meandrites*** *(control fragment)*	366	Illumina HTS (50 million reads minimum)
15-MMEA	***Meandrina meandrites*** *(control fragment)*	240	Illumina HTS (50 million reads minimum)
16-MCAV	***Montastraea cavernosa*** *(Experimental fragment)*	118	Illumina HTS (50 million reads minimum)
17-MCAV	***Montastraea cavernosa*** *(Experimental fragment)*	109	Illumina HTS (50 million reads minimum)
18-MCAV	***Montastraea cavernosa*** *(Control fragment)*	152	Illumina HTS (50 million reads minimum)

3.1.3. Computational analysis of sequencing data

From the first batch of sequenced samples indicated in Table 1/Figure 4, the RNA-seq files were subjected to quality control measures via the bio-informatic tool FASTQC. The raw files were trimmed using FASTQC (Figure 4). We were able to generate 58-70 million trimmed reads for each sample. For the computational analysis, our first approach was to align stony coral genomes available to the trimmed reads and remove them from the pool of reads. This would leave a pool of reads containing microbial and viral sequences which we would then align to NCBI's viral database. There are severe limitations on the availability of the stony coral genomes and transcriptomes available online. We mapped the reads to complete stony coral genomes and were only available to remove about 10-15% or 6-10 million of the reads generated from *C. natans* and *D. labyrinthiformis* transcriptomes. Furthermore, removing Symbiodiniaceae genomes with a reference genome, we were able to remove 900,000-1million reads per sample. We decided to map to the reference genomes available for stony corals using strict parameters to reduce the risk of removing unknown viral reads.

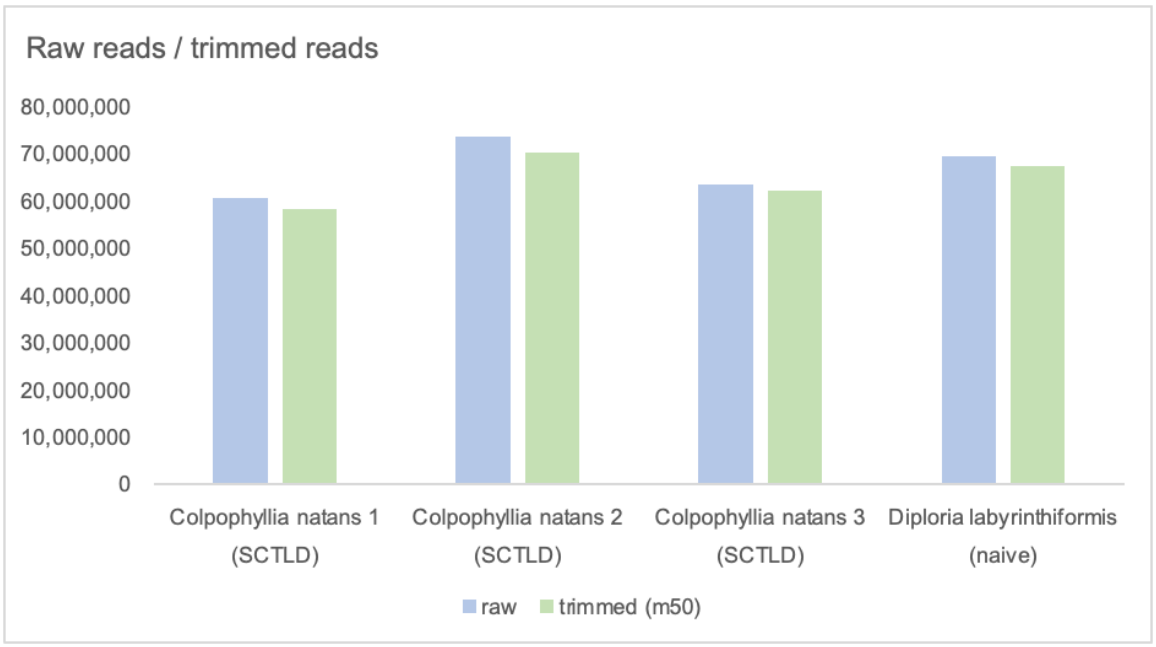

Figure 4. *The number of raw reads of each sequenced sample from Table 1 compared to the trimmed reads.*

After removing a portion of the host and endosymbiont reads, we assembled *de novo* transcriptomes using the program TRINITY, resulting in assembled trinity.fasta files with 500,000+ primary assemblies (Table 3). Using TRINITY we were able to further build into 200,000+ super transcripts from the 500,000+ primary transcripts. We are currently sorting the super transcripts by sample to identify super transcripts present in *D. labyrinthiformis* and not in *C. natans* samples.

