Using size fractionation to determine whether the SCTLD pathogen is bacterial, viral, or other

Final Report

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Contents

Introduction

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 (Precht et al., 2016; Walton et al., 2018). 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 the Florida Reef Tract, and south to Key West in the Lower Florida Keys. The best available information indicates that the disease outbreak is continuing to spread throughout the Caribbean (Weil et al., 2019).

In spite of several years of research and considerable community involvement, the causative agent of stony coral tissue loss disease (SCTLD) remains unidentified. While evidence exists that this is an infectious disease that can be transmitted via water (Aeby et al., 2019), the type of pathogen (e.g. bacterial, viral) is unknown. Antibiotics limiting the disease suggests a bacterial pathogen (Neely et al., 2020), however the observed effects may be due to treatment of secondary opportunistic infections rather than the primary infection. Crystalline inclusion bodies observed in the histological preparations of several diseased corals could be evidence of viral arrays or bacterial toxin proteins (Landsberg et al., 2020). Identification of the pathogen type is critical knowledge that could be used to better direct intervention, treatment, and restoration efforts.

As part of the Coral Disease Technical Workshop held in August 2019, a specific experiment was outlined to use tangential flow filtration (TFF) to concentrate the microbial community from mesocosm water containing diseased corals, size fractionate that microbial community based on filter size (e,g., 0.22-µm filters to capture bacteria and 0.025-µm filters to capture viruses), and then physically apply those filters to healthy corals to see which set of filters (and therefore, which size group of microbes) initiated visible signs of SCTLD. The benefit of using mesocosm water is that the seawater is initially UV-treated and sterile 0.22µm-filtered, substantially reducing the background microbial community of both bacteria and viruses. Incubating corals in these mesocosms should therefore inoculate the water with microbes shed by the corals, and in the case of diseased corals, the SCTLD pathogen (with significantly less background microbes than investigating reef water or coral tissue). Further, using TFF to concentrate the microbial community prior to sequential size fractionation is intended to mitigate the fact that we have no idea what the infectious dose (amount of the pathogen required to initiate disease) is, so it is important to try to capture as many microbes at each stage as possible. Based on this hypothesis, sequencing of the filter size that initiates signs of disease should provide a much smaller pool of potential pathogens, narrowing the suspect list for the causative agent.

The overall goals of this project were 1) to isolate the causative agent of SCTLD to a specific size fraction and 2) to narrow the list of possible suspects within the specific size fraction via sequencing.

Task 1: Size fractionation experiments

Three mesocosm experiments were conducted in collaboration with the Smithsonian Marine Station (SMS) in pursuit of the first goal: a pilot project in October 2019 to provide proof-of-concept prior to Florida DEP funding and two full experiments in November 2020 and March 2021.

Clean seawater (UV-treated and sterile 0.22 µm-filtered) was placed into ~18L mesocosms and then individual healthy or diseased coral colonies were incubated in the mesocosms with air bubblers for 2-5 days to enrich the water with microbes shed from the corals. The hypothesis was that (i) the microbial diversity shed into the water would be less than that of either natural reef water or coral tissues, reducing the microbial 'background noise,' and that (ii) diseased corals would shed the causative agent of SCTLD into the water, since it has been shown to be transmissible through water.

In each of the three experiments, there were 10 healthy (control) and 10 diseased (SCTLD) corals used to inoculate the 20 total mesocosms. All three experiments followed this basic plan (Figure 1): after incubation, corals were removed from the mesocosms and repurposed for other experiments at SMS. Mesocosm water was poured through an ethanol-sterilized mesh screen and into a clean bucket to remove any pieces of coral or sediment that could clog the subsequent filters (and confuse results by including microbes from multiple size fractions). A tangential flow filtration (TFF) system with five 100 kDa filter cassettes was used to concentrate the total microbial community in each mesocosm from a starting volume of ~18 liters down to less than 300 milliliters. This concentrate was then sequentially filtered through different sized filters to physically separate different microbial groups: a 0.8-µm filter to capture larger particles (e.g., diatoms; used in the full experiments only), a 0.22-µm filter to capture bacteria, and a 0.025-µm filter to capture viruses. This sequential sterile filtration resulted in sets of ca. 47-mm diameter cellulose nitrate filters for each size fraction which were then cut into quarters with sterile razor blades. The quarter-filter pieces were applied to healthy "receiver" corals, each in individual containers within water tables. The rest of each filter was promptly frozen at -20 °C for potential sequencing pending experimental results. The controls and treatments were monitored for appearance of disease signs by Smithsonian staff for at least four weeks after filter application.

The specifics of each of the three experiments are provided here:

<u>October 2019 pilot</u>

The pilot experiment was conducted October 28–30, 2019, prior to DEP funding to test the experimental design. Healthy corals (3 *Colpophyllia natans*, 4 *Orbicella faveolata*, 3 *Montastraea cavernosa*) and diseased corals (3 *C. natans*, 4 *O. faveolata*, 3 *M. cavernosa*) from the Florida Keys were incubated in seawater mesocosms for 2–4 days. After removing the corals, the mesocosm water was poured through an ethanol-sterilized 200-µm mesh prefilter to remove any small coral and sediment pieces. The microbial community in the mesocom water was concentrated by TFF and then sequentially filtered through two filter sizes: 0.22-µm (bacteria) and 0.025-µm (viruses).

