Development of physical and sequence bacteria enrichment methods for the detection of species and strain-level variations in SCTLD-associated bacteria

Development of physical and sequence bacteria enrichment methods for the detection of species and strain-level variations in SCTLD-associated bacteria

Final Report

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Management Summary

This project aims to better understand the role of bacteria in stony coral tissue loss disease (SCTLD). While there have been many studies that used 16S rRNA gene data to characterize the bacteria present in this disease these studies can not tell us the potential function of the bacteria and are limited in providing taxonomic and phylogenetic information. To obtain this information shotgun metagenomics needs to be conducted. But this technology is hindered in coral research because the majority of data sequenced is from the coral host and the symbiotic algae – limiting the data that can be collected on the microbial community. To combat this limitation, we proposed to test and develop methods that enrich bacteria sequence data in coral samples to better characterize the bacteria community associated with the disease. In all, we tested enrichment by (1) fluorescence-activated cell sorting (FACS); (2) size fractionation between 5 and 0.1µm; (3) the Silveira lab protocol that couples host depletion and size fraction between 8 and 0.02 µm; (4) the commercially available prokaryotic enrichment kit, HostZERO™; (5) and 2 mm and (6) 0.5 and 1 mm bead beating sizes for DNA extractions using the ZymoBIOMICS MagBead DNA/RNA extraction kit; and (7) selective long-read sequencing with Oxford Nanopore Technologies (ONT). The results for FACS and HostZERO™ did not yield sufficient DNA for sequencing. The 5 to 0.1 µm size fractionation and selective long-read sequencing with Oxford Nanopore Technologies (ONT) did not result in bacteria sequence enrichment. Finally, sequence data is pending from methods: host depletion and 8 to 0.02 µm size fractions, and the 2mm, and 0.5/1mm bead beating sizes for DNA extractions. This project provides guidelines for enriching bacteria in coral samples for shotgun metagenomics. The data generated through these methods could provide a better insight into the role of bacteria in SCTLD.

Executive Summary

Florida's coral reefs are experiencing a massive die-off due to stony coral tissue loss disease (SCTLD), a disease with an unknown causal agent. Infection with SCTLD causes shifts in the bacterial microbiome, but current sequence data is limited in helping understand the role bacteria play in this disease. The aim of our 2022-2023 Florida Department of Environmental Protection grant was to attain bacteria sequencing data to better understand SCTLD. To do so, we used different methods to enrich bacteria from coral samples. In the course of our grant, we tested: (1) fluorescence-activated cell sorting (FACS); (2) size fractionation between 5 and 0.1µm; (3) host depletion coupled with size fraction between 8 and 0.02 µm; (4) the commercially available prokaryotic enrichment kit, HostZERO™; (5) and 2 mm and (6) 0.5 and 1 mm bead beating sizes for DNA extractions using the ZymoBIOMICS MagBead DNA/RNA extraction kit; and (7) selective long-read sequencing with Oxford Nanopore Technologies (ONT). FACS protocols were developed to differentiate coral cells, coral cells containing Symbiodiniaceae, and prokaryotic cells, but the resulting DNA concentrations using these methods were insufficient for whole genome sequencing. Similarly, the bacteria enrichment kit did not provide enough material for whole genome sequencing and were not an option for sequencing in our study. The 5 to 0.1 µm size filtration method also was inconsistent as only two-thirds of the samples processed contained enough DNA for whole genome sequencing. Currently, only the ONT and the 5 to 0.1 µm size fractionation enrichment protocols were sequenced. Both of these resulted in proportionally high abundances of coral host reads and did not enrich for bacteria reads. There is some indication that size fractioning did increase the concentration of bacterial reads, but this increase was insufficient to increase bacterial metagenome-assembled genomes (MAGs). Sequencing data is pending for the host depletion coupled with the 8 and 0.02 µm size fraction method and the two bead beat methods.