Table 3. *Trinity fasta files, with # of sequences from the de novo assembly.*

Trinity assembly *de novo*		
file	# sequences	
Trinity.fasta	526,211	primary assembly
trinity-genes.fasta	262,870	supertranscripts

We aligned the super-transcripts to the Alphaflexiviridae sequences identified in Veglia, et al. 2022 using tBLASTX to search a protein database with a nucleotide input allowing us a more permissive search. Although we did not get direct hits to the putative Alphaflexiviridae sequences, the *C. natans* samples contain transcripts with 36-57% identity to these viruses, with E-values ranging from 9.94E-05 to 1.26E-08. The next step is using BLASTN and tBLASTx to identify viral sequences by aligning the assemblies to NCBI's viral genome database. By using tBLASTx we can identify viruses with more permissive parameters. We are running ‘samtools idxstats’ to quickly pull the number of reads mapped to each trinity-gene. Preliminary checks did not find significant populations of Alphaflexiviridae sequences in the naïve *D. labyrinthiformis* sample. Thus, suggesting that there is a correlation between these sequences and disease, however, more samples are needed to confirm this.

Finally, the second sequencing job (Table 2) is expected to finish in late June and will be processed in the same manner as the samples from the first sequencing run. These sequencing runs will provide valuable information on the microbial and viral communities in SCLTD and naïve samples.

3.1.4. Development of PCR assays for screening samples.

While we continue to analyze our RNA-sequencing data, we aim to develop RT-PCR/PCR assays to screen coral and Symbiodiniaceae samples. From the first batch of samples submitted for RNA-seq (Table 1) we used an aliquot of high-quality RNA (500ng) to create a cDNA library using BioRad iScript Select kit with Oligo dTs. 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 Symbiodiniaceae RNA-seq run, and the CL-Potyvirus and Nlb-Potyvirus universal primers aligning to viruses in the family Potyviridae (Figure 5). In this PCR we were unable to detect any of the viruses. However, the sequences aligning to the Alphaflexiviridae viruses detected in our first RNA-seq run have a low identity to CHFV-1 and CHFV-2, which explains lack of detection in the RT-PCR. We aim to develop primers to the viral sequences detected in our RNA-seq run.

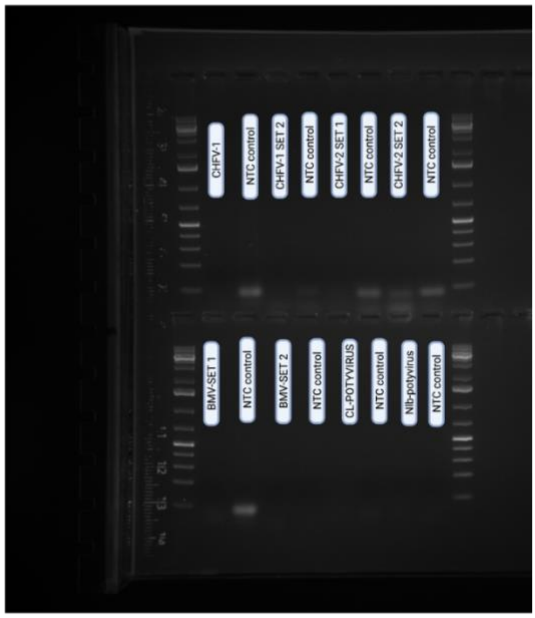

Figure 5. *RT-PCR with extracted coral and Symbiodiniaceae RNA.*

3.2. Task 2: Determine if total viral particle preps from SCTLD lesions will initiate disease in microalgae cultures.

3.2.1. Expose cultures of microalgae to disease samples to assess virulence of potential viruses.

A major issue identified with this approach was that without knowing the identity of the viral pathogen, we had no way of knowing if the endosymbiont cultures were being exposed to a viral pathogen. Another major issue was accounting for the pathogenic bacteria associated with the SCTLD lesions. Although all the cultured bacteria so far appear to be opportunistic pathogens at most, they may confound the results with the endosymbionts.

Algal cultures were obtained from the Smithsonian Marine Station and kept in continuous culture at UNCW. Cultures were originally from Mary-Alice Coffroth. The cultures include *Symbiodinium* sp. Mf 10.02~, *Durusdinium* sp. Mf 2.26-2, and *Breviolum* sp. Mf 1.05b.OI SCI 07-205. All three genera of symbionts were exposed to mucus and filtered bacteria from a diseased *Orbicella faveolata* colony and a healthy *O. faveolata* control colony from Broward County, FL. The diseased *O. faveolata* was originally from Broward County, FL, but was held in captivity for three months at UNCW before showing tissue loss disease signs. This coral was sampled during active tissue loss by collecting mucus and tissue along the lesion.

