Investigation of the Coral Disease Outbreak Affecting Scleractinian Coral Species along the Florida Reef Tract

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Investigation of the Coral Disease Outbreak Affecting Scleractinian Coral Species along the Florida Reef Tract

Final Report

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Executive Summary

From spring 2014 to present, coral disease outbreaks in more than 20 scleractinian (stony coral) species have been reported offshore of the southeast Florida region, within the Upper Florida Keys, and within the Dry Tortugas National Park. Coral disease outbreaks began in late 2014 offshore the Miami-Dade area near Virginia Key. Since those initial reports, further disease outbreaks have been reported in areas north and south of the initial outbreak area. The Florida Fish and Wildlife Conservation Commission (FWC)-Fish and Wildlife Research Institute (FWRI) has been investigating this disease outbreak using a combination of field observations and tissue collections for histological and Transmission Electron Microscopy (TEM) evaluation, archiving tissues for later analyses, and supplying samples to collaborators for diagnostics. This report summarizes the collection of reference (visually healthy) tissue from locations in the southeast Florida region and the Florida Keys, analytical progress to date based on limited evaluation of diseased and reference specimens through histological and TEM examination. The continued evaluation of the histological, pathological and molecular response of the species affected as well as an assessment of normal associated and pathogenic organisms may shed additional light on etiology, as well as differential host response. Much of the data gathered thus far is very preliminary and continued efforts are needed to reappraise priority directions and confirmatory experimental and field plans should be developed to constantly appraise and refocus the ongoing disease investigation as necessary. As analyses progress, FWRI will be able to recommend specific collection and analytical efforts to address the ongoing disease outbreak.

Acknowledgements

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Table of Contents

List of Figures

List of Tables

List of Acronyms

CLO: chlamydia-like organisms
CNAT: *Colpophyllia natans*
CREMP: Coral Reef Evaluation and Monitoring Program
DLAB: *Diploria labyrinthiformis*
DNA: deoxyribonucleic acid
EDTA: ethylenediaminetetraacetic acid
DEP: Florida Department of Environmental Protection
FKNMS: Florida Keys National Marine Sanctuary
FWC: Florida Fish and Wildlife Conservation Commission
FWRI: Fish and Wildlife Research Institute
GMU: George Mason University
GMS: Grocott's methenamine silver
H&E: hematoxylin & eosin
HD: histology sample from diseased colony
HH: histology sample from healthy coral colony
HU: unaffected histology sample from diseased coral colony
MCAV: *Montastraea cavernosa*
MC3: Martin County 3, a SECREMP site
MDC: Miami-Dade County
MGP: methyl green pyronin, for microbes
NOAA: National Oceanographic and Atmospheric Administration
OFAV: *Orbicella faveolata*
PAS: periodic-acid-Schiff
PAST: *Porites astreoides*
PCLI: *Pseudodiploria clivosa*
QAQC: Quality Assurance Quality Control
RLO: rickettsia-like organisms
RTL: rapid tissue loss
SECREMP: Southeast Florida Coral Reef Evaluation and Monitoring Program
SEFCRI TAC: Southeast Florida Coral Reef Initiative's Technical Advisory Committee
SOW: scope of work
SSID: *Siderastrea siderea*
TEM: transmission electron microscopy

1. INTRODUCTION

1.1. Background

Disease is recognized as a major cause of reef-building coral mortality and reef degradation. The first reports of coral disease in the Florida Keys and Caribbean emerged in the 1970's (reviewed by Richardson 1998). Since that time, worldwide reports have been increasing in frequency and many coral reefs are being decimated (Bruno et al. 2007). The Florida Reef Tract is currently experiencing one of the largest disease outbreaks on record. Multiple diseases have been reported affecting at least 20 species of scleractinian (stony) coral, including primary reef builders and species listed as Threatened under the Endangered Species Act. Coral disease outbreaks began in late 2014 offshore the Miami-Dade area, with increased reports peaking in spring 2015 and the outbreak area expanding to the north and south (DEP disease call reporting). Disease prevalence values as high as 80% of all colonies present at a given site were reported offshore of southeast Florida (Miami-Dade, Broward, and Palm Beach Counties) during this time (DEP reporting). By winter 2015, reports of similar outbreaks were affecting reefs within the Florida Keys National Marine Sanctuary in the northern area of the Upper Keys. Throughout 2016 and into 2017, the affected areas spread north through Martin County and south through the Upper Keys.