Filters were attached to *O. faveolata* coral medallions obtained from the Coral Restoration Foundation (Project ID CRF-2019-011) using sterile plastic forceps (Figure 1) with ten 0.22-µm (bacteria) treatments, ten 0.025-µm (viruses) treatments, and ten combination treatments that included filter pieces from both size fractions. If the virus fraction had not filtered to completion after 5 hours, the remaining liquid was added to the corresponding treatment container to maintain a full mesocosm's 'dose' of that size fraction. The treatments were observed for 4 weeks.

Results

Four out of ten diseased corals (mesocosms Ofav16, Mcav8, Mcav17, and Mcav18) were Vcp+ (positive test for the presence of *Vibrio coralliilyticus*).

There were no effects to the receiver corals from any of the treatments derived from healthy coral mesocosms. In other words, the receiver corals did not show any adverse reaction (e.g., bleaching or tissue loss) in response to physical contact with filters or exposure to concentrated microbes shed by healthy corals into seawater.

Microbes (bacteria and viruses) were shed into the mesocosm water by both healthy and diseased corals and successfully concentrated and size fractionated by our method. This was confirmed by epifluorescence microscopy spot-checks of the water at each step of the process. Additionally, microscopy revealed the presence of pennate diatoms in the diseased coral mesocosms. This resulted in the addition of a 0.8-µm filter size to the experimental design for the full experiments to separate out any eukaryotes like diatoms from the bacterial size fraction.

The 0.22-µm size fraction filters from two of the ten diseased samples (Mcav17 and Mcav18) initiated signs of SCTLD after 12 days. None of the other controls or treatments showed any disease signs.

November 2020 Experiment

The first full experiment was conducted November 4–8, 2020. Healthy corals (1 *Diploria labyrinthiformis*, 2 *O. faveolata*, 3 *C. natans*, and 4 *M. cavernosa*) originated from the Dry Tortugas and Key West nursery (but had been held for some time in tanks at SMS), and diseased corals (1 *D. labyrinthiformis*, 2 *O. annularis*, 3 *C. natans*, and 4 *M. cavernosa*) were collected from reefs off Marathon and Broward immediately prior to the experiment. The corals were incubated in seawater mesocosms for 3–4 days. After coral fragment removal, the mesocosm water was poured through an ethanol-sterilized 106-µm mesh prefilter to remove any small coral and sediment pieces. The microbial community in the mesocosm water was concentrated by TFF and then sequentially filtered through three filter sizes: 0.8-µm (microeukaryotes), 0.22-µm (bacteria) and 0.025-µm (viruses). Additionally, the TFF filtrate (< 100 kDa) was also collected to use as a non-microbial treatment to test for small molecule/chemical signal effects. Filters were attached to *O. faveolata* coral medallions obtained from the Coral Restoration Foundation (Project ID CRF-2020-004) using sterile plastic forceps (Figure 1) with ten 0.8-µm treatments, ten 0.22-µm (bacteria) treatments, ten 0.025-µm (viruses) treatments, ten combination bacterial/viral treatments, and ten treatments where a coral fragment was immersed in the TFF filtrate instead of seawater. If the virus fraction had not filtered to completion after 5 hours, the remaining liquid was added to the corresponding treatment container to maintain a full mesocosm's 'dose' of that size fraction. The treatments were observed for 4 weeks, at which point the filters were removed, and the observation continued for an additional 3 weeks.

Results

All of the November 2020 diseased corals were Vcp- (negative for the presence of *Vibrio coralliilyticus*).

Examination of duplicate microscopy slides made for each stage of the process (Figure 2) confirmed that the concentration and size fractionation occurred as expected. Ten different genotypes of *O. faveolata* medallions received filters during the treatments. After 7 weeks, none of the treatments had shown signs of disease (unlike the October 2019 pilot experiment). Subsequent experiments conducted by Valerie Paul and Kelly Pitts of the Smithsonian Marine Station tested the susceptibility of these *O. faveolata* genotypes to SCTLD by placing representative corals in direct contact with healthy or SCTLD-affected coral fragments. While one of the 7 genotypes tested succumbed to SCTLD after approximately 12 days of direct contact, 3 genotypes took over 2 months to become diseased and 3 genotypes never became visibly infected. None of the genotypes in contact with healthy coral were affected.

