The use of algal symbiont cultures (Family Symbiodiniaceae) as model systems to study stony coral tissue loss disease (SCTLD): Use of fractionated disease isolates to help with pathogen identification

The use of algal symbiont cultures (Family Symbiodiniaceae) as model systems to study stony coral tissue loss disease (SCTLD): Use of fractionated disease isolates to help with pathogen identification

Final Report

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Management Summary (300 words or less)

We investigated the role of algal symbionts (Family Symbiodiniaceae) in stony coral tissue loss disease (SCTLD) and furthered the use of algal symbiont cultures as model systems for pathogen identification by systematically exposing five strains of cultured Symbiodiniaceae in four genera to six different SCTLD exposure treatments. We found that cultures of *Breviolum minutum*, *Cladocopium goreaui*, and *Durusdinium trenchii* were all negatively affected by seawater sourced from around diseased corals that had been filtered to between 0.2 and 0.8 μm, exhibiting a rapid (3-6 days) reduction in photochemical efficiency, reductions in cell abundance, increased vacuolization and loss of chloroplasts (visualized by transmission electron microscopy), and eventual degradation (after 6-8 days). In contrast, cultures of *Breviolum psygmophilium* and *Symbiodinium microadriaticum* were unaffected by the same SCTLD exposures. These findings support our previous DEP-funded work (Dennison et al. 2021) that found that hosting particular types of algal symbiont was a primary risk factor in SCTLD susceptibility. Taken together with those findings and the results of our FY22 work, these data strongly support that algal symbionts are in fact the primary target of the SCTLD pathogen. Furthermore, our findings suggest that the pathogen is between 0.2 μm and 0.8 μm in size (and likely the lower end of this size range, given that some 0.2 μm fractions still transmitted the disease). We hypothesize that the pathogen may be a giant virus replicating inside affected algal symbionts or, perhaps, a very small bacterium. In collaboration with Dr. Tony Goldberg (University of Wisconsin-Madison) we plan to use samples collected in this study to further characterize the pathogen via transcriptomics and/or metagenomics. Since coral colonies are a valuable resource and each coral colony acts independently as a result of its specific genetic identity, particular environmental history, individual holobiome composition, and prior (and often unknown) history of SCTLD exposure, using clonal cultures of algal symbionts as model systems for the study of SCTLD could help accelerate research progress. We strongly advocate for the use of cultured Symbiodiniaceae as model systems for future efforts to identify and characterize the SCTLD pathogen and test the efficacy of interventions and/or palliative treatments.

Executive Summary (max 1 page)

Since its initial appearance in late summer 2014, Florida's Coral Reef has experienced unprecedented losses of brain and boulder corals due to stony coral tissue loss disease (SCTLD). These coral species appear to be unusually susceptible to this disease, but the factors underlying differential susceptibility (at both the species and colony level) are not well understood. Studies have shown that algal symbionts (Family Symbiodiniaceae) are implicated in disease etiology (Landsberg et al., 2020) and that different algal symbionts can modulate susceptibility, both within and between species (Dennison et al., 2021). Subsequent work on algal symbiont cultures showed that some algal symbionts, particularly certain taxa in the genus *Breviolum* and *Durusdinium*, but also members of *Effrenium* (but not *Symbiodinium* or *Fugacium*) may be the direct targets of a SCTLD pathogen(s) when exposed to seawater from disease colonies filtered to 10 μm, suggesting that cultured symbionts may be informative model systems for understanding SCTLD, due to the relative ease of maintaining clonal lineages in the laboratory under standard conditions that can be easily replicated for comparative experiments across space and time (Karp et al., 2022). Here, we expanded on this approach by fractionating the disease-exposed seawater to identify the lowest size fraction that elicits this disease response in algal symbiont cultures. We systematically exposed five strains (*Symbiodinium microadriaticum*, *Breviolum minutum*, *Breviolum psygmophilium*, *Cladocopium goreaui*, and *Durusdinium trenchii*) to six treatments (control 0.2 μm f/2, "healthy" coral water filtered to 10 μm, and seawater sourced from around diseased corals that was filtered to 10 μm, 3 μm, 0.8 μm, and 0.2 μm). We monitored cell culture response for 10 days using light microscopy and chlorophyll fluorescence, and preserved samples for downstream analysis using TEM, fluorescence microscopy, and transcriptomic analyses. Furthermore, we preserved samples for other collaborators to study metabolomics (Dr. Neha Garg, Georgia Tech) and screen for the SCTLD pathogen through metagenomics (Dr. Tony Goldberg, University of Wisconsin-Madison). Our findings show that *B. minutum*, *C. goreaui*, and *D. trenchii* are directly impacted by seawater surrounding coral colonies presenting with active SCTLD lesions. Furthermore, the responses of these cultures are similar even when the source water is filtered to 0.8 μm (but generally not when filtered to 0.2 μm), which is consistent with the pathogen being a very small bacterium or a giant virus. Algal symbiont cultures thus may be useful laboratory models to further identify the pathogen through targeted studies, removing the complications of using corals with varied environmental histories, holobiome compositions, and (often unknown) SCTLD exposure histories, while also limited the extraction or use of valuable coral resources for routine SCTLD experiments.

Acknowledgements

Dr. Thierry Work was immensely helpful in TEM image analysis. We thank Dr. Javier del Campo, Dr. Mike Schmale, and Dr. Mohammad Moniruzzaman for equipment support and aid in characterizing quantification methods. We thank Alexandra Wen, Emily Yeager, Anthony Bonacolta, Fabrizio Lepiz Conejo, Danae Stephens, Owen Bornhorst, and Tiana Acito for help with sample preservation. Furthermore, we thank Vania Almeida at the Miller School of Medicine's TEM core for TEM sample processing. We also thank Mary Alice Coffroth, John Parkinson, and Madeleine van Oppen for sharing Symbiodiniaceae cultures and identifications. We remain very grateful to Dr. Karen Neely, for her continued provision of diseased coral colonies. This project would not have been possible without the support from the Florida Department of Environmental Protection's Office of Resilience and Coastal Protection, the Florida Keys National Marine Sanctuary (FKNMS), and the Florida Fish and Wildlife Conservation Commission (FWC) who permitted the collection of corals. Finally, we would like to acknowledge the help of Victoria Barker, Samantha Cook, Kristi Kerrigan, Joanna Walczak, and Maurizio Martinelli for their assistance throughout this project.

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1. DESCRIPTION

1.1. Overview

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. Approximately 21 species of coral, including both Endangered Species Act-listed and primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, stony coral tissue loss disease (SCTLD) is now endemic to all of Florida's Coral Reef, including the Dry Tortugas. SCTLD has also presented on corals in The Bahamas, Puerto Rico, and the US Virgin Islands, with the best available information indicating this outbreak is continuing to spread throughout the Caribbean (AGGRA, 2021). Recent studies have found that SCTLD has reduced live tissue by more than 60% on some Florida reefs (Walton et al. 2018) and other studies noted significant changes to coral community composition throughout the Caribbean (Alvarez-Filip et al. 2019; Estrada-Saldívar et al. 2021; Heres et al. 2021).