The coral diseases reported include white plague, several uncharacterized diseases including "white blotch", "white bleaching band" and an irregular tissue loss that is similar to rapid tissue loss (RTL) which previously was used to describe irregular patterns of RTL on acroporids (Work and Aeby 2006). Other typical background level diseases have also been reported (i.e., black band, dark spot disease) but they are not as consistent throughout the affected areas as the other diseases previously mentioned.

Initial sampling efforts began in late 2015 targeting diseased coral tissues from several species from the offshore North Miami-Fort Lauderdale area for histological analysis (K. Bohnsack, DEP; S. Tanner, MDC). Tissues were shipped to the Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute (FWC FWRI) for processing and analysis. In June and July 2016, additional disease outbreaks were reported from the Coral Reef Evaluation and Monitoring Project (CREMP) at historical sites in the upper Florida Keys including Carysfort Reef and Grecian Rocks. The CREMP team, part of FWC FWRI, conducted a directed sampling effort at Grecian Rocks targeting both apparently healthy and disease colonies for comparison and collected paired molecular and histology tissue samples for analysis. Prevalence surveys conducted at the time of sampling revealed that 100% of *Meandrina meandrites* colonies, 66.7% of *Diploria labyrinthiformis* colonies, 53.3% of *Montastraea cavernosa* colonies, 50% of *Dichocoenia stokesii* colonies, 50% of *Pseudodiploria strigosa* colonies, 42.3% of *Siderastrea siderea* colonies, 33.3% of *Colpophyllia natans* colonies, and 33.3% of *Eusmilia fastigiata* colonies were actively diseased or recently dead.

FWRI has been coordinating and conducting field efforts, permitting, sample processing, slide reading and collaborative analysis with multiple partners and agencies. These efforts

have resulted in field reports, prevalence data, and preliminary histopathology results that have been communicated to the Coral Disease Coordination Calls coordinated by Florida's Department of Environmental Protection (DEP) as part of the state-wide collaborative effort to gain insight into this event. Diagnostic samples have also been provided to a wide number of collaborating partners and institutions that are assisting with the disease investigation. A suite of diagnostic analyses are aimed to identify potential pathogens and pathologies associated with the disease(s) and are a first step towards determining the cause of the outbreak. This project supports the ongoing response effort.

1.2. Goals and Objectives

The goal of this work is to support DEP and the ongoing multi-agency, multi-collaborator investigation into the widespread disease outbreak along the Florida Reef Tract. The following objectives have been met or are ongoing:

1. Development of standardized protocols for coral tissue sample collections for histopathology, ultrastructural, and molecular analyses.
2. Collection of reference samples from a disease-free site in southeast Florida (with advisory assistance from the Southeast Florida Coral Reef Evaluation and Monitoring Program, or SECREMP) and from a disease-free site south of the disease outbreak area in the Middle Florida Keys.
3. Histological slide preparation of collected samples, including preparing and shipping two sets of standard histology slides for collaborators (Thierry Work and Dr. Esther Peters) and a subset of special slides for fluorescent in-situ hybridization (Dr. Esther Peters).
4. Continuation of this investigation to provide insight of the disease(s) and potential pathogens from gross field observations using detailed morphological descriptors, and from histopathology and ultrastructure (transmission electron microscopy [TEM]) for microscopic and pathologic diagnosis in the laboratory.
5. Summary of preliminary results in a final report and outreach summary.

2. METHODS

2.1. Field and Laboratory Protocol Development

Field protocols, including those outlined below, were adapted and developed from: NOAA's established protocols in the Coral Disease and Health Consortium's *Field Manual for Investigating Coral Disease Outbreaks* (Woodley et al. 2008); George Mason University (GMU) Histology Laboratory protocols for coral sample collection by coring tube (Peters 2012a); and Smithsonian Research Station molecular sample preservation (V. Paul personal communication, November 21, 2016). Histological laboratory protocols for sample preservation and processing were adapted from: GMU Histology Laboratory established protocols for decalcification of coral tissues, and tissue enrobing (Peters 2012b; Peters 2012c); and standard histology preparation and staining manual (Luna 1968). Data were recorded on a FWRI Coral Tissue Collection data sheet.

2.2. Sample Collection

Each Sampling Team consisted of two members: the Sampler and the Sample Handler. The Sampler collected the prescribed samples from coral colonies from a maximum of six species (i.e., *M. cavernosa*, *D. labyrinthiformis*, *C. natans*, *S. siderea*, *O. faveolata*, *A. agaricites*) under approved collection permits (i.e., FKNMS-2016-078-A1, SAL-16-1702A-SRP) and photographed the pre-and post- biopsy site (Figure 1). The Sample Handler assisted the Sampler by keeping track of collection bags and tubes, verifying the labeling, and securing the samples once taken. The Sample Handler was responsible for filling out the Coral Tissue Collection data sheet and taking/estimating measurements. Target specimens were representative of the species affected by disease elsewhere, and were suitable for further laboratory analyses. Paired samples were taken for histology, ultrastructure, and molecular analyses.