March 2021 Experiment

The second full experiment was conducted March 25–29. 2021. Healthy corals (3 *D. labyrinthiformis*, 3 *C. natans*, and 4 *Pseudodiploria strigosa*) originated from the Dry Tortugas and Key West nursery (but had been held for some time in tanks at SMS), and diseased corals (1 *P. strigosa*, and 9 *C. natans*) were collected from reefs off Marathon. The corals were incubated in seawater mesocosms for 4–5 days. Following removal of the corals from the mesocosm, the mesocosm water was poured through an ethanol-sterilized 106-µm mesh prefilter to remove any small coral and sediment pieces. The microbial community in the mesocosm water was concentrated by TFF and then sequentially filtered through three filter sizes: 0.8-µm, 0.22-µm (bacteria) and 0.025-µm (viruses). Additionally, the TFF filtrate (< 100 kDa) was also collected to use as a non-microbial treatment to test for small molecule/chemical signal effects. Filters were attached to *O. faveolata* coral fragments obtained from Mote Marine Laboratory using sterile plastic forceps (Figure 1) with ten 0.8-µm (microeukaryotes) treatments, ten 0.22-µm (bacteria) treatments, ten 0.025-µm (viruses) treatments, and ten treatments where a coral fragment was immersed in the TFF filtrate instead of seawater. If the virus fraction had not filtered to completion after 5 hours, the remaining liquid was added to the corresponding treatment container to maintain a full mesocosm's 'dose' of that size fraction. The treatments were observed for 4 weeks.

Results

All of the March 2021 diseased corals were Vcp- (negative for the presence of *Vibrio coralliilyticus*).

The 0.22-µm size fraction filters from the following diseased samples initiated signs of SCTLD: PsD-5 after 8 days, CnD-23 after 16 days, and CnD-20 after 23 days. None of the corresponding control coral mesocosm 0.22-µm size fraction filters showed any signs of disease. This corroborates what was seen in the October 2019 pilot experiment (and removes the potential confounding factor of diatoms or other eukaryotes that were included in this size fraction during the 2019 pilot).

Unfortunately, the signal is not entirely clean, since several of the other treatments (0.8-µm, 0.025-µm, and the < 100 kDa filtrate) resulted in tissue loss symptoms (although not consistent with SCTLD) in both the control and diseased treatments. In an effort to avoid corals that might be resistant to SCTLD, we obtained naïve *O. faveolata* fragments from Mote Marine Laboratory

that had only been housed in tanks and therefore were never exposed to SCTLD in the wild. However, approximately 3 weeks before our experiment, the Mote tank housing these coral fragments began to show signs of a disease. Coral fragments were removed immediately and quarantined at SMS in advance of our experiment. The *O. faveolata* fragments appeared healthy; however, the indiscriminate responses across most of the treatments (including controls) suggest the fragments were already compromised. Diseased receiver coral fragments have been preserved for histology since it may be possible to confirm SCTLD as distinguished from the other tissue loss disease at the cellular level.

Task 2: Identification of limited pathogen pool

The October 2019 pilot resulted in SCTLD transmission to two receiver corals in the 0.22-µm size fraction. The November 2020 experiment had zero SCTLD transmission in any treatment (disease resistance of many the receiver corals was confirmed subsequently). The March 2021 experiment had probable SCTLD transmission to three receiver corals in the 0.22-µm size fraction (to be confirmed via histological examination).

In early March, based on the Oct 2019 and November 2020 experiments, we chose a small number of 0.22-µm filters for exploratory sequencing (Table 1). We chose not to wait until the conclusion of the March 2021 experiment (end of April 2021) because sequencing core facilities were experiencing 4-6 week backlogs due to the coronavirus pandemic and we could not be certain of receiving and analyzing the data in time to meet the June 1 deliverable deadline if we waited to include all the filters.

Microbial DNA was extracted from the filters using the Qiagen DNeasy PowerBiofilm kit, following the manufacturer's protocols. The bacterial community present on the filters was surveyed by using polymerase chain reaction (PCR) primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012) which target hypervariable region 4 (V4) of the 16S ribosomal RNA gene. Additional controls included an extraction kit blank and inclusion of a mock community. The kit blank is a tube of the Qiagen DNeasy PowerBiofilm kit that was processed without the addition of any sample as a control for trace DNA contamination in the kit reagents (Salter et al., 2014; Glassing et al., 2016). The mock community is a sequencing control of known composition and was purchased from the American Type Culture Collection: ABRF-MGRG 10 Strain Even Mix Genomic Material (ATCC MSA-3001) (Yeh et al., 2018). Sequencing was performed by the Michigan State University RTSF Genomics Core using an Illumina MiSeq v2 Nano flow cell in a 2x250 base pair paired-end format using a v2 500 cycle reagent cartridge. Sequences were analyzed using the QIIME2 platform (Bolyen et al., 2019; Estaki et al., 2020) and taxonomy was assigned using the Silva database release silva-132-99-515-806 (Quast et al., 2013). For comparison, the sequences were also analyzed using the OneCodex platform and their proprietary curated databases (Siegwald et al., 2017). The raw data from this exploratory dataset is available via USGS data release at https://doi.org/10.5066/P9B13K8N or from NCBI as BioProject PRJNA731170.