Early observations of this disease noted differential disease susceptibility, both among and within affected species (FKNMS case definition) and our 2020-2021 DEP-funded work demonstrated the role that different algal symbionts (Family Symbiodiniaceae) play in disease susceptibility. By manipulating corals to host different algal symbionts and then exposing them to SCTLD, we found that corals which predominantly or exclusively associate with *Breviolum* were the most susceptible to SCTLD, followed by corals associated with *Cladocopium,* which are slightly more susceptible than those with *Durusdinium* (Dennison et al. 2021). Based on field observations (Precht et al. 2016; Walton et al. 2018; Aeby et al. 2019; Muller et al. 2020), all three of these genera are more susceptible than corals that associate with *Symbiodinium* such as acroporids, which do not appear to be susceptible to this disease. In fact, corals that exclusively associate with *Breviolum* are 2.5x more likely to present with SCTLD than those exclusively associated with *Durusdinium*. This relative risk model suggests that disease susceptibility increases as the proportion of *Breviolum* increases (Dennison et al. 2021). This suggests that either the disease affects the algal symbionts directly, or that corals that host these symbionts are affected in such a way that they become more susceptible (e.g., as a result of coral host immunosuppression by certain algal symbionts, Fuess et al. 2020). The former hypothesis is supported by a recent report which suggests that SCTLD may be the result of a virus affecting the algal symbionts (Work et al. 2021, Aine Hawthorn DAC Presentation August 25, 2021). Moreover, damage to algal symbionts has also been observed in histological examinations of disease lesions and has more recently been associated through transcriptomic analysis (Landsberg et al. 2020, Beavers et al. 2023).

Our 2021-2022 work then built on these findings by exposing isolated cultured algal symbionts to disease seawater. We found that the same algal symbiont genera that are affected in corals are also susceptible in the cultured symbionts, with *Breviolum*, and

Durusdinium cultures declining significantly in both cell abundance and photochemical performance compared to symbionts in the genera *Symbiodinium* and *Fugacium* (as well as unexposed controls) showing little (or no) response. Transmission electron microscopy (TEM), further showed declines in internal cellular function when Symbiodiniaceae were exposed to pathogen water.

These findings suggest that particular algal symbionts are indeed the targets of the disease agent, and indicate that Symbiodiniaceae cultures may be useful model systems for future investigations of the disease, since (unlike studies involving coral hosts) they facilitate experimental trials that can be easily replicated and repeated with the same genotype and physiological status, allowing direct comparison between different studies.

1.2. Project Goals

The primary goal of this research is to further investigate the role of algal symbionts in SCTLD through experimental disease trials and systemically identify the size of the pathogen to set up future work to identify the causative agent and find alternative intervention strategies. Secondarily, we expand on the use of algal symbiont cultures as model systems for the study of coral diseases by identifying best practices and metrics to identify symbiont decline.

Specifically, we tested whether isolated Symbiodiniaceae cultures exhibit the same or similar response to disease inoculates that have been subjected to different levels of filtration. We challenged cultures of five different species from four different Symbiodiniaceae genera that vary in their susceptibility to SCTLD (based on our 2021–2022 results) by exposing them to disease-exposed seawater filtered to 0.2 µm, 0.8 µm, 3 µm, and 10 µm as well as a 0.2 µm f/2 culture medium to determine the approximate size of the disease pathogen. Our previous results (which filtered all disease treatments to 10 µm) indicate the pathogen is waterborne and <10 µm in size, but did not distinguish between viral, bacterial, or other planktonic size classes.

To achieve these objectives, we used a combination of physiological (chlorophyll fluorometry), microscopical (TEM, histopathology), and molecular techniques (transcriptomics) to characterize differential response among treatments. We also collected additional samples to share with collaborators for downstream questions to ensure the experiment had the widest possible use (e.g., the role of viruses in the observed response and further investigations aiming to identify the pathogen. These research questions have immediate applications to both understanding SCTLD and informing interventions using both asexual and sexual coral restoration practices to counter the impacts and spread of the disease.

2. METHODS

2.1. Symbiont Disease Exposures

Experiment 1:

In December 2022, we conducted a 10-day disease exposure experiment, of thirty replicate culture wells per symbiont per treatment belonging to five different strains of Symbiodiniaceae in four genera (*Symbiodinium microadriaticum*, *Breviolum minutum*, *B. psygmophilium*, *Cladocopium goreaui*, and *Durusdinium trenchii*) using six treatments (control 0.2 µm f/2 medium, "healthy" coral water filtered to 10 µm, seawater sourced from a tank containing corals with active SCTLD lesions filtered to 10 µm, 3 µm, 0.8 µm, and 0.2 µm) for a total of 6 treatments x 5 symbiont strains x 30 replicate wells = 900 experimental culture wells, each containing 4 mL of symbiont culture in f/2 medium. Both disease-presenting, and healthy coral colonies were established in 300 L flow-through aquaria systems with seawater from Bear Cut, Miami, FL, that had been filtered to 1 µm, UV-sterilized, and supplied at a rate of ~200mL per minute (turnover rate of water in tank ~24 h). The "diseased coral" tank contained colonies of *Colpophylia natans*, *Montastraea cavernosa*, *Orbicella faveolata*, *Pseudodiploria clivosa*, and *Pseudodiploria strigosa* showing active signs of SCTLD sourced from the Florida Keys National Marine Sanctuary (FKNMS) under permit FKNMS-2021-134-A1. The control tank was set up with conspecifics sourced from Miami-Dade County with no active lesions that were obtained as corals of opportunity under Special Activities License SAL-22-2443-SCRP from the Florida Fish and Wildlife Conservation Commission. After seven days of incubation in these systems, water from both the "diseased" and "control" tank was collected, filtered to 10 µm (to remove coral cells, mucus and tissue debris) to form two of the six treatments. An aliquot of the 10 µm-filtered water from the diseased tanks was then taken and filtered to 3 µm (to test for nanoplankton as the pathogen), 0.8 µm (for picoplankton and large viral particles), and 0.2 µm (for bacterioplankton versus viroplankton) for an additional three treatments. An f/2 control (growth medium) treatment was also used as a standard of autoclaved 0.2 µm filtered seawater. To each experimental culture well, we added 0.5 mL of these treatments for a standardized dose of ~11% (v/v). Water filters and an aliquot of each treatment water was preserved in DNA/RNA Shield and stored for future analysis. Additionally, treatment water was treated with concentrated iron chloride to chelate any potential viral particles or nucleic acids based on the protocol described by John et al. (2011). Pelleted iron was dissolved in ascorbate EDTA buffer and stored in DNA/RNA shield for downstream extraction.

Following inoculation, a subset of culture wells were monitored daily at 40x magnification using light microscopy (VWR VistaVision Inverted Microscope equipped with an iPhone 12 Mini lens adapter) and every well was monitored using dark-adapted chlorophyll fluorometry (with an Imaging-Pulse Amplitude Modulated fluorometer, Walz, GmbH). A total of 712 IPAM images and 7,799 5-second videos were used to quantify *in situ* culture conditions and prioritize samples for future downstream analysis.