- **Tissue Biopsy:** Cross-contamination minimization measures outlined under QAQC considerations were followed for all sampling activities. Paired 1" circular cores were collected using a stainless steel corer/punch. For each apparently healthy or reference coral colony, one histology tissue core and one molecular tissue core were collected. Each tissue core was placed in a pre-labeled Whirl Pack and recorded. Histology samples were returned to the boat and kept in a cooler at ambient temperature (kept in shade on the boat) during transportation to the lab. Molecular samples were frozen as soon as possible and were given sample processing priority. Samples were frozen on the boat using a Dewar with liquid nitrogen.

Figure 1: *An Orbicella faveolata colony located at Grecian Rocks in the Florida Keys, pre and post-biopsy.*

2.3. Data Management

All field data were stored and managed at FWRI. In addition, the samples will be stored at FWRI for future analyses (i.e., histology paraffin blocks, TEM blocks, representative frozen tissue samples for molecular).

2.4. QAQC Considerations

- **Minimizing Cross Contamination**

  All sampling followed established protocols developed for this project to ensure quality and integrity of the samples. Only visually healthy/unaffected sites were visited for this project. All sampling equipment was sterilized on land before use and placed in separate numbered collection bags for each sample target. Each numbered collection bag, one for each colony to be sampled, contained sterile corer, a pair of nitrile gloves, and pre-labeled Whirl Packs. To minimize cross contamination between colonies, each pair of nitrile gloves was discarded in a separate designated sealable bag after each colony was sampled. To minimize cross contamination between sites, all collection equipment was sterilized on the boat in a 5-10% sodium hypochlorite (bleach) solution for 20 minutes, while traveling between sampling sites.

- **Slide Stain Quality**

  Histology slides were produced and stained following standard protocols for Quality Assurance (Luna 1968). Slides were stained in batches. For Quality Control, staining levels and times were checked between batches to avoid over staining tissues. Time in the stain was adjusted as needed for subsequent batches.

- **Data Entry**

  All field data was entered in an excel spreadsheet, verified and crosschecked. Excel spreadsheets were double checked by a different observer to ensure data were entered correctly and any typos corrected. Field data sheets were photocopied and scanned to ensure there are multiple backup copies of the data. Data are stored on a secure FWC server which is backed up regularly by FWC FWRI. Copies of the data will be submitted to DEP.

2.5. Histology and TEM Processing

- **Gross Examination**

  Following field collections, fixation (either with 10% seawater formalin or Z-Fix), and delivery to FWC-FWRI, St. Petersburg, coral samples were grossly evaluated and photographically documented (top and bottom, and as relevant in side view, together with the labeled sample container [with health status, specimen number and site code] to ensure accuracy in sample specimen documentation, see Figure 2) prior to being processed for histology and TEM. Digital gross images were recorded of the coral sample external surface and internal views. If areas of interest were found (e.g. lesions, fouling organisms, unusual structures), then a dissecting microscope equipped with a digital camera was applied for higher magnification observation and recording.