Algal samples were plated in triplicate in 96 well plates and fluorescence was measured using a plate reader at the emission 680 nm and excitation 465 nm. Baseline fluorescence was obtained before cultures were exposed in triplicate to mucus. Plates were then incubated at 28°C overnight. Fluorescence was then measured, 10 µL of RO water was added to each well to prevent evaporation, and plates were then incubated in a humidified light algal incubator at 27°C for the duration of the experiment. Plates were also measured on days two and six.

There was no difference between fluorescence in the various dilutions for either the mucus, the filter-captured bacteria, or filtrate (Figure 6). The fluorescence did not change in response to the treatments when compared to the control for either experiment.

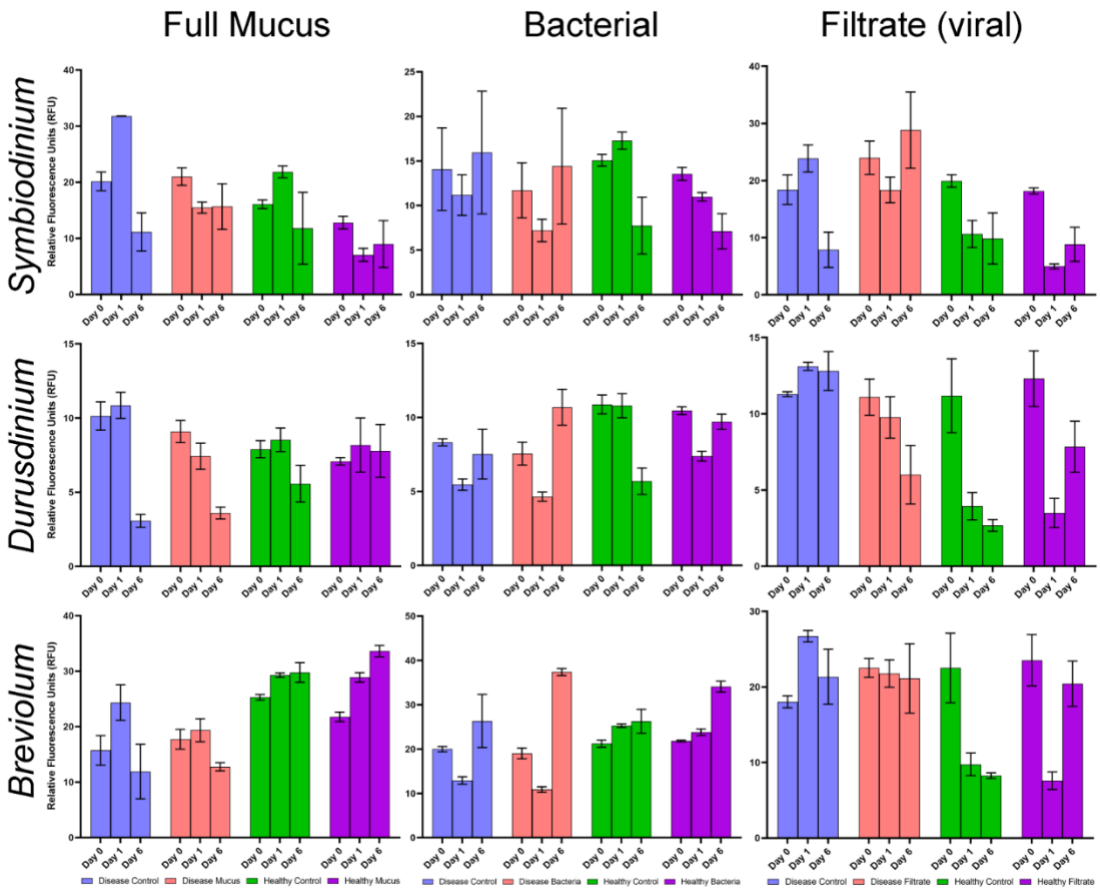

Figure 6. *Fluorescence of endosymbiont cultures exposed to samples from healthy and diseased coral. Each replicate had a corresponding control on the same plate to control for plate effects, therefore, there are separate controls for replicates exposed to healthy or disease samples. The control replicates were only exposed to artificial seawater solution instead of sample. Blue bars represent the controls for the disease treatments, while green bars represent controls for the healthy sample treatments. The pink bars represent the disease experimental treatments, while the purple bars represent the healthy sample treatments. Error bars represent the standard error for three independent replicates.*