Experiment 2:

A second, smaller disease exposure experiment was conducted on *B. minutum*, *B. psygmophilium*, and *D. trenchii* to test the effects of cell density and help standardize disease exposure trials. Prior to disease dosing, standardized aliquots of each culture were counted on a hemocytometer and ~1.25±0.23 million cells were added to sterilized f/2 medium and the final volume was brought to 4 mL. 0.5 mL of treatment water was then added to each well for an 11% (v/v) disease dose. To test for an effect of cell density and facilitate the comparison of symbiont cultures, a control treatment of f/2 medium with autoclaved seawater filtered to 0.2 μm was then used, together with two different disease pathogen fractions (0.8 μm and 0.2 μm) from a tank containing SCTLD-presenting colonies of *M. cavernosa* and *P. strigosa* water.

Following exposure to these inoculates, a subset of culture wells were monitored daily at 40x magnification using light microscopy (VWR VistaVision Inverted Microscope equipped with an iPhone 12 Mini lens adapter) and every well monitored through dark-adapted chlorophyll fluorometry of (using an Imaging-Pulse Amplitude Modulated fluorometer, Walz, GmbH). Cell density samples were also preserved in 10% Lugols by removing 0.45 mL of culture from triplicate wells daily for future cell counting.

2.2. Sample preservation

Triplicate wells from each culture and treatment were sacrificed daily and preserved for future analysis of epifluorescence, transmission electron microscopy (TEM), metabolomics, metagenomics, and transcriptomics. For epifluorescence analysis, 0.9 mL of culture was fixed in 0.1 mL of 10% glutaraldehyde, stained with 10 μL DAPI (0.5 mg/mL), and passed through a 0.8-um polycarbonate membrane filter. Filters were analyzed in duplicate using Leica DM 2500 at 40x magnification.

TEM samples were collected by pulling 1-mL aliquots from each well centrifuging at 2,000 g for 10 minutes, and removing the supernatant. Replicate cells from each symbiont and treatment were then combined, centrifuged again at 2,000 g for 10 minutes, the supernatant was removed, and stored in 0.4 mL of 2% glutaraldehyde at 4^{o}C. Metabolomics and metagenomic samples were processed by removing 1-mL of culture from each well and flash-freezing in liquid nitrogen before being stored at -80^{o}C. Transcriptomic samples were preserved by removing 1 mL of culture from each well, centrifuging at 2,000 g for 10 minutes at 4^{o}C, removing the supernatant, and flash-freezing the cell pellet in liquid nitrogen before being stored at -80^{o}C. This sample preservation scheme generated triplicate independent samples for each treatment and symbiont over the course of the 10-day experiment and generated 771 samples for transcriptomic, metabolomic, metagenomic, and epifluorescence microscopy to aid in further disease response quantification and pathogen detection. It also yielded 257 independent samples to process for TEM analysis. Samples were then prioritized for future downstream analysis based on *in situ* experimental observations of light microscopy and chlorophyll fluorometry.

2.3. Transmission electron microscopy processing (TEM)

A total of 51 samples were sectioned into 100 nm grids and stained with uranyl acetate and lead citrate. Grids were then imaged at the University of Miami's TEM core facility at 1,400x, 5,000x, and 8,000x magnification.

2.4. Symbiodiniaceae identification and quantification

Genomic DNA was extracted using a modified organic extraction protocol (Baker & Cunning, 2016) and the associated Symbiodiniaceae genera in each colony/core was characterized using real-time PCR (qPCR) assays. TaqMan environmental master mix (Thermo Fisher Scientific) assays were used to amplify the actin gene in *Symbiodinium* (Winter, 2017), *Breviolum* (Cunning et al., 2015), *Cladocopium*, *Durusdinium* (Cunning & Baker, 2013), *Effrenium* and *Fugacium* (Meisterzheim et al., 2019). qPCR data were used to identify associated algal symbionts and their relative abundance using the StepOne package for R (Cunning et al., 2013) adapted for these data.

2.5. Transcriptomic Sample Processing

The Quick-RNA Fungal/Bacterial Microprep Kit with DNase I treatment (Zymo) and 10 minute 0.1 mm & 0.5 mm bead beating step on a Vortex Genie was used to extract RNA from 384 samples for transcriptomic analysis. Following extraction, all samples were quantified using Quant-iT high sensitivity RNA quantitation assay (Life Technology) on an Infinite 200 Pro plate reader (Tecan).

384 samples of extracted mRNA were prepared for sequencing using a QuantSeq 3' mRNA-Seq V2 Library Prep Kit FWD kit (Lexogen). Samples are in the pipeline for sequencing at the sequencing core facility of the John P. Hussman Institute for Human Genomics at the University of Miami's Miller School of Medicine, using an Illumina NovaSeq with 150 base single end reads at a sequencing depth of ~5 million reads per sample.

Sequences will then go through standard bioinformatics pipelines for host/symbiont gene expression analysis. Raw 3' reads will be trimmed of their adapters and quality-filtered using BBDuk from the BBTools software suite. Cleaned reads will be quantified using Salmon with previously generated de novo transcriptomes as reference. Quantified reads will be imported into the R statistical environment using tximport and differential expression calculated with DESeq2. KEGG and GO ontology enrichment analysis of differentially expressed transcripts will be performed using the clusterProfiler R package with previously generated annotations for each transcriptome. The R implementation of Expression Variance Evolution (EVE) Model will be used to compare results among species to parse out the effects due to phylogeny on differential expression to compare transcriptional response to treatments among taxa. Finally, the WGCNA R package will be used to generate co-expression modules for each taxon to identify gene expression networks and the effects of treatments on their connectivity and expression.

2.6. Data Analysis

All data analysis was performed in Rstudio version 4.2.1. Linear regression and cubic polynomial regression assessed differences between symbionts and treatment. The emmeans package was used to assess pairwise differences in differential response. ImageJ pipelines were developed to analyze microscopy images using the “Analyze particle” plugin.

3. RESULTS

3.1. Chlorophyll fluorometry

Six wells from each treatment and symbiont type were monitored daily, and two different-12 replicate wells were monitored every other day, yielding 18 chlorophyll fluorometry samples per symbiont per treatment per experimental day. Experimental wells were removed from analysis after being sacrificially sampled. Additionally, data points were removed if diatoms became dominant in culture and the photochemical signal was no longer generated by symbionts, as determined by observational analysis. The cubic polynomial regression of daily, dark-adapted, chlorophyll fluorometry data showed a significant decline based on days in treatment, symbiont strain, and treatment ($P<0.01$, $R^2=0.7769$). The photochemical efficiency of *B. minutum*, *C. goreaui*, and *D. trenchii* strains inoculated with seawater from around diseased corals filtered to 0.8 µm and larger fractions showed statistically different linear declines compared to control and 0.2 µm-dosed cultures. *C. goreaui* and *D. trenchii* also declined with seawater filtered to 0.2 µm, but the rate of decline was slower from that of the 10 µm, 3 µm, and 0.8 µm treatments. *B. psygmophilium* and *S. microadriaticum* were unaffected by any of the treatments (Figure 1). Due to cell density differences, treatments could be assessed within symbiont types, but not among them.