Acknowledgments

We would like to thank Aliyah True, and David Ehrens from the Nikki Traylor-Knowles lab at the University of Miami for assisting in running the fluorescence-activated cell sorting machine. We also thank Cynthia Silveira and Bailey Wallace for sharing and conducting their size filtration DNA extraction protocol. Lastly, we like to thank the Smithsonian Marine Station in Ft. Pierce for providing the archived DNA samples and Blake Ushijima and his lab for providing freshly collected disease coral samples. We also like to thank Caroline Dennison for collecting coral for DNA extractions.

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4′,6-diamidino-2-phenylindole (DAPI)
Autoclaved seawater (ASW)
Back-scatter by area (BSC-A)
Basic Local Alignment Search Tool (BLAST)
Ethylenediaminetetraacetic acid (EDTA)
Fluorescence-activated cell sorting (FACS)
Forward scatter by area (FSC-A)
number (n)
Oxford Nanopore Technologies (ONT)
Peridinin-Chlorophyll-protein (PerCP)
Phosphate Buffered Saline (PBS)
University of Wisconsin Biotechnology Center (UWBC)

1.1. Description:

To date, numerous studies have showcased the relevance of the bacteria community in SCTLD. However, currently, most data characterized what bacteria groups are present in a sample. To better understand the role of bacteria associated with SCTLD, studies are needed to characterize the function, characterize phylogeny, and characterize these bacteria to finer taxonomic groups such as species and strain. To generate these data shotgun metagenomics needs to be conducted. Although, shotgun metagenomes from coral samples can often be computationally difficult to analyze and to extract meaningful bacteria information due to the high abundance of host and symbiont reads in the data (upwards of 99% of host sequences). The aim of this project was to enrich bacteria sequences from coral samples to increase the number of bacteria reads sequenced to obtain robust information on SCTLD-associated bacteria. To do this we performed seven different bacteria enrichment methods. Our results showed that two methods did not generate enough DNA for sequencing, two other methods did not reduce the number of coral reads, and three methods are currently pending sequencing and will be analyzed upon receiving the data.

2. Methods:

In total seven different bacteria enrichment methods were tested at the University of Miami. Each method is detailed below. The sample information is available in the metadata file; the Smithsonian samples were used to evaluate FACS, size fractionation, and selective sequencing, and the samples provided by the Ushijima laboratory were used to evaluate the HostZERO™, host depletion coupled with 8 to 0.02µm size fractionation, and the ZymoBIOMICS MagBead DNA/RNA bashing bead sizes 2 mm and 0.1 and 0.5 mm protocol.

2.1. Method: Fluorescence-activated cell sorting (FACS)

FACS buffer was filtered through a 0.22 µm filter before use and stored at 4 °C to preserve for later use. Healthy coral samples from *Montastrea cavernosa* from the Traylor-Knowles lab were used to troubleshoot the protocol. Fresh *M. cavernosa* fragments were aspirated using filtered FACS buffer and stored in sterile 50mL falcon tubes until sorting. Samples were visualized using the Sony SH800Z Flow cytometer with the main methods used to detect cells as follows:

- Forward scatter by area (FSC-A)
- Back-scatter by area (BSC-A)
- The fluorescence range for 4′,6-diamidino-2-phenylindole (DAPI) stain detection (Brilliant Violet-421-A-Compenstated)
- The fluorescence range for chlorophyll-a detection (PerCPCy [Peridinin-Chlorophyll-protein] 5.5-A-Compensated)

The first step is to differentiate cells from machine noise. The filtered FACS buffer was run through the FACS to identify machine-generated artifacts, which were found to reside entirely in the box labeled “A” (Figure 1) and below “AM” (Figure 2) when the cells were visualized by BSC-A and FSC-A.