3.2.2. Expose cultures of microalgae to purified or partially purified virus.

In addition to the exposure of endosymbiont cultures with full coral samples, we also attempted to partially purify the bacterial and viral fractions of the samples using filtration of the coral samples with a 0.22 µm pore membrane filter. The total bacteria were resuspended off the filter using a volume of artificial seawater (ASW) identical to the original sample. The filtrate was also saved because it theoretically should only contain viral particles and little to no bacterial cells. Like the results with the disease samples, there was no significant negative effect from exposure to the bacterial or viral fractions (Figure 6).

These results are consistent with the two independent trials conducted by the Smithsonian Marine Station as well as similar trials conducted by the Ushijima lab in 2021 with these same three endosymbiont strains using samples from a diseased *M. cavernosa* and *D. labyrinthiformis* colony. In those previous trials (not part of this current project) there was no evidence of negative effects from exposure to SCTLD

samples. It should be noted that all disease samples used in these endosymbiont experiments (past and current) had tested negative for the presence of *Vibrio coralliilyticus* ($VcpA^-$), which is known to be toxic to endosymbiont cultures.

4. DISCUSSION

There appears to be a correlation of VLPs observed in corals with TEM present in SCTLD endemic zones, regardless of health state. Therefore, initial efforts should begin to check captive corals for potential latent viral infections. While no immediate treatment is known at this time, it would be important to know the extent of potential infections in the precious captive populations. Caution must still be exercised in ruling out other causes of SCTLD and focusing solely on a putative viral pathogen. Other types of pathogens (i.e., bacteria) have not been ruled out as a primary cause of SCTLD, while they could be responsible for dangerous opportunistic infections or the main drivers for tissue loss lesions. The effect of various classes of antibiotics does suggest that pathogenic bacteria still play some role in SCTLD, therefore additional means of treatment targeting this group of pathogens and investigations into them should be continued. We highly recommend continued investigations into the potential involvement of viral pathogens with SCTLD. If a viral pathogen is involved that would require revaluating biosecurity measures and treatments.

The difference in the Alphaflexiviridae sequences from Florida versus the USVI does suggest that are different viruses from this group associated with the lesions in these regions. However, this does call into question if these viruses are (1) important for SCTLD lesion initiation (primary pathogens) or (2) if the disease in the USVI has a similar microbial make up as the disease in Florida. More investigations are recommended to further characterize the viral communities in Florida corals but establishing causation between specific viruses and SCTLD should be established before making any solid conclusions.

The lack of negative effects from the infection experiments was not completely surprising due to the previous trials run by the Ushijima lab as well as by collaborator Dr. Val Paul. At this stage of the project, we have various hypotheses on this matter:
(1) The endosymbionts are being infected by a viral agent, but it is a latent infection and will manifest in cell lysis after exposure to specific stressors. This is consistent with previous studies on viral infections of endosymbionts where disease manifests only after environmental stressors like high temperature or high UV exposure [3–5]; however, various labs can get repeatable SCTLD transmission with corals under controlled laboratory conditions.
(2) Viral infection alone is asymptomatic; however, this may make the endosymbionts more susceptible to other infections. This is still possible, but we would need a purified viral agent to test this effectively. Though, the study in Evans et al. 2022 did test bacterial and viral fractions from SCTLD lesions, but only the bacterial fraction appeared to initiate disease [6]. This could have been due to the healthy corals already carrying latent viral infections.
(3) The putative viral partials are an opportunistic infection that is endemic to Florida's waters. This is still possible, but there appears to be a correlation between these VLPs and the presence of SCTLD in the environment or susceptibility to the disease (see Task 1 results).

(4) The endosymbionts are susceptible to infection by these viruses only when they are within coral cells (symbiotic state vs. free-swimming state). The physiology of these symbionts changes as they transition from a non-motile, and larger endosymbiotic cell to a smaller, motile free-living cell (in culture). Further, the use of endosymbiont cultures has also been called into question by various labs on how representative they are to the endosymbiotic state [7]. Studying this would require a different experimental approach, and we proposal using a time-course experiment with whole corals run in parallel with endosymbiont cultures exposed to the same disease material. A combination of TEM and sequencing (or qPCR specific to putative viral agents) could then be applied to determine if the precedence of VLPs, viral titer, and disease signs correlate with infection.

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