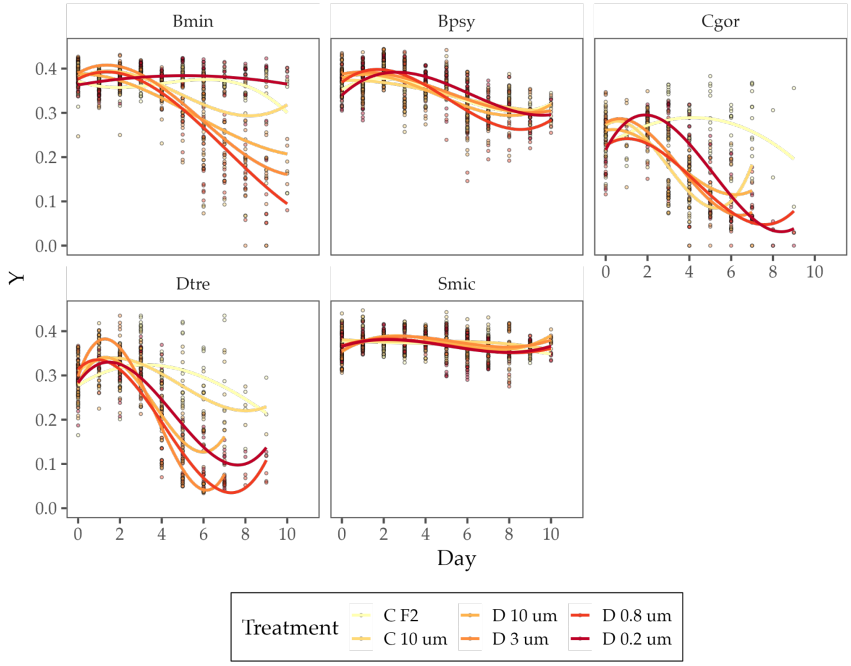

Figure 1. Decline in photochemical efficiency of symbiont cultures by treatment during first experiment.

In the second, smaller experiment with three strains, three treatments, we sampled 24 replicate wells daily and controlled for initial symbiont density in the cultures. A cubic polynomial regression showed a statistical difference between symbionts, treatment, and day (Figure 2, $P<0.01$, $R^2=0.5565$). When symbiont density was controlled for, *B. minutum* dosed with water sourced from around corals presenting with SCTLD that had been filtered to 0.8 µm declined significantly more rapidly than that of either *B. psygmophilium* or *D. trenchii*, suggesting higher susceptibility.

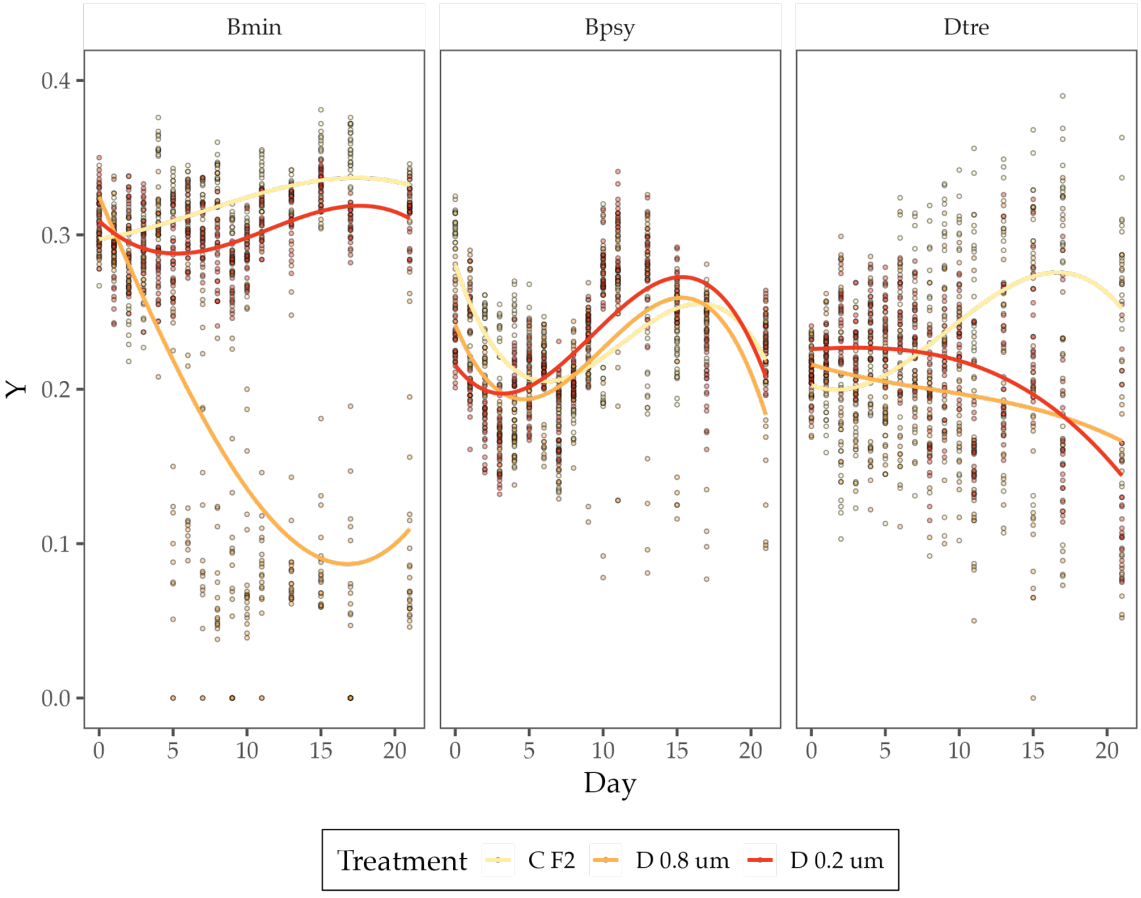

Figure 2. Decline in photochemical efficiency of symbiont cultures by treatment in the second experiment.

3.2. Cell Counts

Still images were extracted every 1 second from 5-second videos taken in duplicate daily of six replicate culture wells and the three sacrificial culture wells per treatment per symbiont for nine independent well measurements each day. Still images were then extracted using a Python script prior to cell counting using an ImageJ. The

ImageJ pipeline utilized the Analyze Particle plugin with a watershed filter to disentangle cells that were close to one another. Triplicate images from each day for each symbiont for each treatment were also classified to determine relative cell density (low, medium, high) and confirm presence of only symbionts. Cubic polynomial regression on cell counts showed a significant effect of symbiont, treatment, and days (Figure 2, $P<0.01$, R^2: 0.723). Initial cell densities differed significantly between symbiont types, with both *C. goreaui* and *D. trenchii* having fewer cells than those of *Breviolum* or *Symbiodinium*. Cell abundance trends, however, showed similar results to those of chlorophyll fluorescence where *B. minutum*, *C. goreaui,* and *D. trenchii* all significantly decreased their cell abundance when exposed to treatments below 0.8 µm. Linear decreases in cellular abundance were statistically different between disease treatments of *B. minutum*, *C. goreaui*, and *D. trenchii* compared to controls. Similar to photochemical analysis, *S. microadriaticum* and *B. psymophilium* did not decline differentially between treatments when compared to disease treatments.