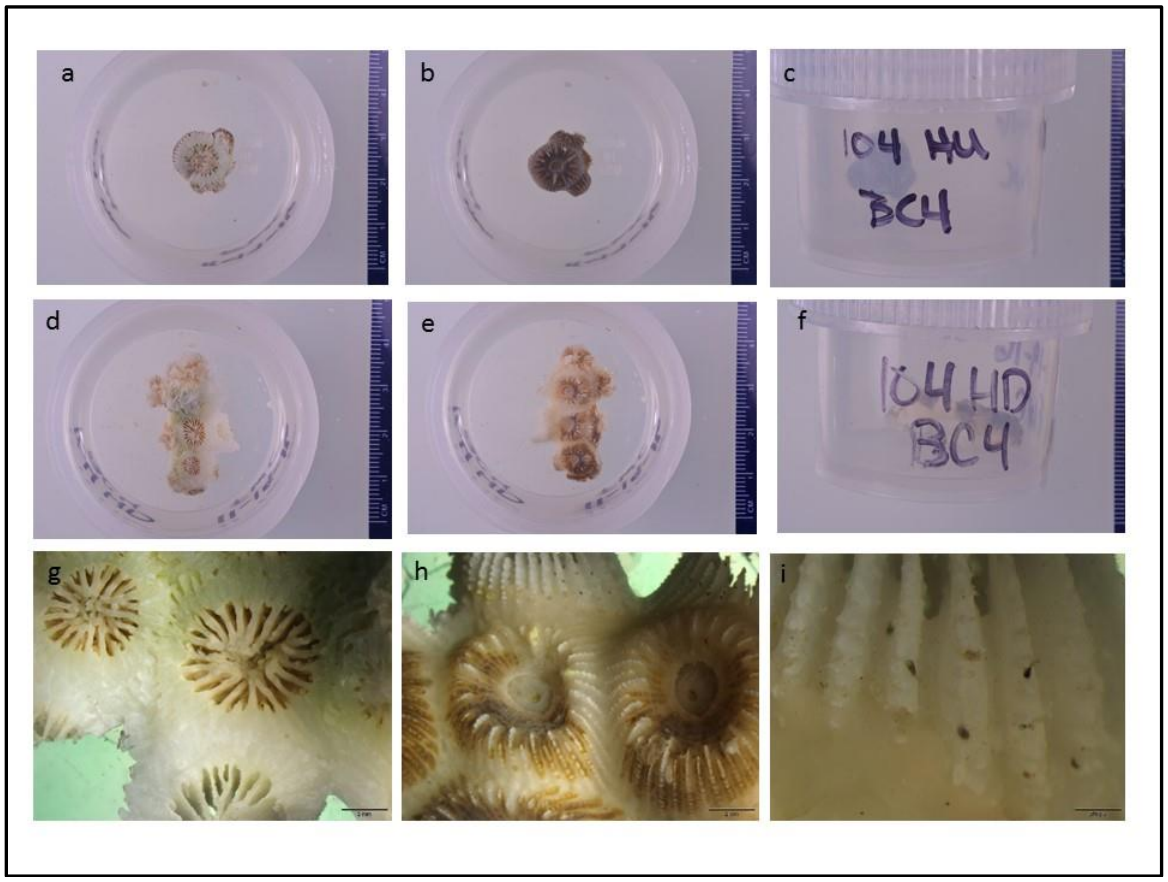

Figure 2: *Macrophotography series of MCAV specimen example #104 showing unaffected (HU) sample bottom (a) and top (b) views, specimen container with label (c), diseased (HD) sample bottom (d) and top (e) views and sample container (f), and higher magnification photos of diseased bottom (g) and top views (h) and a close up of a Halofolliculina infection on skeletal surface (i).*

- **Histology**
  After at least 48–96 h of fixation, the fixative was discarded (in accordance with appropriate safety and environmental guidelines), and the specimen was gently rinsed with running tap water (for at least 30 min) to remove any residual formalin. For the purposes of this contract, both previously-collected diseased samples (collected in Broward County during November 2016) and the reference samples collected during the period covered by this Scope of Work will be evaluated through histology. For coral tissue exhibiting gross lesions (recorded as 'disease [HD]'), the tissue was enrobed with 2% agarose under vacuum pressure, followed by placing the tissue in 10% EDTA solution for decalcification (Peters 2012). The volume of the tissue to the EDTA solution ratio was at least 1:10 to allow for adequate penetration. The internal tissue side of the agarose was trimmed off with a razor blade to allow the tissue to contact the solution. For healthy tissue, recorded as 'healthy (H)' or for tissue exhibiting

no signs of gross lesions and recorded as 'unaffected (U),' the enrobing process was omitted and tissues were directly immersed into the EDTA. The duration of decalcification varied by approximately 4–5 weeks or longer, depending on whether the tissues were agarose enrobed and on their size. Larger, enrobed tissues took longer to decalcify than smaller tissue samples that were not enrobed. During decalcification, fresh EDTA solution was exchanged every 2–3 days. The decalcification endpoint was determined by test cutting the tissue with using a sharp razor blade to assess if there was any resistance. After appropriate decalcification in EDTA solution, the EDTA was thoroughly rinsed off the coral sample with running tap water. The coral samples were placed in their labeled containers in tap water, and then fresh tap water was exchanged several times after allowing the tissues to soak for approximately 30 min. The HH (healthy) or HU (unaffected) tissues or enrobed HD (diseased) tissues (approximately < 30mm long × 26mm wide × 5mm thick) were placed into a tissue processing cassette for longitudinal sectioning. The cassettes were then placed into to a holding container with 70% ethanol. Tissues were processed for gradual dehydration with an ethanol series and then cleared with an artificial solvent, CitriSolv (Fisher Scientific), followed by routine paraffin embedding and sectioning with a rotary microtome at 4 µm thickness (Luna 1968). Sectioned slides were stained with a general routine stain (H&E) or with other special stains (Luna 1968) listed below following methods development and testing. Stained slides were observed with light microscopy, and the digitized images were captured. Digital images were recorded using standard coding for FWRI histology microphotographs with the sample date, species, histological stain, and magnification.