Results

Over 100,000 sequencing reads were obtained for each of the six filter samples. Alpha- and Gammaproteobacteria dominated the samples, with unclassified members of families Alteromonadaceae and Rhodobacteraceae being common (Figure 3). The genera *Marivita* and *Phaeodactylibacter* had a higher relative abundance in samples Mcav17 and Mcav18 compared to the rest of the samples (Figure 3).

In a Principal Coordinate Analysis based upon unweighted UniFrac (Lozupone and Knight, 2005), there was clear segregation of the filter bacterial communities by both treatment (healthy vs. diseased) and by year (Figure 4). Unweighted UniFrac considers the phylogenetic composition of the community but not the relative abundance of its constituent taxa. In contrast, a higher percentage of the variability is described by weighted UniFrac, which does include relative abundance, and separates the samples by year (Figure 5).

Researchers examining diseased tissues from corals experiencing SCTLD have identified a number of bacteria as only being present in diseased but not healthy corals or present at higher relative abundance in diseased corals. An ongoing USGS study (Iwanowicz et al., 2020) has identified seven such 'bacteria of interest': *Clostridioides difficile*, *Algicola bacteriolytica*, *Arcobacter bivalviorum*, *Romboutsia lituseburensis*, *Shimia aquaeponti*, *Burkholderia gladioli*, and *Pseudoalteromonas haloplanktis*. Bacterial sequence identification is dependent on both the method of classification and the reference database (Siegwald et al., 2017). Earlier microbial studies that examined healthy and SCTLD-infected coral tissues used a cluster-first approach (e.g., QIIME2) and the Silva database (Meyer et al., 2019; Rosales et al., 2020). However, the USGS study employed a classification-first approach and a curated database by using ONE CODEX. To take these different approaches into consideration, we analyzed our exploratory dataset both ways. ONE CODEX now offers two database options, one based on over 100,000 curated genomes and one based on targeted loci specific to 16S amplicon data. While the targeted loci database is more appropriate to our data, we ran our dataset against both databases to compare the results against the more commonly used open-source Silva database. As an illustration, we then screened the results of each of the three classifications to see if our dataset contained the seven bacteria identified as "always" or "commonly" associated with SCTLD in coral tissues (Table 2). We also screened our exploratory dataset for other bacterial genera (Table 3) that have been identified by SCTLD studies of coral tissues from the Florida Keys and U.S. Virgin Islands (Meyer et al., 2019; Becker et al., 2021).

Future Directions

We are in the process of submitting the rest of the 0.22-µm filters from October 2019, November 2020, and March 2021 for bacterial amplicon sequencing to increase the statistical power of differential findings. Those data will become publicly available as a separate data release (in addition to the exploratory dataset described in this report: USGS data release at https://doi.org/10.5066/P9B13K8N). Both the exploratory dataset and the future complete filter dataset will be described in a peer-reviewed journal publication.

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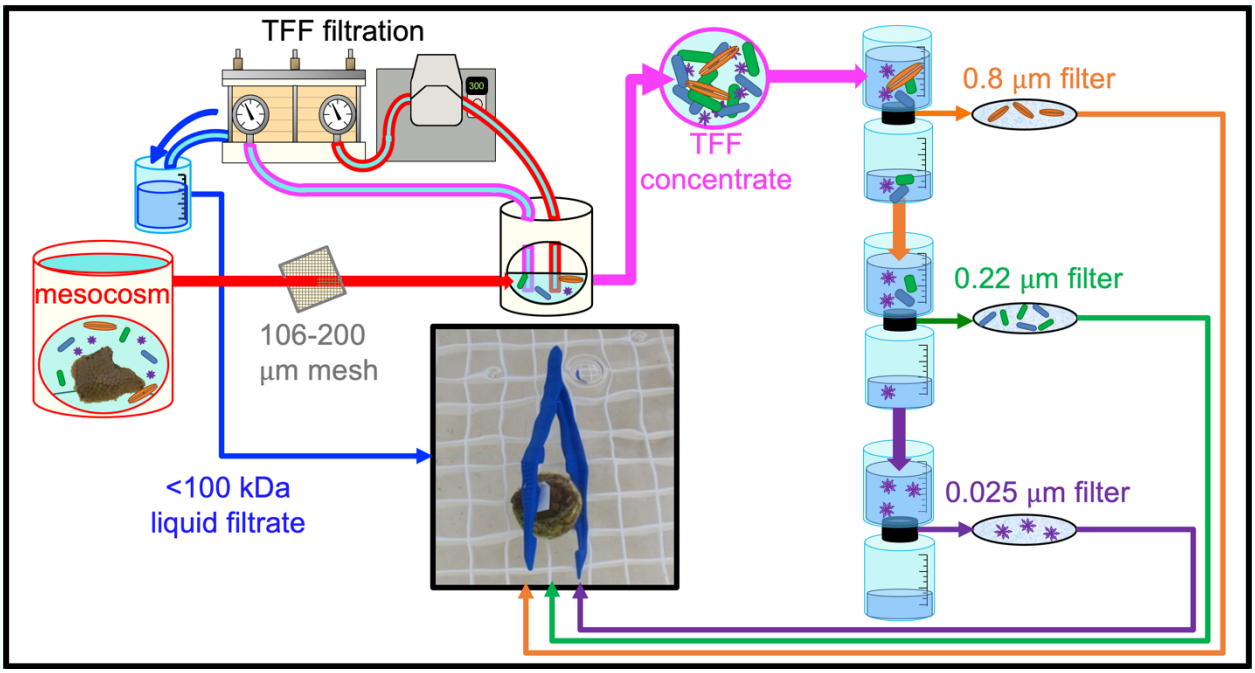