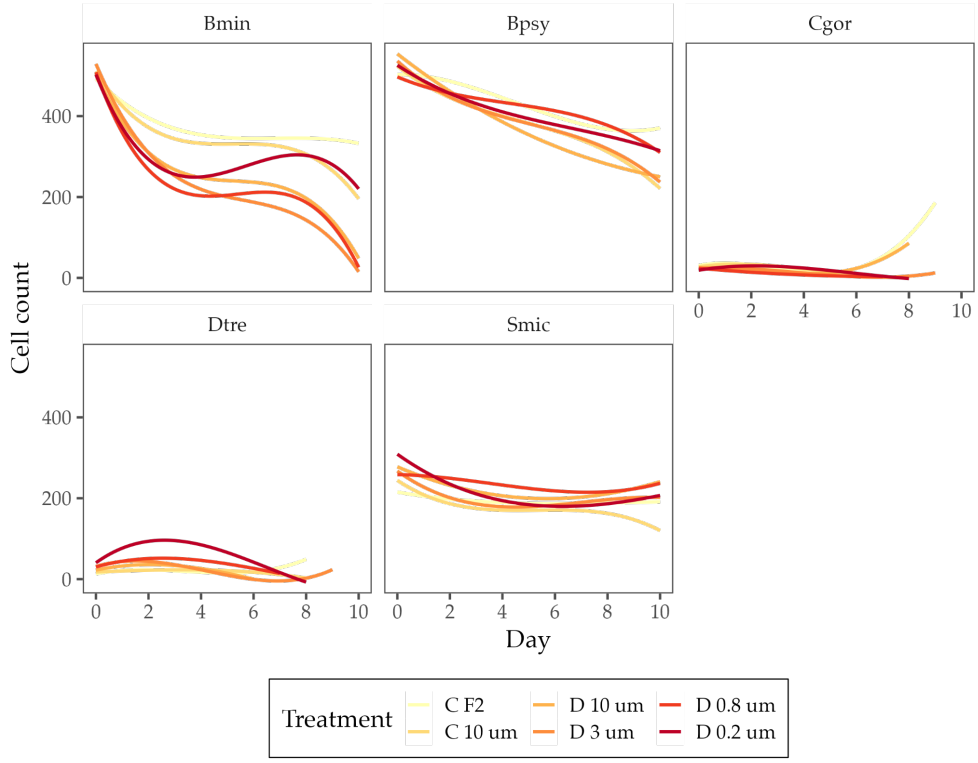

Figure 3. Video cell counts of each symbiont and treatment over the course of the experiment.

3.3. Epifluorescence microscopy

Fluorescence data analysis is still ongoing, but 240 filters have been imaged in duplicate for chlorophyll and positive nuclei with DAPI staining using a Leica DM 2500

microscope at 40x magnification. ImageJ was used to count the number of nuclei and chlorophyll cells, and we are working on developing a reliable pipeline to assess overlap between them. Preliminary analyses suggest a decrease in the number of symbiont and DAPI-stained cells in disease treatments of 10 µm, 3 µm, and 0.8 µm compared to controls, with *B. psygmophilium* remaining unaffected. However, these decreases are not significant, and we continue to try to refine our methods to increase accuracy of this new (and somewhat automated) method. Further analysis will help to determine the overlap between cells and test for cell abundances of *D. trenchii* throughout the course of the experiment. Assessing chlorophyll to DAPI overlap may better elucidate when symbiont cells are impaired, but still intact. Additionally, these fixed slides can also be used to produce other stains of symbiont cells, which may be useful to define new metrics of symbionts in culture and better assess the pathogen of interest such as RNA viruses, with Dr. Samantha Coy at Texas A&M University.

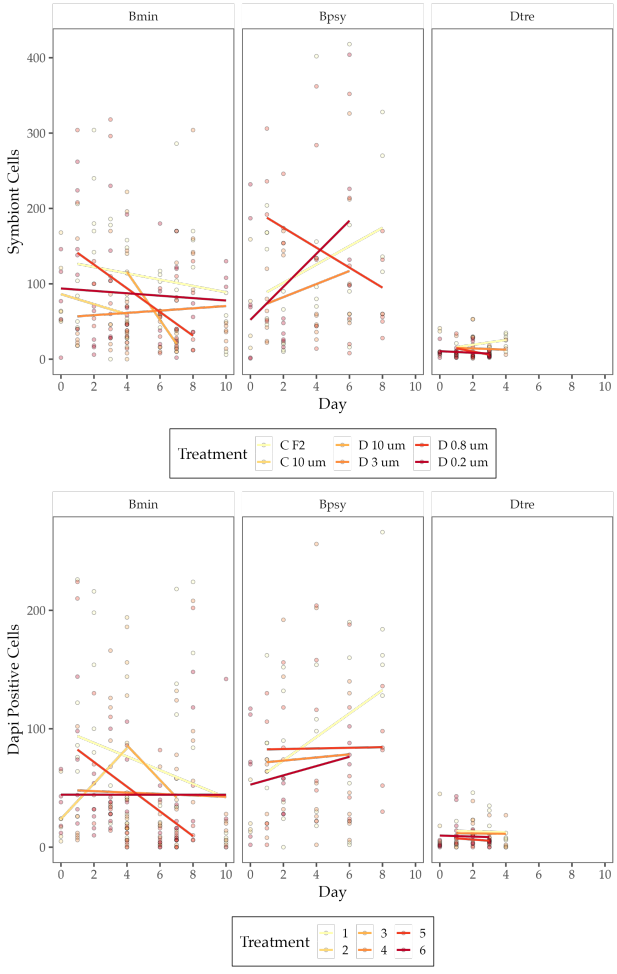

Figure 4. Cell counts through epifluorescence microscopy of A) chlorophyll autofluorescence and B) DAPI stained positive nuclei.

3.4. TEM analyses

A total of 51 samples have been prepared for TEM imaging and 32 have been imaged. Due to low cell density pellets, neither *C. goreaui* nor *D. trenchii* could be processed for TEM. All processed disease treatment samples of both *B. minutum* and *B. psygmophilium* appear to be affected throughout the course of the experiment with lower cell abundance, loss of cell structure, and the presence of more electron-dense inclusions and disrupted electron dense inclusions (Figure 5 and 6). Diseased samples of *B. minutum*, however, declined the most compared to f/2 control samples, with a visible loss of chloroplasts, pyrenoids, and other cellular organelles. In later time points, internal small bacteria <0.8 μm also become more present in fractions exposed to diseased seawater filtered to less than 0.8 μm. Expert analysis is needed to confirm whether anisometric viral-like particles (AVLPs) are present, but presence of electron-dense regions, which may be viroplasms, appear to vary considerably by symbiont strain and increase throughout the course of the experiment (Work et al. 2021).