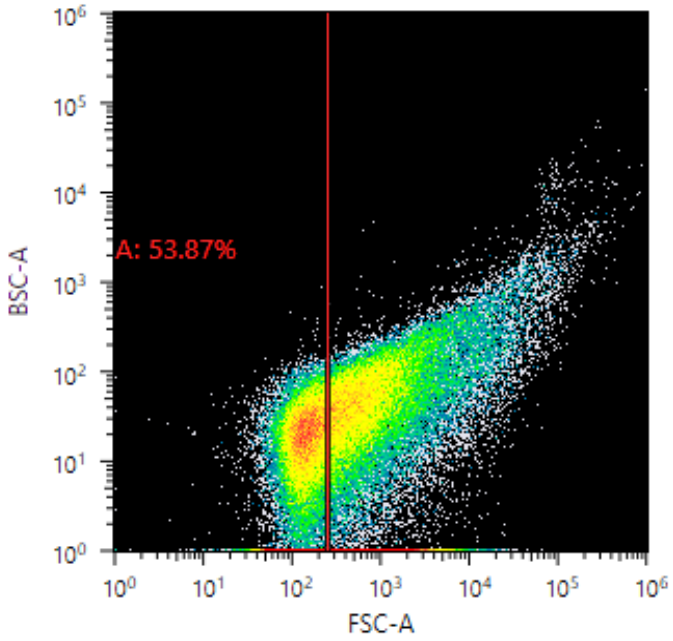

Figure 1: *Step 1 of the FACS protocol is removing false positives generated by machine scatter.*

A new gating protocol was created which represented only signals selected in A, the cells were visualized by a DAPI stain by area (Brilliant Violet 421-A-Compenstated; Figure 2). Using microscopy, cells were determined to be greater than $10^{4.1}$ in the Brilliant Violet detection range – labeled “AL” (Figure 2). The other signals detected below this range were determined to be cellular and other debris that contain DNA and thus are also stained with DAPI; this gate was labeled “AM”. The gate labeled “AM” are likely machine noise, as a similar signal is observed when a deionized water sample was run (Figure 2).

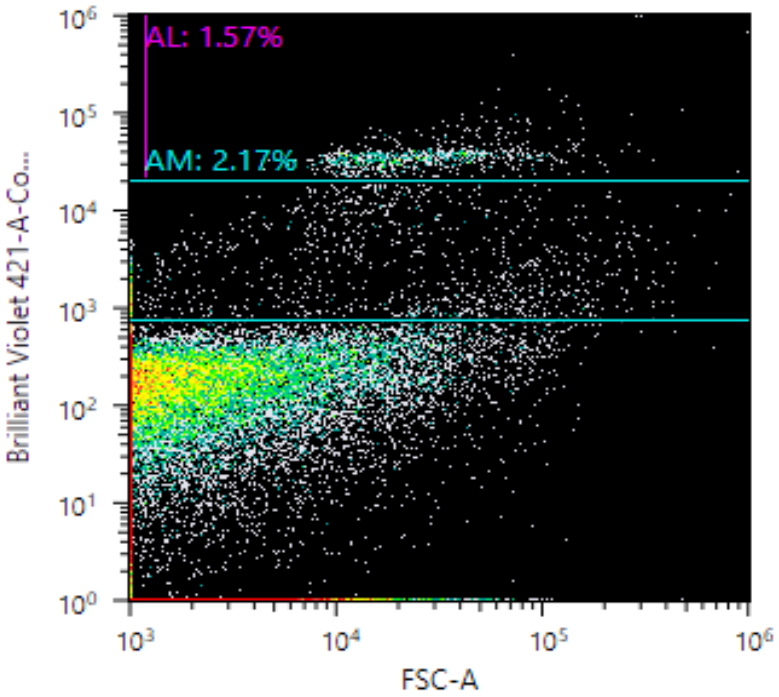

Figure 2: *Step 2 of the FACs is removing false positives resulting from DAPI staining of non-cellular material.*

Finally, we differentiated between larger cells with and without Symbiodinacae, "A" and smaller particles were excluded and shown in "AM" using PerCP-Cy5.5A-Compensated and Brilliant Violet 421-A-Compensated (Figure 3). Larger cells fluorescing in higher PerCP range are likely autofluorescing chlorophyll; these were confirmed by microscopy to be coral cells containing Symbiodinacae. Larger cells that did not fluoresce highly in PerCP, labeled "AO", were confirmed to contain larger cells that did not contain Symbiodinacae. Smaller cells that were not fluorescing highly in PerCP were labeled "AQ" and were confirmed to be prokaryotic cells.