Figure 1. General experimental design followed by all three experiments. Corals were incubated in individual mesocosms for several days, then mesocosm water (red) was passed through a mesh screen and then concentrated through tangential flow filtration (TFF). The TFF-concentrated microbial community (pink) was then passed through a series of different-sized filters: 0.8 µm (orange; 2020, 2021 experiments only), 0.22 µm (green), and 0.025 µm (purple). Microeukaryotes are represented by orange rods, bacteria by blue and green rods, and viruses by purple stars. Quarter portions of these filters were applied as treatments to 'receiver' corals using sterile plastic forceps. TFF filtrate (blue) was also retained as a treatment (2020, 2021 experiments only).

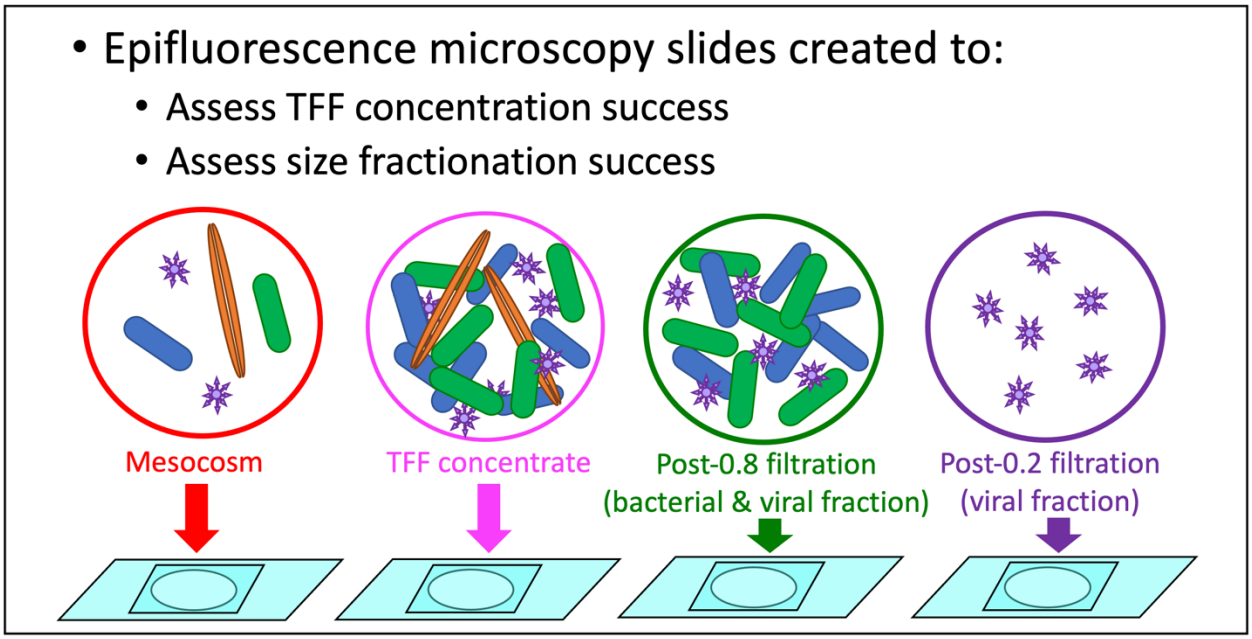

Figure 2. The success of the experimental design was confirmed using epifluorescence microscopy. Examination of initial mesocosm water (red circle) and the TFF-concentrated mesocosm water (pink circle) confirmed that the TFF method concentrated the mesocosm microbial community. Examination of filtrate following 0.8-µm (green circle) and 0.2-µm (purple circle) filtration confirmed that microeukaryotes (orange rods) and bacteria (green and blue rods) were removed by these subsequent filtration steps, leaving only viruses (purple stars) in the last fraction.