Preliminary analysis of the number of chloroplasts in *B. minutum* and *B. psygmophilium* shows that control samples of both symbionts have significantly more functional chloroplasts than 0.8 μm disease-treated samples at later time points (Figure 9a, ANOVA, $p<0.05$). Additionally, disease-exposed samples of *B. minutum* significantly increase the amount of Type IA inclusions (disrupted Type I electron dense regions) and increase in internal bacterial abundance (Figure 9bc, ANOVA, $p<0.05$).

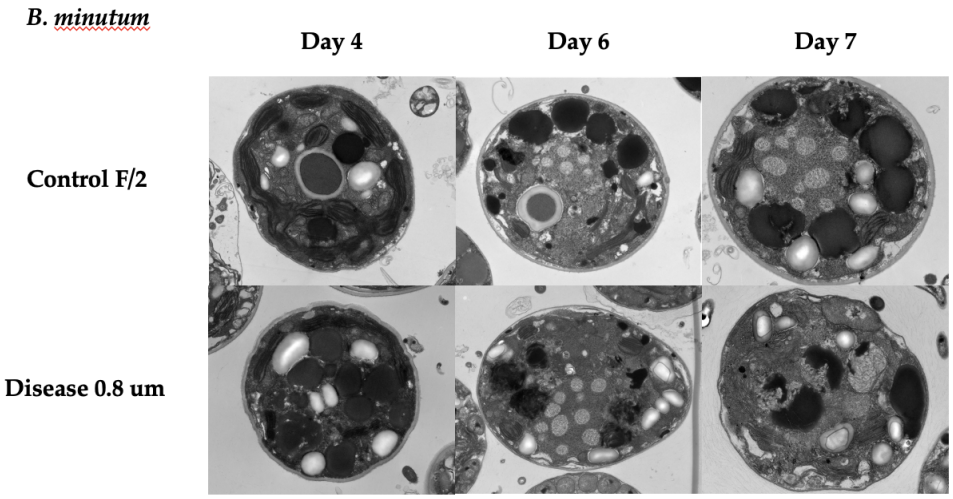

Figure 5. Representative images of *B. minutum* at 4,000x magnification through transmission electron microscopy. The top row is control cells at days 4, 6, and 7. The bottom row shows the decline of symbionts in disease treatments over the same time period.

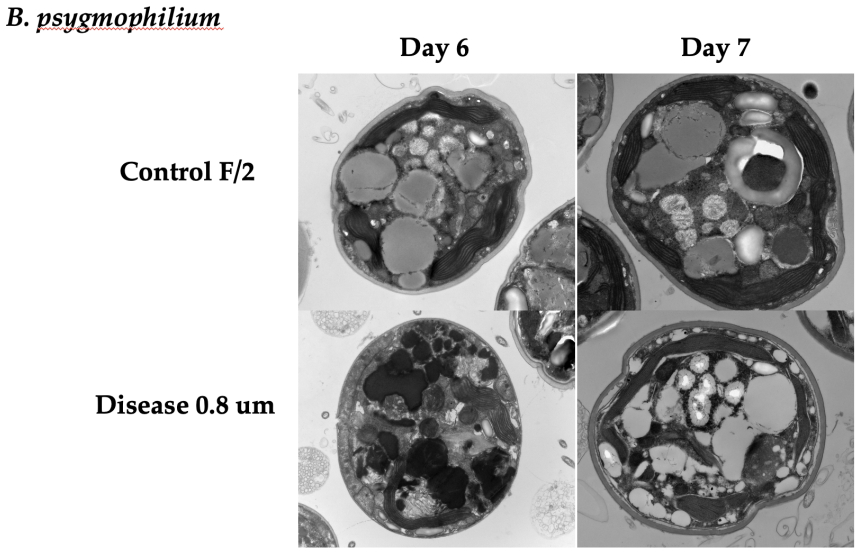

Figure 6. Representative images of *B. psygmophilium* at 4,000x magnification through transmission electron microscopy. The top row is control cells at days 6 and 7. The bottom row shows the decline of symbionts in disease treatments over the same time period.