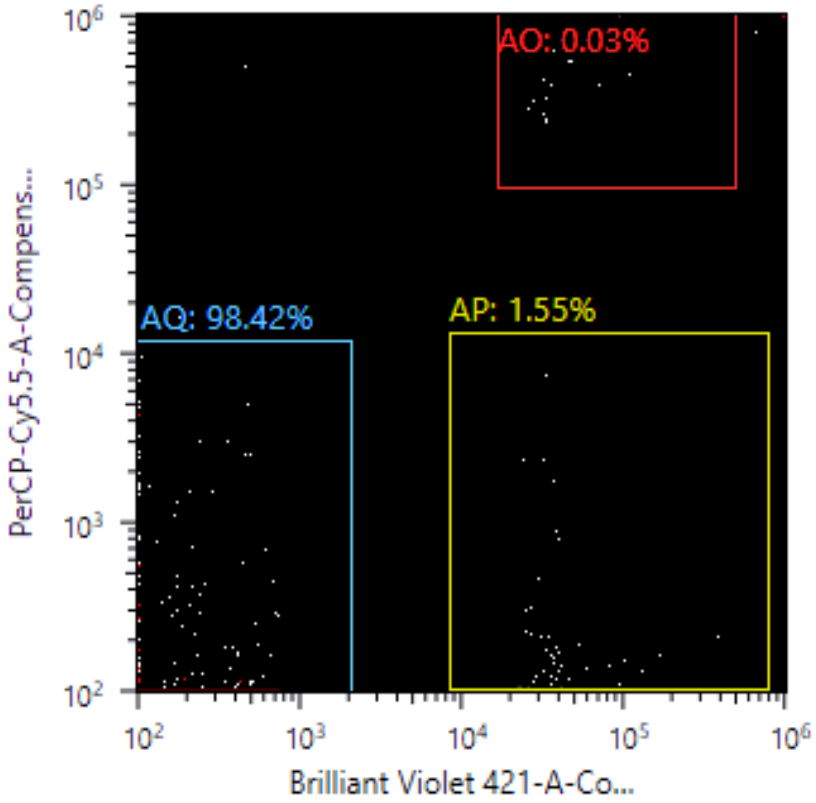

Figure 3: *Step 3 of the FACs protocol is the final gating for three cell types. The following designations were confirmed by microscopy: "AO" was coral cells containing Symbiodinacae; "AP" was coral cells without Symbiodinacae; "AQ" was smaller cells, that are likely prokaryotic.*

The aforementioned gates will shift slightly (approximately $10^{0.2}$) both within a coral species and between coral species and whether the sample is fresh or preserved. Aspiration also had a significant impact on the concentration of cells and so did the time required to process the samples. For example, a sample preserved in RNAlater was processed twice in one day; the first sample required <90 minutes and required <5mL but the second sample required >9 hours and required >10mL.

The protocol was also tested on archived/RNAlater preserved coral samples with SCTLD. Sorting bacterial cells was a relatively fast protocol for the majority of fresh and preserved coral samples (<15 minutes), however, sorting coral cells and coral cells containing Symbiodinium even when optimized could take more than 90 minutes. Due to this method requiring open Eppendorf tubes during the sorting period, in the absence of a DNA-clean facility, this is not advisable for the purposes of detecting bacteria attached to coral or Symbiodinum cells due to the

risk of contamination. Sorted cells were DNA extracted using the ZymoBIOMICS MagBead DNA/RNA kit and quantified using the NanoDrop and the HS DNA protocol for Qubit Fluorometric Quantification (Qubit).