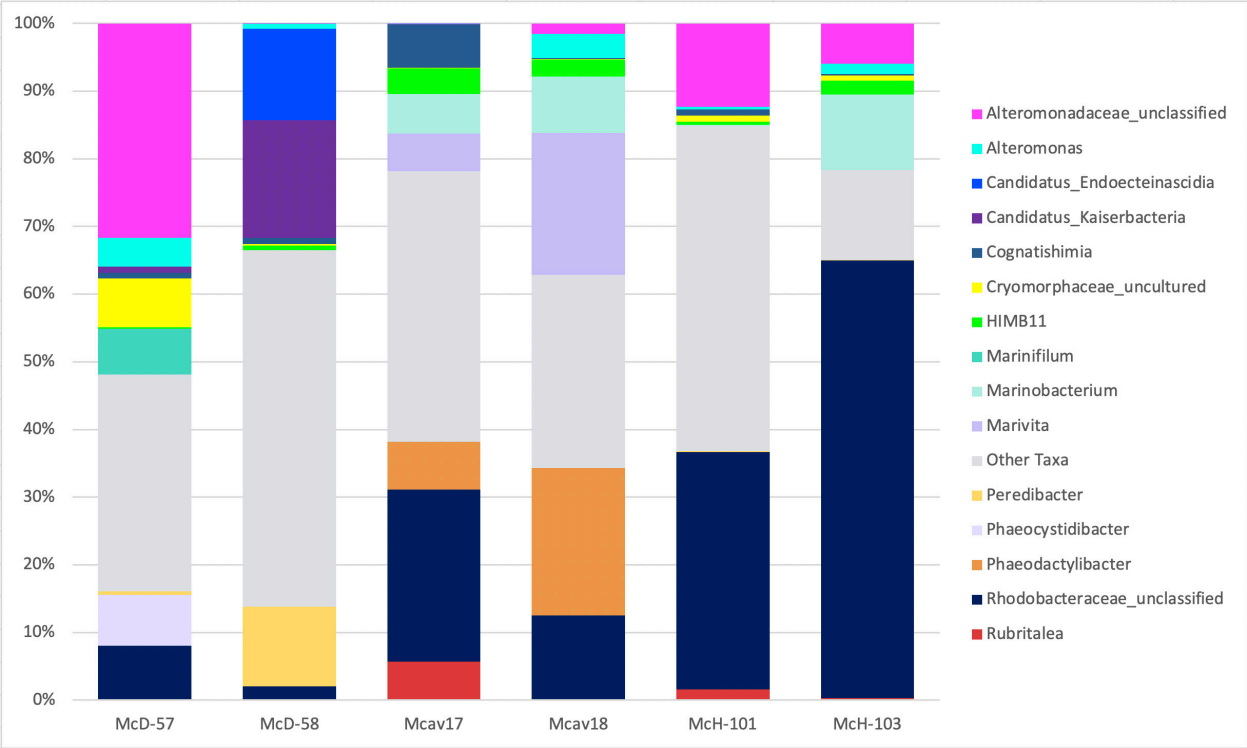

Figure 3. Relative abundance of common bacterial genera or unclassified families on the 0.22-µm filters in the exploratory dataset based on Silva taxonomy. Sample information as listed in Table 1. Other taxa includes all genera containing <1% of total sequence reads (n=559,758) across the entire dataset.