- **TEM**

For the TEM samples (Peters et al. 1983), coral tissues collected with Trump's fixative (4% formaldehyde, 1% glutaraldehyde, 50 mM sodium phosphate, pH 7.2) were transported and held under chilled conditions at the FWRI laboratory. Tissues were then decalcified with 10% EDTA as described above similar to that for the histology process. However, agarose enrobing was omitted for all samples, and therefore decalcification took about three weeks because of the smaller tissue size compared to those samples processed for histology. At the completion of the decalcification, tissue was cut into smaller, appropriately-sized pieces (approximately 1 mm^3). Samples were then postfixed with 1% osmium tetraoxide for 1 h. Tissues were then processed following routine TEM processing. Briefly, tissues were dehydrated in a graded ethanol series, infiltrated with epoxy propylene oxide, and embedded in epoxy resin. The epoxy block was then sectioned with an ultramicrotome (Leica, EM UC6), stained with uranyl acetate followed by lead citrate, and examined with a transmission electron microscope (JEOL, JEM-1400) equipped with a digital CCD camera (Gatan, Orius SC1000). Semithin (1 µm) sections of the epoxy block were also prepared, stained with toluidine blue, examined under light microscope for anatomical confirmations and sectioning conditions. For the

November 2016 and April 2017collections, TEM samples were collected from all corals sampled in parallel with samples to be processed for histology and molecular diagnostics.

2.6. Preservation for Molecular Analysis

The tissue core collected for molecular analyses was frozen as soon as possible (on the boat) in a Dewar filled with liquid nitrogen. Currently, these samples are being stored frozen (in a -80ºC freezer at FWRI for long term storage) and will be shipped on dry ice to collaborative partners for analysis. Results from molecular sampling will not be discussed in this report.

3. SUMMARY OF FIELD RESPONSE EFFORTS

To complement previous diseased coral collection efforts conducted by FWRI, a suite of reference samples were obtained over the course of two field days from three visually healthy reef sites. On 25 April 2017, FWRI and DEP divers visited the reef site "MC3," located in Martin County. This site has been historically monitored by SECREMP. Using established FWRI standardized protocols, paired reference samples were taken from four scleractinian (stony) coral species. FWRI divers also visited two visually healthy sites in the Florida Keys, including "West Turtle" (sampled on 26 April 2017) and "Dustan Rocks" (sampled 27 April 2017). Total collection efforts are summarized in Tables 1-3.

***Table 2**: Summary of Field Collection Efforts of Reference Samples from Martin County*

Species	Reference		
	Histology	Molecular	TEM
S. siderea (SSID)	5	5	5
M. cavernosa (MCAV)	5	5	5
D. labyrinthiformis (DLAB)	0	0	0
C. natans (CNAT)	0	0	0
O. faveolata (OFAV)	0	0	0

***Table 2**: Summary of Field Collection Efforts of Reference Samples from West Turtle*

Species	Reference		
	Histology	Molecular	TEM
S. siderea (SSID)	2	2	2
M. cavernosa (MCAV)	2	2	2
D. labyrinthiformis (DLAB)	0	0	0
C. natans (CNAT)	2	2	2
O. faveolata (OFAV)	1	1	1

***Table 3**: Summary of Field Collection Efforts of Reference Samples from Dustan Rocks*

Species	Reference		
	Histology	Molecular	TEM
S. siderea (SSID)	3	3	3
M. cavernosa (MCAV)	3	3	3
D. labyrinthiformis (DLAB)	5	5	5
C. natans (CNAT)	3	3	3
O. faveolata (OFAV)	4	4	4

4. PRELIMINARY HISTOLOGY AND TEM RESULTS

Tissue samples from five scleractinian species have been prepared (or are in process) for histology and TEM from two field collections made during 2016:

1) July, Grecian Rocks (*Diploria labyrinthiformis* [DLAB], *Colpophyllia natans* [CNAT], *Montastraea cavernosa* [MCAV], *Siderastrea siderea* [SSID])
2) November, Broward County (*Orbicella faveolata* [OFAV], SSID, MCAV)

For each specimen surveyed and sampled from a previous November 2016 collection event (Table 1), we evaluated either diseased (HD) or unaffected (HU) tissues taken from the same diseased colony exhibiting lesions or examined samples taken from corals appearing to be healthy (HH).