2.2. Method: Size fractionation capturing 5 and 0.1 µm

A cell separation protocol was developed based on the general size differences that are observable between eukaryotic and prokaryotic fractions. Prokaryotic cells are generally between 0.1 and 5 µm and eukaryotic cells are generally greater than 5 µm; therefore size fractioning the sample using polycarbonate filters should separate these two populations. To evaluate this, a total of 10 mL of *M. cavernosa* aspirate from healthy and SCTLD infected samples were filtered through the 5 µm filter to capture the eukaryotic cells. That filter was washed with 10 mL of 0.2 µm filtered and autoclaved seawater to dislodge any bacteria likely to have become stuck to or are loosely associated with eukaryotic cells. The filtrate and wash were then passed through a 0.1 µm polycarbonate filter and extracted using the ZymoBIOMICS MagBead DNA/RNA extraction protocol. Samples were quantified using Qubit. The samples were then submitted to the University of Wisconsin Biotechnology Center (UWBC) core facility for sequencing on the Illumina NovaSeq 6000.

2.2.1 Method: Bioinformatics for Illumina

Illumina reads were trimmed and adaptors removed using scripts bbduk.sh and normalized with bbnorm.sh both in BBMap version 39.01. Cleaned and normalized reads were assembled using the program spades v3.15.5 and assembled reads were binned using metabat2 v.2:2.15, which depends on the programs bwa v.0.7.17-r1188 and samtools v.1.6. Binned genomes were taxonomy evaluated using the programs GTDB-Tk - v1.7.0 (available through www.kbase.us) and Basic Local Alignment Search Tool (BLAST V.2.13.0). Binned data were evaluated for completeness and contamination using checkm v1.2.2. Phyloflash v.3.4.2 was used to determine the total proportion of reads that were bacterial compared to eukaryotic.

2.3. Method: Host depletion and Size fractionation capturing 8 and 0.02 µm

Another size fraction protocol was used based on a newly developed method by the Silveira lab at the University of Miami. Using a sterilized mortar and pestle, coral samples were ground and transferred to a tube with acid-washed 0.6 mm glass beads and autoclaved seawater (ASW). The tubes with beads and coral samples were vortexed for 3-5 min and then centrifuged. Samples were then treated with DNase and stopped with Ethylenediaminetetraacetic acid (EDTA)

after 2hrs. Each sample was filtered through an 8 µm filter and 5 mL of ASW was added to bring the total volume high enough to pass through the syringe attached to the filter. The samples were then transferred to a Vivaspin® 20 Centrifugal Concentrator and centrifuged at max speed. The filter was rinsed with T1 Lysis Buffer and Proteinase K from the DNeasy® Blood and Tissue extraction kit and incubated at 55 °C for 1 hour. The samples were then processed following the DNeasy® manufacturer's instructions. Samples were quantified using Qubit and submitted for sequencing at the John P. Hussman Institute for Human Genomics at the University of Miami core facility.

2.4. Method: Zymo HostZERO™

Coral samples were homogenized in 2 mL tubes filled with 2mm beads and Phosphate Buffered Saline (PBS) buffer. Samples were homogenized for 45 minutes using a vortex-adaptor at maximum speed. The samples were spun down at 10,000 g for 30 seconds and the supernatant was transferred to a new 1.5 mL tube before being processed as outlined in the HostZERO™ manufacturer's protocol. Samples were quantified using Qubit.

2.5. Method: Bashing bead sizes 0.1 and 0.5 mm

A 1-2cm^2 coral sample was collected with sterilized bone cutters. Each sample was placed in DNA/RNA shield in ZR BashingBead Lysis tubes with 0.1 and 0.5 mm beads. They were then homogenized for 45 minutes using a vortex adaptor at maximum speed. DNA was extracted following the ZymoBIOMICS MagBead DNA/RNA kit and quantified using the NanoDrop and Qubit. This was performed on both Smithsonian samples to extract DNA for selective sequencing (described in 1.2.7 below) and the Ushijima samples (sequencing results pending from the University of Miami).

2.6. Method: Bashing bead sizes 2mm

A 1-2cm^2 coral sample was collected with sterilized bone cutters. Each sample was placed in DNA/RNA shield in ZR BashingBead Lysis tubes with 2 mm beads. They were then homogenized for 45 minutes using a vortex adaptor at maximum speed. DNA was extracted using the ZymoBIOMICS MagBead DNA/RNA kit, quantified using a Qubit, and submitted to the John P. Hussman Institute for Human Genomics at the University of Miami core facility.