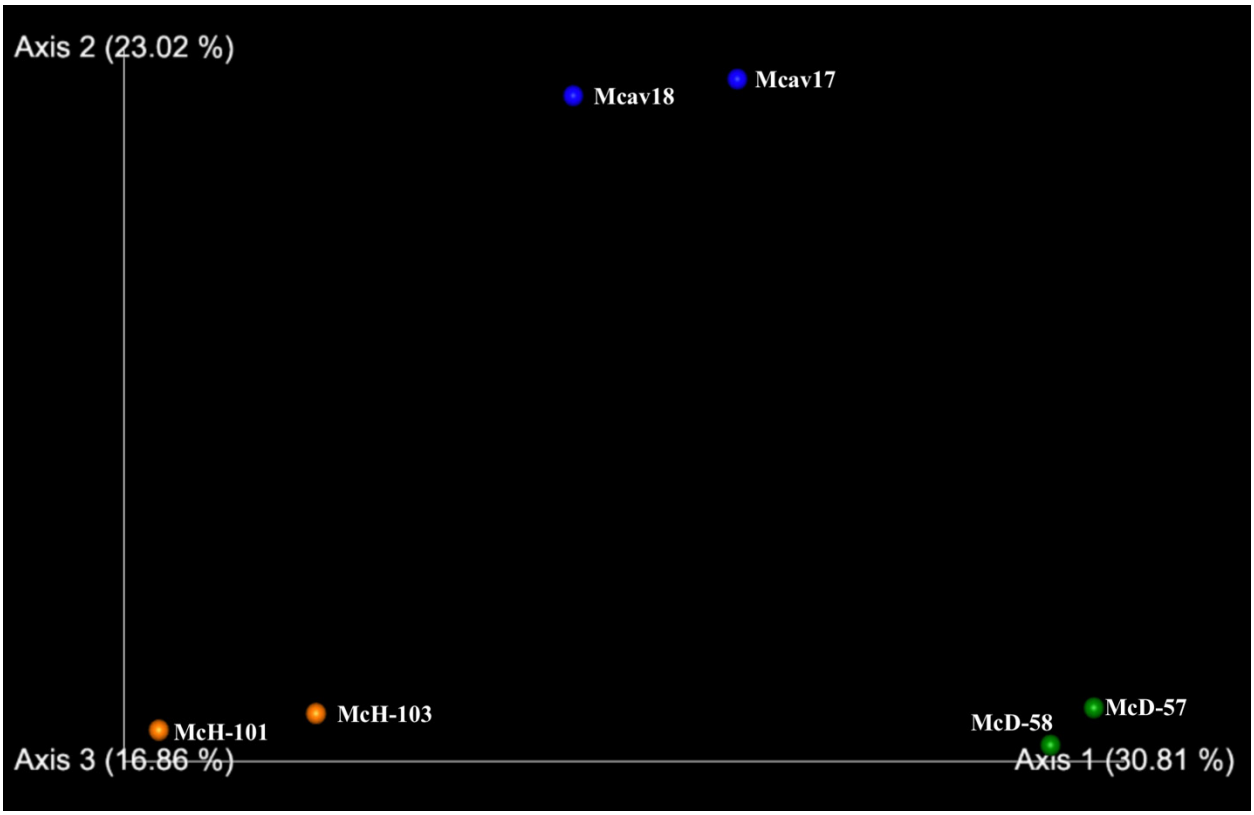

Figure 4. Principal coordinate analysis of the bacterial communities from the 0.22-µm filters based on unweighted UniFrac. Blue symbols (top) are diseased samples from October 2019. Orange symbols (lower left) are healthy samples from October 2019. Green symbols (lower right) are diseased samples from November 2020.

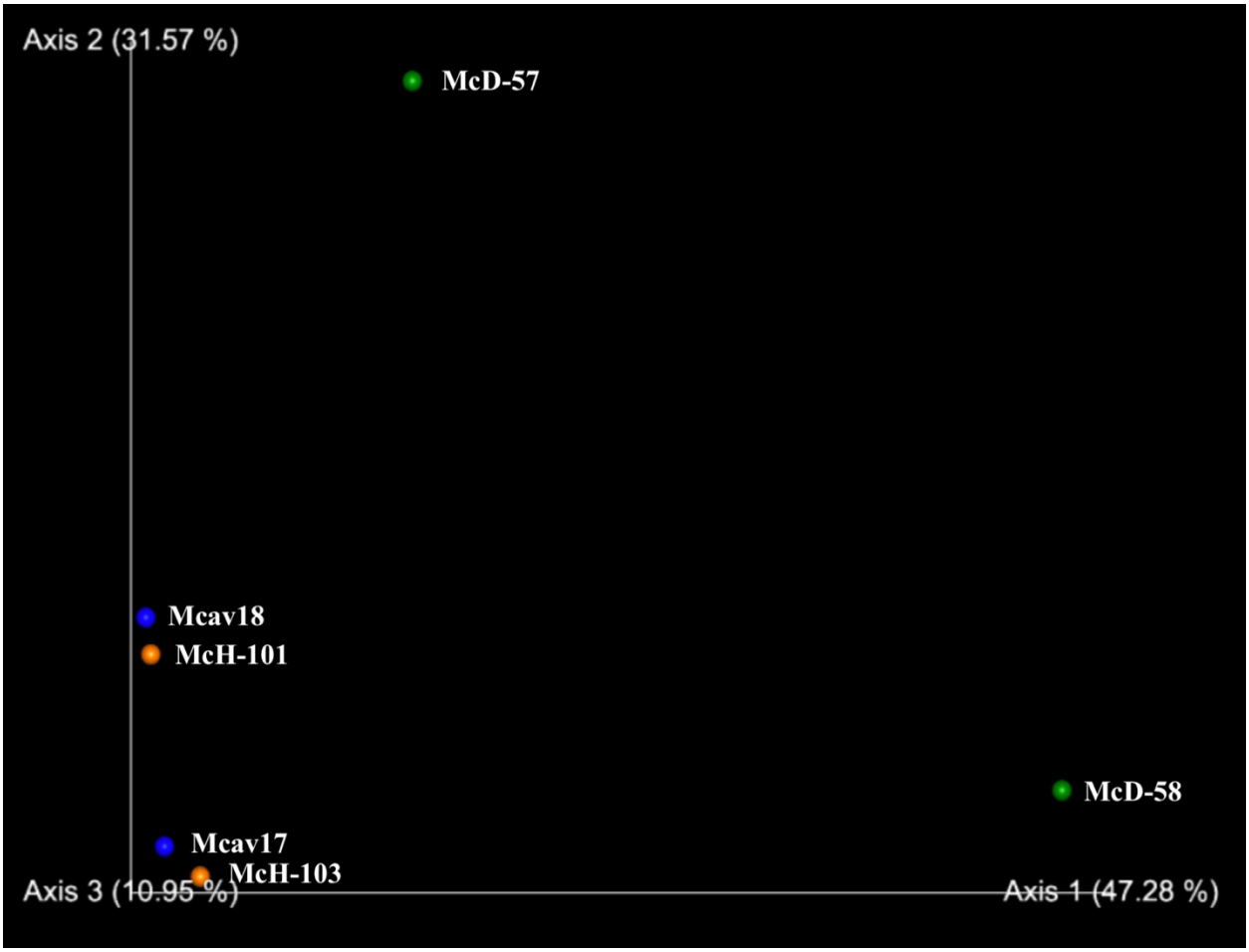

Figure 5. Principal coordinate analysis of the bacterial communities from the 0.22-µm filters based on weighted UniFrac. Blue symbols (Mcav17 and Mcav18) are diseased samples from October 2019. Orange symbols (McH-101 and McH-103) are healthy samples from October 2019. Green symbols (McD-57 and McD-58) are diseased samples from November 2020.