***Table 4**: List of coral samples collected in November 2016 from Broward County shown by the species, specimen number, and health status.*

Species (code)	Specimen #	Health status
Montastraea cavernosa (MCAV)	101	HD, HU
	102	HD, HU
	103	HD, HU
	104	HD, HU
	105	HD, HU
	107	HD, HU
	108	HD, HU
	109	HD, HU
	110	HD, HU
Siderastrea siderea (SSID)	111	HD, HU
	112	HD, HU
	113	HD, HU
Montastraea cavernosa (MCAV)	114	HD, HU
Orbicella faveolata (OFAV)	115	HD, HU
	116	HD, HU
	117	HD, HU
Montastraea cavernosa (MCAV)	131	HH
	132	HH
	133	HH
	134	HH
	135	HH
Siderastrea siderea (SSID)	136	HH
	137	HH
	138	HH
Orbicella faveolata (OFAV)	139	HH
	140	HH
	141	HH

Following initial screening observations of histological slides stained routinely with H&E, thionin, or periodic-acid-Schiff (PAS); special histological stains were used to review specific cell types (e.g. mucocytes [mucus cells]) or to highlight e.g. DNA (possible pathogens) or areas of interest. Histological stains included:

- hematoxylin & Eosin (H&E, routine tissues, pathology)
- thionin (DNA)
- periodic-acid-Schiff (PAS, e.g. polysaccharides)
- alcian blue (e.g. for mucus, mucocytes)
- Fontana-Masson (e.g. for melanin, fungi)

- Grocott's methenamine silver (GMS, e.g. for fungi)
- Giemsa (for parasites in general)
- gram stain (for bacteria)
- Gimenez and/or Macchiavello (for microbes, especially chlamydia/rickettsia)
- methyl green pyronin (MGP for microbes)
- Feulgen (for DNA, potential pathogens)

Some of these special stains (e.g. MGP, Macchiavello) have not routinely been used for coral disease assessment and may hold promise for future evaluations. Several special stains (MGP, Macchiavello) took several months to develop through trial and error troubleshooting on coral sections as they were adapted from mammalian veterinary or human medical diagnostics.

The adaptation of routine histological slides using special stains was decided based on preliminary evaluation of some samples provided to us by DEP and MDC from 2015 collections made of five coral species in Dade County. Early determination that samples had potential pathogens or organisms of interest such as intracellular bacteria (or putative Rickettsia-like organisms [RLOs] or Chlamydia-like organisms [CLOs]) warranted the use of special stains. Status reports about these early findings were presented on the DEP coral disease coordination conference calls (see products).

Ongoing evaluation of histological slides (CNAT [n = 8); DLAB [n = 5]; MCAV [n = 7]; and SSID [n = 7]) and TEM specimens (CNAT [n = 1]; DLAB [n = 1]; MCAV [n = 2]; and SSID [n = 2]) from the July 2016 collection at Grecian Rocks (not included under this SOW) has determined the presence of a few potential pathogens (that need confirmation by molecular analyses), but at this stage it is not possible to distinguish between background organism presence, likely normal symbionts, and any potential pathogens causing any obvious pathologies. Findings are still preliminary and subject to change.

TEM samples are still being processed (delcalcification with EDTA has just been completed for all samples collected). We will target a subset of specimens for TEM based on an initial screening of histological slides and will identify if there are any specific priority specimens of interest. There are >25 samples in preparation for TEM that could be evaluated. Prioritization of a subset is the appropriate strategy for moving forward.

The reference histology and TEM samples collected in April 2017 from three different sites (Table 1 –3) are still being processed under decalcification with EDTA.

Preliminary findings (from the July 2016 collections at Grecian Rocks, additional data provided than that requested by the SOW) are:

- Endolithic fungi (Figure 3a) and algae (Figure 3b) are commonly found in the skeleton of all species (but these are likely at greater or lesser abundance and presumptively comprise multiple taxa in different coral species evaluated thus far). There may be some associated pathology with the endolithic community and this is one area of interest to explore further.

- Putative CLOs were observed in many specimens examined. These are found commonly in the cnidoglandular band and the epidermal area. Generally these do not appear to be associated with any obvious pathology.

- Putative CLO-organisms in SSID (TEM, n = 1) (Figure 3c).

- A putative stramenopile-like organism in CNAT (TEM, n = 1) (Figure 3d).