2.7. Method: Selective sequences

The samples collected as described in 1.2.5, were submitted to the University of Wisconsin Biotechnology Center (UWBC) core facility. At UWBC sequence DNA libraries were prepared using the Ligation Sequencing Kit SQK-LSK109

and EXP-NBD196 (Oxford Nanopore, UK), following the manufacturer's recommendations. The libraries were then sequenced on a MinION flow cell (R9.4.1), run on GridION. Guppy basecaller v6.3.9 was used with a high-accuracy model (read filtering, minimum Qscore=7). Adaptive sampling was accomplished through the creation of a minimap file from a fasta file containing Symbiodinacea and *M. Cavernosa* genome. Adaptive sampling parameters were set to deplete the sequences in the minimap file. These samples were also sequenced on the Illumina NovaSeq 6000 platform to generate 150 base pair sequences ("short reads") to complement the long-read sequences which have a higher rate of sequencing error compared to short reads.

2.7.1 Method: Bioinformatics for long reads

Sequence data was processed both uncleaned (transforming fastq to fasta reads) and quality-controlled data. Long-read data was processed using Galaxy pipelines described on our Github. Briefly, the program Porechop (Galaxy Version 0.2.4+galaxy0) was used to clean and quality control the data using the default settings. The quality of Porechop cleaned data were evaluated using NanoPlot. Assemblies were constructed using Unicycler on Galaxy with default settings (Galaxy Version 1.36.2+galaxy1) as well as locally using Flye v.2.9.1.

2.7.2 Method: Bioinformatics for Illumina sequencing

Reads were processed as described in section *1.2.2.1 Method: Bioinformatics for Illumina*.

3. Results:

3.1 Results: Fluorescence-activated cell sorting (FACS)

Through various troubleshooting, we determined that Fluorescence-activated cell sorting (FACS) did not provide adequate concentrations of bacterial cells for genomic analysis. Bacterial cell sorting was tested on archived samples from an SCTLD transmission study conducted by Smithsonian scientists. Three genotypes characterized as either healthy or diseased were used to test our protocol. The flow cytometer was able to sort 50,000 bacterial cells but the resulting concentration of cells was not sufficient to provide enough DNA for whole genome sequencing (0.5 µg) (Table 1). The time to sort coral and Symbiodinacea-containing cells was also found to be both a risk for contamination and unlikely to provide the concentration of DNA necessary for genome sequencing.

Table 1*: Nanodrop and Qubit result of FACS processing of one SCTLD healthy sample from a Smithsonian transmission study. Readings of "0" are below the detectable DNA range using Qubit.*

Sample	Date	Nanodrop ng/µL	Nanodrop A260/A280	Nanodrop A260/A230	Qubit ng/ult
FACs Coral + symbiodinium	10-31-22	0.9	0.82	0.01	0
FACs Coral	10-31-22	10.9	0.82	0.01	0
FACs Bacteria	10-31-22	3.1	2.38	0.01	0
FACs blank	10-31-22	5.3	1.22	0.01	0
DNA filtered seawater	10-31-22	2.3	1.91	0.01	0

3.2. Results: Size fractionation capturing between 5 and 0.1 µm

Size fractioning resulted in adequate DNA concentrations for sequencing only 2/6 samples (0.5 µg total; Mcav3 control and diseased). This is compared to the whole coral samples taken of the same sample, which had consistently high DNA concentrations (Table 2).

Table 2*: The quantity of DNA resulting from samples of SCTLD healthy and diseased samples from a Smithsonian transmission experiment. The samples were extracted using the method size fractionation capturing between 5 and 0.1 µm (Filter) and method bashing bead sizes 0.1 and 0.5 mm (Whole coral).*

Sample	Filter Qubit ng/µl	Whole coral Qubit ng/µl
CN2 disease	1.15	29.9
CN2 control	0.151	41.1
T8 disease	0.286	35
T8 control	0.121	40.9

Mcav3 control	3.4	48.3
Mcav3 disease	3.91	44.8

3.3. Results: Host depletion and size fractionation capturing 8 to 0.02 µm
DNA from 16 samples from freshly collected diseased colonies (n= 8 lesions, and n=8 unaffected) were submitted to the John P. Hussman Institute for Human Genomics at the University of Miami core facility. Samples are currently pending sequencing data.