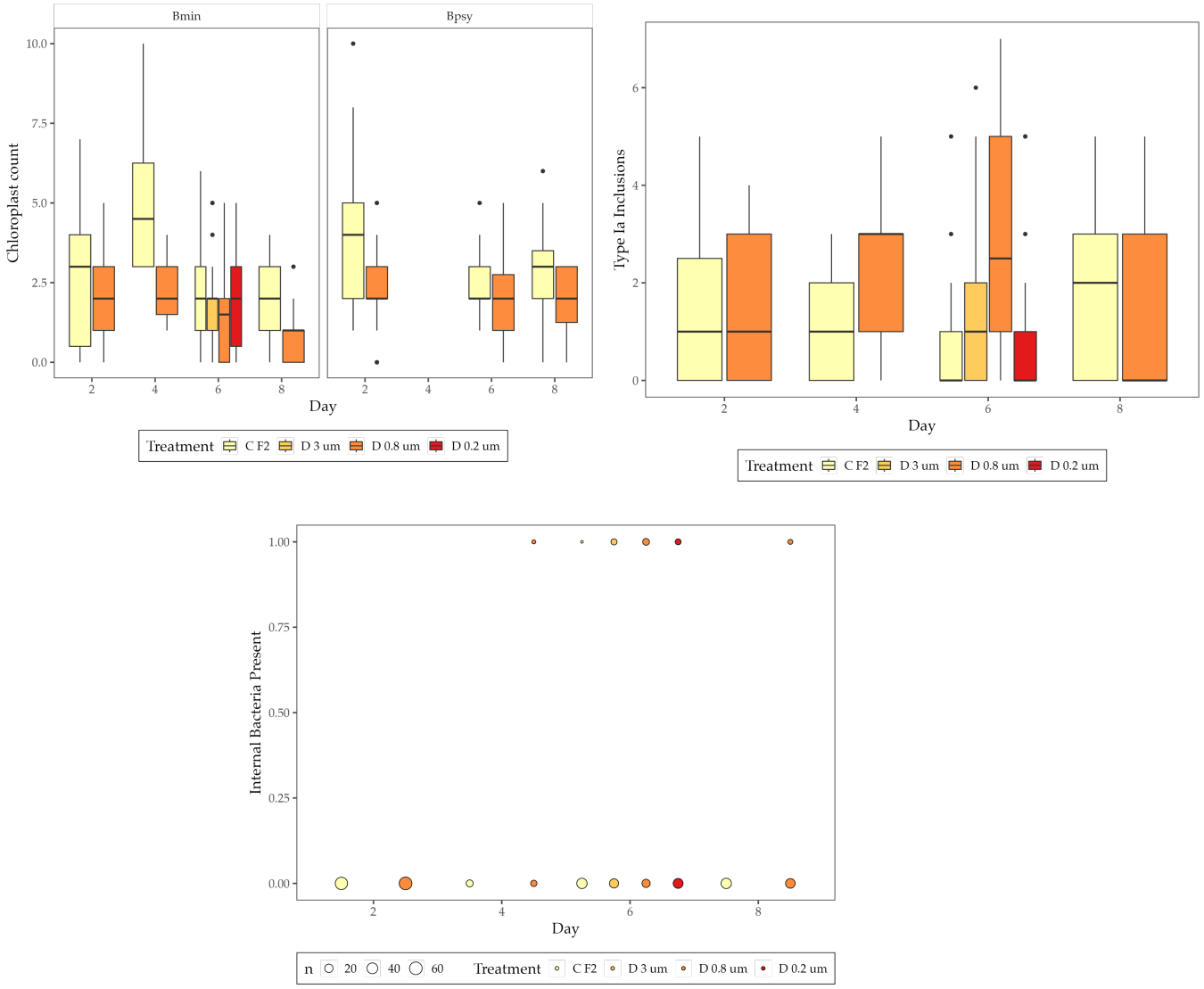

Figure 7. Statistical analysis of TEM data. A) Loss of chloroplasts in disease treatments of *B. minutum* and *B. pysgomophilium*. B) Increase of Type IA inclusions in *B. minutum* disease treatments. C) Increase of internal bacteria of disease treatments in *B. minutum*.

3.5. RNA Sequencing

RNA analysis will help inform the molecular mechanisms by which symbionts are affected by exposure to the SCTLD pathogen(s) and may help to identify the

pathogens if sufficient pathogen reads are detected. A total of 384 samples across treatments, symbionts, and timepoints (See Table 1) were extracted with the Quick-RNA Fungal/Bacterial Microprep Kit and RNA yield was quantified using a Quant-iT high sensitivity RNA assay. The samples are in the pipeline for sequencing with the QuantSeq 3' mRNA-Seq V2 Library Prep Kit FWD kit (Lexogen). Due to low cell abundance, no *Cladopium* samples were generated for sequencing. Samples are being sequenced at the University of Miami Sequencing Core using an Illumina NovaSeq with 150 base single end reads at a sequencing depth of 5-10 million reads per sample. Four samples with high RNA concentration will be prepared from starter cultures for de novo assembly of symbiont transcriptomes. De novo assembly of culture transcriptomes is necessary to properly identify and map to transcript 3' untranslated regions for symbionts without species level transcriptomes or high quality genomes.

Symbiont	Experiment Day	Column1	C F/2	C 10 µm	D 10 µm	D 3 µm	D 0.8 µm	D 0.2 µm	#N/A	Grand Total
Grand Total		**0**	**101**	**41**	**30**	**55**	**102**	**68**	**0**	**397**
Bmin (2)	0			3				3		6
	1		3	3	3	3	3	3		18
	2		3	3	3	3	3	3		18
	3		3	3	3	3	3	3		18
	5		3	3	3	3	3	3		18
	6		3	3	3	3	3	3		18
	7		3	3	3	3	3	3		18
	8		3	3	3	3	3	3		18
	9		3	3	3	3	3	3		18
	10		3	3	3	3	3	3		18
Bmin (2) Total			27	30	27	27	27	30		**168**
Bpsy (16)	0			3				3		6
	1		3	1		3	3	3		13
	2		3			3	3	3		12
	3		3			3	3	3		12
	4		3			3	3	3		12
	6		3			3	3	2		11
	7		3			3	3	3		12
	8		3			3	3	3		12
	9		3			3	3	3		12
	10		3	1		3	3	3		13
Bpsy(16) Total			27	5		27	27	29		115
Dtre (6)	0			3				9		12
	1		3	1		1	3			8
	2		3	2	2		3			10
	3		3				3			6
	4		3				3			6
	5		3				3			6
	6		3				3			6
	7		3				3			6
Dtre (6) Total			21	6	2	1	21	9		60
Smic (26)	0		3				3			6
	1		3				3			6
	2		3				3			6
	3		3		1		3			7
	5		3				3			6
	6		3				3			6
	7		3				3			6
	8		2				3			5
	9		3				3			6
Smic (26) Total			26		1		27			54

Table 1. Symbiont samples extracted by day and treatment for RNA.

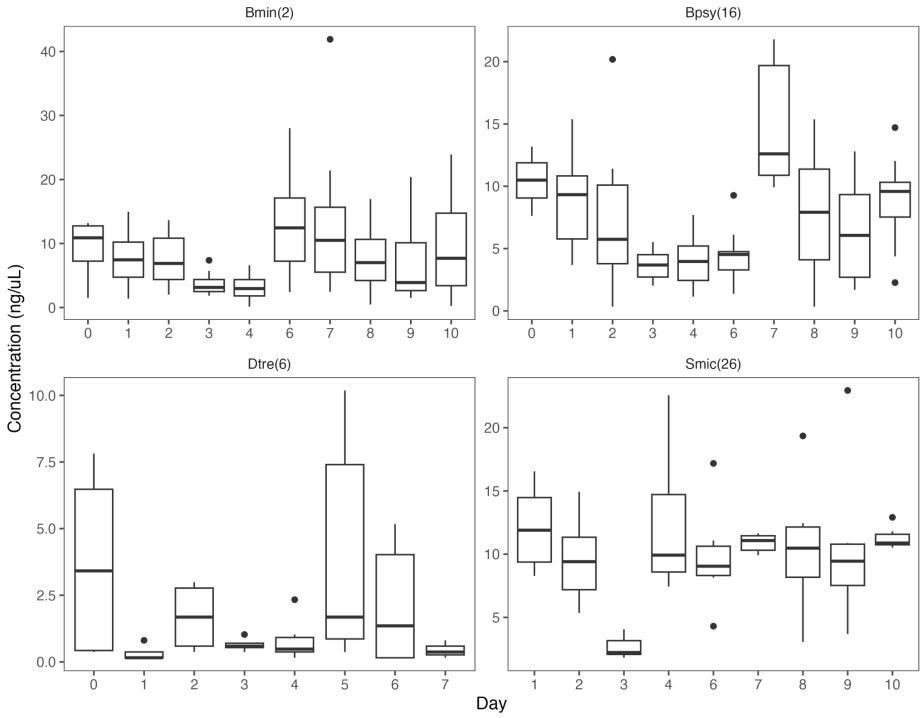

Figure 8. Library preparation input RNA concentrations.

3.6. qPCR and Sanger sequencing for symbiont identity

qPCR analysis of the actin gene for each symbiont genera confirmed the identities of the cultures to the genus level (*Symbiodinium*, *Breviolum*, *Durusdinium*, and *Cladocopium*). Extracted DNA from parent cultures were sent for Sanger sequencing to identify the cultures at the ITS2 (species) level and BLAST sequences confirmed (at >99% identity) the cultures at the strain level as *S. microadriatiucum*, *B. minutum*, *B. psygmophilium*, and *D. trenchii*. Due to limited cell abundance, *C. goreaui* has not been confirmed on our end, but has been confirmed by Australian collaborators (Madeleine van Oppen, AIMS).