- Putative coccoid-like organisms in MCAV (TEM, n = 1).

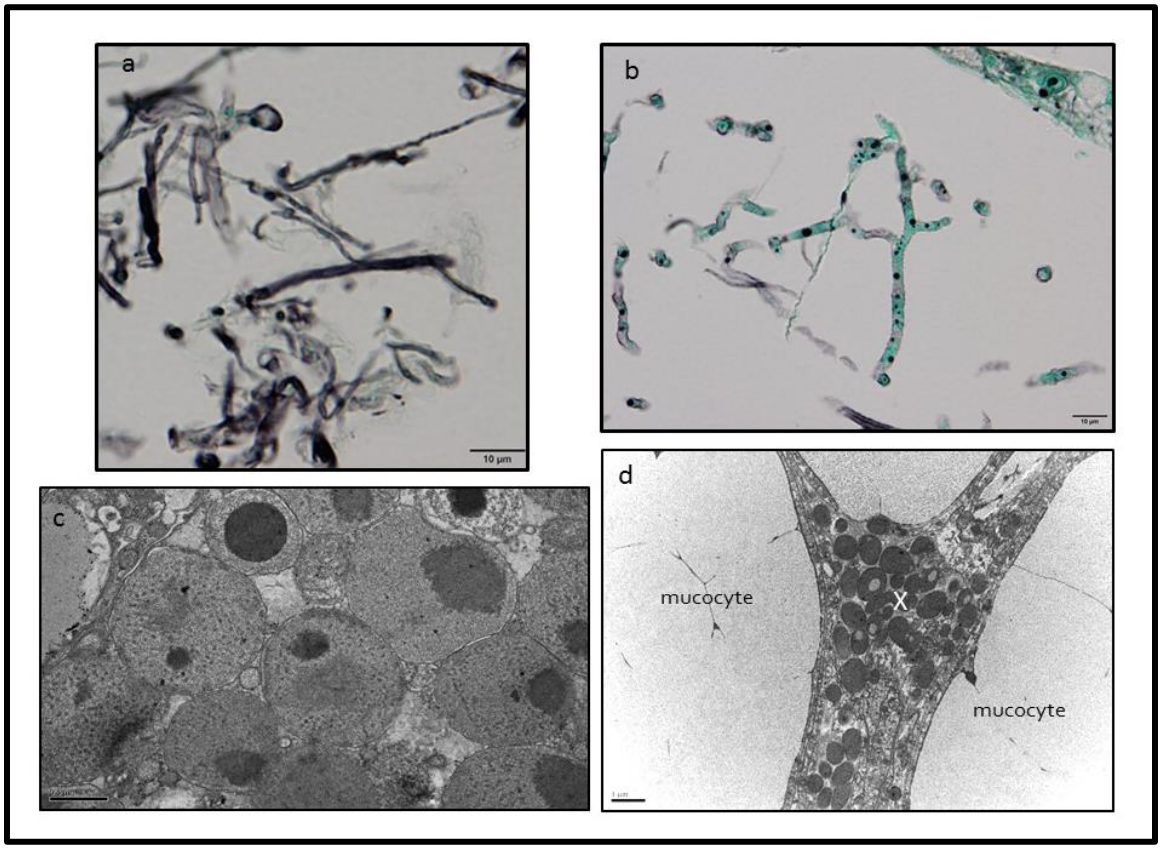

Figure 3: *Photographs of putative organisms as seen in histology (a, b) and TEM (c, d). a) endolithic fungi in skeleton of MCAV, stain GMS b) endolithic algae in skeleton of DLAB, stain GMS, c) TEM of putative CLOs in SSID, d) putative stramenopile in CNAT.*

At this stage, until definitive identifications have been made and an assessment made of possible significance with respect to their relationship with the ongoing disease outbreak, it is not possible to determine the potential role of these putative organisms as pathogens. Endolithic algae and fungi are commonly reported in corals, and in some cases have been considered to be pathogenic (Le Campion-Alsumard et al., 1995,

Fine et al., 2010; Gutiérrez-Isaza et al., 2015). Similarly stramenopiles have been reported in *Favia* sp. and were associated with the formation of a white coating on the surface, but they are not necessarily pathogenic (Kramarsky-Winter et al., 2006; Siboni et al., 2010). More data is needed to determine if these organisms are normal symbionts or if they play a direct or indirect role in the disease outbreak.