3.4. Results: Zymo HostZERO™
Using samples from freshly collected disease corals, this kit did not generate enough DNA for sequencing.

3.5. Results: Bashing bead sizes 0.1mm and 0.5mm
DNA from 16 samples from freshly collected diseased colonies (n= 8 lesions, and n=8 unaffected) were submitted to the John P. Hussman Institute for Human Genomics at the University of Miami core facility. Samples are currently pending sequencing data.

3.6. Results: Bashing bead sizes 2mm
DNA from 16 samples from freshly collected diseased colonies (n= 8 lesions, and n=8 unaffected) were submitted to the John P. Hussman Institute for Human Genomics at the University of Miami core facility. Samples are currently pending sequencing data.

3.7. Results: Selective sequences
Selective sequencing data were analyzed from two samples (Mcav3 diseased and healthy). Quality control using the program porkchop resulted in an average of 5.6 x 10^6 reads per sample. The average and median read length was generally lower for the diseased samples, with the healthy Mcav3 samples being nearly twice as long on average (Figure 4). Despite the quality controlled reads from healthy samples generally being of higher quality (data on GitHub), neither the healthy nor diseased sequences had bacterial reads. All quality-controlled reads and subsequently assembled reads were identified in BLAST as coral. Reads that were not quality controlled were found to be more diverse, with only a subset of those reads being identified to bacteria genus level in more than one read per sample. A count table of the taxa identified can be found on GitHub. Notably, BLAST identified Mycolicibacterium reads in healthy (n=140) and diseased (n=1183) samples. Diseased samples also had multiple reads identified to the following

bacteria: *Kiebsiella*, *Escherichia*, *Staphylococcus*, *Vibrio*, *Stieleria*, and *Streptomyces*. Unprocessed Eukaryotic reads that were not coral included fungal genera (such as *Trichoderma* and *Saccharomyces*). Reads were determined to be Symbiodinium as well, but these were in equal proportion to individual fungal genera. Most notably, *Plasmodium* was found in similar proportions to coral reads in both healthy and diseased samples. It should be noted that limited sampling and low-quality reads produced these sequences and results.

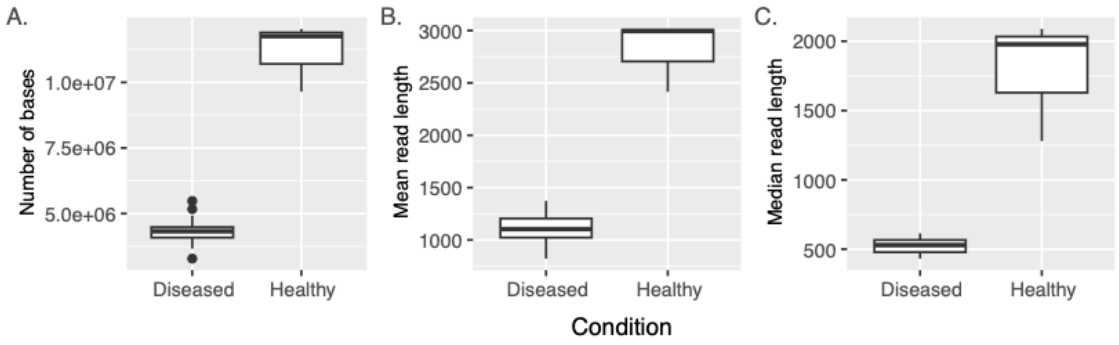

Figure 4*: The quality results for the selectively sequenced Mcav3 samples, comparing both diseased and healthy. The average number of bases sequenced for the diseased samples was fewer than the healthy (A), as was the mean (B) and median (C) read length. Data can be found on Github.*

3.8. Results: Illumina sequencing

For both the filtered material and the whole coral samples, only 1-2 MAGs were produced for each, but were not able to be taxonomically classified with GTDB-Tk - v1.7.0. Individual contigs from the MAGs were all found to be coral with the BLAST database. Unbinned spades contigs were more taxonomically diverse, but of the total base pairs assembled, over 99% of the total base pairs assembled were determined to be Eukaryotic (Table 3). The total base pairs assembled that were identified as bacteria was < 1.5% and was not consistently greater in filtered samples compared to the whole coral.