4. DISCUSSION

In this experiment, we systemically exposed different algal symbiont species to fractionated seawater surrounding coral colonies presenting with active SCTLD lesions to determine the size of SCTLD pathogen and refine methods to better identify it outside the coral host. Results of this study continue to support the discovery that algal symbionts are affected by exposure to SCTLD in isolated cultures (Karp et al. 2022). Again, we show that the susceptibility of isolated Symbiodiniaceae cultures is similar to that of Symbiodiniaceae *in hospite,* with both *B. minutum* and *D. trenchii* being susceptible to SCTLD relative to other strains (Dennison et al. 2021). When we were further control for cell density, *B. minutum* is significantly more susceptible than *D. trenchii* and mirrors the hierarchy found in the coral host. This study also expanded on previous work by including additional Symbiodiniaceae cultures (*C. goreaui* and *B. psygmophilium*), and the use of water fractionation to better understand the size of the SCTLD pathogen(s). These additional species allowed for direct comparison of two different *Breviolum* species, which show differential susceptibility in the coral host, and also test the susceptibility of *C. goreaui* in culture. Using physiological (chlorophyll fluorometry), and microscopic (light, epifluorescence, TEM) approaches, we conclude that different Symbiodiniaceae vary considerably in their susceptibility to SCTLD following exposure to seawater surrounding corals affected by the disease. Sample preservation from this experiment will be coupled with advanced molecular techniques to identify disease etiology such as metabolomics (with Dr. Neha Garg, Georgia Tech), or the pathogen itself either through this grant (transcriptomics) or other collaborations, such as RNA viral detection in preserved samples (with Dr. Samantha Coy, Texas A&M University), and/or metagenomic (with Dr. Tony Goldberg, University of Wisconsin-Madison).

Cultures of *B. minutum*, *C. goreaui*, and *D. trenchii*, which were affected by diseased seawater showed a similar response in cellular abundance and loss of photosynthetic function in the 10 μm, 3 μm, and 0.8 μm seawater treatments. These results indicate that the likely SCTLD pathogen(s) are between 0.2 μm and 0.8 μm in size and support previous work that the pathogen may be a virus and is spread through water (since we used shared seawater as the direct mode of transmission). Both *C. goreaui* and *D. trenchii* were also affected by the 0.2 μm treatment, but in a slightly different manner, with both photophysiology and cell abundances declining slightly later than that of the other three disease treatments. However, these two cultures contained fewer cells (~25%) compared to the other three strains, and it is possible that this may have affected relative susceptibility. We hypothesize that the pathogen may be close in size to the threshold of the 0.2 μm filter, allowing some pathogen particles through, but decreasing the overall disease dose to which cultures are exposed. Additionally, *C. goreaui* was originally isolated from the Great Barrier Reef and is typically grown in Daigo's IMK culture medium rather than the f/2 medium used here (which we used for ease of comparison across cultures). Originating from a different ocean basin and growing in a new medium may explain its eventual decline across all treatments. Additionally, the cultures that were exposed to water from a healthy coral tank that had been filtered to 10 μm also declined in health in both *C. goreaui* and *D. trenchii*, which could have been due to low cell abundance reflecting poor culture quality to begin with. Additionally, at the end of the experiment (nine days after dosing) one of the putatively

healthy corals began to show signs of disease, and it is therefore possible that a low dose of pathogen may have been present in the control treatments that were prepared nine days earlier.

To further develop algal symbionts as model systems and try to better compare between algal symbiont strains, we controlled for initial cell density in a smaller experiment where exposed *B. minutum*, *B. psygmophillium*, and *D. trenchii* to three different treatments. We found that no cultures (including *D. trenchii*) were affected by seawater less than 0.2 μm, but *B. minutum* was more susceptible than *D. trenchii* when exposed to 0.8 μm seawater. Although the pathogenic agent(s) have not been identified, these results suggest the pathogen(s) may be either a giant virus or small bacterium that infects the Symbiodiniaceae.

Transmission electron microscopy images of *B. minutum* show an increase in electron-dense regions in disease treatments and an eventual disruption of these electron-dense regions before *B. minutum* is disrupted entirely and begin to show increases in intracellular bacteria. *B. psymophilium* also shows a similar TEM pattern, with loss of chloroplasts and increased electron-dense regions in disease treatments, but appears unaffected by disease treatments in terms of cell abundance or photophysiology. This further indicates different symbionts vary in their SCTLD response and highlight an immune response that may be further elucidated by transcriptomics. We interpret the proliferation of internal bacteria as a secondary infection of (potentially endogenous) bacteria which consume rapidly degrading algal symbionts and their host cells, potentially explaining why amoxicillin treatments of affected coral hosts are effective, but do not entirely stop the spread of SCTLD in corals (Neely et al., 2021).

This work paves the way to accelerate efforts to identify the SCTLD pathogen identification and trial palliative treatments by using lab-raised symbiont cultures, rather than coral animals, as the primary target of the disease agent (Soffer et al., 2010). Using clonal cultures of algal symbionts as model systems to study SCTLD has several advantages because they can be raised under laboratory conditions (incubators) that can be standardized across laboratories, they have known environmental histories, and, if properly maintained, can be guaranteed to be naive to SCTLD. Experiments undertaken by different laboratories can therefore be directly compared in ways that might be impossible with corals as the experimental targets. Indeed, the only uncontrolled variable in disease trials of symbiont cultures (such as the ones described here) is the pathogen load created in the disease bath.

In conclusion, we suggest that algal symbiont cultures may be useful model systems for the advanced study of stony coral tissue loss disease. We have now undertaken multiple experiments that have shown that algal symbiont are differentially susceptible to a rapidly developing detrimental condition when exposed to seawater sourced from tanks containing coral colonies with active SCTLD lesions, but not when exposed to seawater from control tanks containing healthy corals. The hierarchy of susceptibility of algal symbionts to this condition is remarkably predictive of the hierarchy of coral species' susceptibility to SCTLD when viewed in light of the algal symbionts they host (Dennison et al. 2021). Here, we expand on this novel approach to

studying “algal symbiont degenerative disease” (ASDD) by successively exposing algal cultures to fractionated disease doses and show that the pathogen is relatively small (perhaps ~200 nm in diameter), and likely represents either a small bacterium or giant virus. The pathogen is too small to be the bacteria that we observed proliferating in affected symbionts, and we suggest these larger bacteria are opportunistic consumers of the organic carbon resulting from large pools of rapidly degrading algal symbionts. Application of antibiotics may arrest this proliferation and halt the spread of disease, but may not be treating the pathogen itself. Finally, we suggest that these findings open the door to considering whether other diseases, particularly white syndromes, might also be diseases of algal symbionts or other components of the coral microbiome, which may benefit from a similar model-systems approach (Traylor-Knowles et al. 2022).

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