4.1. Outreach Contributions

Archived samples and histological slides are potentially available for researchers. FWRI has been actively engaged in collaborative discussions with other researchers addressing complementary disease response collections, experiments, and analyses. Presentations to date have included two webinars on initial observations on 2015 samples and July 2016 samples respectively, on the DEP coral disease coordination conference calls (presented on 11/03/16, conference call #4, 1/18/17, conference call #5). Additionally, a status report talk was presented to the SEFCRI TAC at the invitation of DEP (4/20/17, Nova University, Dania Beach, talk entitled, Coral disease investigation at Grecian Rocks, by Jan H.

Landsberg, Vanessa Brinkhuis, Yasu Kiryu, Patrick Wilson, Clark Gray, and Lindsay Huebner).

4.2. Future Recommendations

FWRI recommends convening a working group of researchers and managers to synthesize response efforts and analyses conducted to date, and to then determine and prioritize future steps. Ongoing response efforts to this disease outbreak event should continue to include prevalence surveys, gross species observations, sample collection, diagnostic evaluations, and spatial extent documentation. Investigations into transmission pathways, as well as treatment options, are also recommended as high priorities for funding opportunities. The continued evaluation of the histological, pathological and molecular response of the species affected as well as an assessment of normal associated and pathogenic organisms may shed additional light on etiology, as well as differential host response. Much of the data gathered thus far is very preliminary and continued efforts are needed to reappraise priority directions and confirmatory experimental and field plans should be developed to constantly appraise and refocus the ongoing disease investigation as necessary.

5. LITERATURE CITED

Bruno, J.F., Selig, E.R., Casey, K.S., Page, C.A., Willis, B.L, Harvell, C.D., Sweatman, H., Melendy, A.M. 2007. Thermal stress and coral cover as drivers of coral disease outbreaks. PLoS Biology. 5:6 e124.

Fine, M., Roff, G., Ainsworth, T.D., Hoegh-Guldberg, O., 2006. Phototrophic microendoliths bloom during coral "white syndrome". Coral Reefs 25, 577-581.

Gutiérrez-Isaza, N., Espinoza-Avalos, J., León-Tejera, H., González-Solís, D., 2015. Endolithic community composition of *Orbicella faveolata* (Scleractinia) underneath the interface between coral tissue and turf algae. Coral Reefs 34, 625.

Kramarsky-Winter, E., Harel, M., Siboni, N., Ben Dov, E., Brickner, I., Loya, Y., Kushmaro, A., 2006. Identification of a protist-coral association and its possible ecological role. Mar. Ecol. Prog. Ser. 317, 67-73.

Le Campion-Alsumard, T., Golubic, S., Priess, K., 1995. Fungi in corals: symbiosis or disease? Interaction between polyps and fungi causes pearl-like skeleton biomineralization. Mar. Ecol. Prog. Ser. 117, 137-147.

Luna, L.G. 1968. Manual of histologic staining methods of the Armed Forces Institutes of Pathology, 3rd edition. McGraw-Hill, New York

Peters, E. 2012a. *Coral sample collection by coring tube.* George Mason University Histology Laboratory Standard Operating Procedure. George Mason University, Fairfax, VA 8pp.

Peters, E. 2012b. *Decalcification*. George Mason University Histology Laboratory Standard Operating Procedure. George Mason University, Fairfax, VA 8pp.

Peters, E. 2012c. *Tissue Enrobing*. George Mason University Histology Laboratory Standard Operating Procedure. George Mason University, Fairfax, VA 7pp.

Peters, E.C., Oprandy, J.J., Yevich, P.P. 1983. Possible causal agent of 'white band disease' in Caribbean acroporid corals. J. Invertebr. Pathol. 41, 394-396.

Richardson, L.L. 1998. Coral diseases: What is really known? Trends Ecol Evol 13: 438-443.

Siboni, N., Rasoulouniriana, D., Ben-Dov, E., Kramarsky-Winter, E., Sivan, A., Loya, Y., Hoegh-Guldberg, O., Kushmaro, A., 2010. Stramenopile microorganisms associated with the massive coral Favia sp. J. Eukaryot. Microbiol. 57, 236-244.

Woodley, C.M., Bruckner, A.W., McLenon, A.L., Higgins, J.L., Galloway, S.B. and Nicholson, J.H. 2008. *Field Manual for Investigating Coral Disease Outbreaks*. NOAA Technical Memorandum NOS NCCOS 80 and CRCP 6. National Oceanic and Atmospheric Administration, Silver Spring, MD 85pp.

Work, T.M., Aeby, G.S. 2006. Systematically describing gross lesions in corals. Dis. Aquat. Org. 70(1-2): 155-160.