Filter ID	Treatment	Experiment	Donor Coral Collection Site, Date	Reason
Mcav18	Diseased	Oct-19	FL Keys, Oct. 2019	SCLD transmission
Mcav17	Diseased	Oct-19	FL Keys, Oct. 2019	SCTLD transmission
McH-101	Healthy	Oct-19	FL Keys, April 2018	Matching control
McH-103(4)	Healthy	Oct-19	Key West Nursery, Jan. 2019	Matching control
McD-57	Diseased	Nov-20	Marathon, Nov. 2020	Potential carrier
McD-58	Diseased	Nov-20	Marathon, Nov. 2020	Potential carrier

Table 1. Bacterial (0.22 µm) filters from selected *Montastraea cavernosa*-inoculated mesocosms that were extracted for exploratory sequencing

SILVA DATABASE:	Mcav 17	Mcav 18	McD-57	McD-58	McH-101	McH-103
Clostridioides difficile	G	G	-	-	G	-
Romboutsia lituseburensis	-	-	-	-	-	-
Arcobacter bivalviorium	G	-	G	-	-	-
Algicola bacteriolytica	-	-	S	-	S	-
Shimia aquaeponti	G	G	-	-	G	G
Burkholderia gladioli	-	-	-	-	-	-
Pseudoalteromonas haloplanktis	G	-	-	G	G	G
ONE CODEX DATABASE:	Mcav 17	Mcav 18	McD-57	McD-58	McH-101	McH-103
Clostridioides difficile	S	S	S	S	S	S
Romboutsia lituseburensis	G	G	-	-	G	-
Arcobacter bivalviorium	G	G	G	G	G	G
Algicola bacteriolytica	-	-	-	-	-	-
Shimia aquaeponti	G	G	G	G	G	G
Burkholderia gladioli	G	G	G	G	G	G
Pseudoalteromonas haloplanktis	G	G	G	G	G	G
ONE CODEX TARGETED LOCI:	Mcav 17	Mcav 18	McD-57	McD-58	McH-101	McH-103
Clostridioides difficile	S	S	G	G	G	-
Romboutsia lituseburensis	-	-	-	-	-	-
Arcobacter bivalviorium	S	S	S	S	G	-
Algicola bacteriolytica	-	-	S	-	-	-
Shimia aquaeponti	G	-	G	G	G	G
Burkholderia gladioli	-	-	-	-	-	-
Pseudoalteromonas haloplanktis	-	G	-	-	G	G

Table 2. Comparing the presence or absence of seven 'bacteria of interest' (left column) in our exploratory dataset based on three different taxonomic reference databases: Silva (top table; yellow), One Codex (middle table, pink), and the One Codex targeted loci database (bottom table, aqua). Blue columns correspond to diseased corals from the October 2019 experiment, green columns to diseased corals from the November 2020 experiment, and orange columns to healthy corals from the October 2019 experiment. "S" means the species was identified in our data set, "G" indicates the genus was present in our data set, and "-" indicates neither was found in our data set.

	Mcav 17	Mcav 18	McD-57	McD-58	McH-101	McH-103
Arcobacter	+	-	+	-	-	-
Desulfovibrio	-	+	-	-	+	-
Halodesulfovibrio	+	+	+	+	+	+
Fusibacter	+	+	+	+	-	+
Wenyingzhuangia	-	+	+	+	-	+
Vallitalea	+	+	-	-	+	-
Marinifilum	+	+	+	+	+	-
Tepidibacter	+	+	-	-	+	-
Roseimarinus	+	+	+	-	-	-
Algicola	-	-	+	-	+	-
Cohaesibacter	+	+	+	+	-	-
Shimia	+	+	-	-	+	+
Thalassobius	-	-	-	-	-	-
Vibrio	+	+	+	+	+	+
Marinovum	-	-	-	-	-	-

Table 3. Comparing the presence or absence of 15 bacterial genera identified by studies in both Florida and the U.S. Virgin Islands as being associated with SCTLD (left column) in our exploratory dataset based on the Silva database. Blue columns correspond to diseased corals from the October 2019 experiment, green columns to diseased corals from the November 2020 experiment, and orange columns to healthy corals from the October 2019 experiment. A plus sign (+) indicates the genus was present in a sample, a minus sign (-) means the taxon was not found in the sample.