***Table 3**: The proportions of the total assembled reads that were identified in BLAST as being either Archaea, Bacteria, Eukaryota, Virus, or that were not classified (N/A). This table compares the results between Illumina sequencing and assembly using DNA from either "Filter" (size fractionation capturing between 5 and 0.1 µm) or "Whole" (bead-beating protocol using 0.1 and 0.5µm beads) protocols evaluating Healthy and Diseased Mcav3 samples.*

ID	Filter (Healthy Mcav3)	Whole (Healthy Mcav3)	Filter (Diseased Mcav3)	Whole (Diseased Mcav3)
Archaea	0.01	0.01	0.01	0.01
Bacteria	0.36	1.27	0.46	0.28
Eukaryota	99.61	99.68	99.48	99.68
Viruses	0.01	0.02	0.03	0.002
N/A	0.01	0.02	0.02	0.01

Further investigation to determine if bacteria enrichment efforts were successful was analyzed with the program phyloflash, a program that uses 16S rRNA and 18S rRNA to estimate the total makeup of a sample that is from each Kingdom (results available on Github). This software was only able to process 3 of the 4 samples for reasons we were unable to determine (unable to process the healthy whole coral sample). From this we found that bacteria made up 0.19% of the reads from each of the filtered samples and 0.08% of the one whole coral sample (Mcav3 diseased), thus showing a doubling of bacterial reads that can be achieved using the filtering protocol relative to whole coral. However, the majority of the sample (>99%) was still made up of eukaryotic cells for both methods.

4. Discussion:

The methods originally proposed in this project were assessed and found to provide inconsistent results that do not markedly improve our prospects of concentrating bacterial sequences in coral samples. Thus flow cytometry, size fractionation, and selective

sequencing using Oxford Nanopore GridION Technology are not recommended as they are not reliable nor consistent methods of enriching coral-associated bacteria.

FACS did not provide the high concentration of cells needed for metagenomic sequencing. The time needed to sort a high number of cells (> 50,000) was also likely to lead to contamination of these low-biomass samples and even with clean technologies would be unlikely to result in high enough concentrations of DNA for sequencing. While not a viable option for sequencing, the developed FACS protocol may be useful for the development of coral, bacterial, or Symbiodinium cell culturing, as sorting individual cells into a 96-well plate takes <15 minutes and thus requires less material and is less prone to contamination. This application may help to aid in the effort to get these cell types into culture for *in situ* SCTLD experiments, which may help to better determine causal agents or contributing factors.

Selective sequencing using Oxford Nanopore GridION is also not recommended due to the propensity of coral sequences that were found in our samples. Although we had reference genomes, the quality of the *M. cavernosa* and Symbiont genomes may not be of sufficient quality for this technology. Draft genomes may lead to the removal of wanted sequences since they likely contain microbial reads that will then be targeted for removal. It is also probable that *M. cavernosa* genomes are relatively diverse between genotypes making selective sequencing technologies suboptimal to select for or against coral genomes. In a follow-up analysis, we plan to find the proportion of long reads that aligned to the reference genomes.

As FACS, selective sequencing, and the 5 to 0.1 µm size fractionation methods did not yield the desired results, we are testing additional methods. The methodologies still to be evaluated include one developed by the Silveira lab that degrades free DNA using DNase and targets a filtrate from 8 to 0.02 µm. We are also evaluating if 0.1 and 0.5 µm beads will increase bacteria yields compared to 2mm beads as the former target bacteria cells and the later eukaryotes.