Florida Fish and Wildlife Conservation Commission
Florida's State Wildlife Grants Program
Application Form

Project Title: Disease investigation: in search of a cause for the widespread coral mortality event in Florida

Principal Investigator

Name: Dr. Jan Landsberg

Department: Fish & Wildlife Research Institute

Institution: Florida Fish and Wildlife Conservation C

Address: 100 Eighth Avenue SE

City: Saint Petersburg

State: FL ZIP: 33701

Phone: (727) 502-4880

E-Mail: jan.landsberg@myfwc.com

Partners

	Name	Affiliation	Co-PI?
+ -	E. Peters	George Mason University	☑
+ -	Y.Kiryu	FWC/FWRI	☑
+ -	E. Muller	Mote Marine Lab	☑
+ -	R. Ruzicka	FWC/FWRI	☑
+ -	L. Huebner	FWC/FWRI	☑
+ -			☐

Authorizing Official

Name: Gil McRae

Title: Director, FWRI

Phone: (727) 896-8626

E-Mail: Gil.McRae@myFWC.com

Project Start Date: 7/1/2018

Project End Date: 6/30/2021

Maximum Three Year Duration

Target Species: Acropora palmata (archived specimens), Acropora cervicornis (archived specimens), Montastraea cavernosa, Orbicella faveolata, Siderastrea siderea

Target Habitats: Coral Reef

Project Location: Florida Reef Tract (Martin, Palm Beach, Broward, Miami-Dade, and/or Monroe Counties)

☐ No Fieldwork

Match Source: FWC Marine Resources Conservation Trust Fund. ~35% salary match each from Jan Landsberg and Yasu Kiryu.

Requested Grant Funding	$305,181.00
FWC Match	$186,701.14
Non-FWC Match	
Total Project Cost	$491,882.14

Relevance to Florida's State Wildlife Action Plan and Florida's Wildlife Legacy Initiative Implementation Goals

This project directly addresses a number of actions identified in the 2012 SWAP, which follow:

"Improve capabilities/sophistication for inspection, recognition, and treatment of aquatic organism diseases and parasites."
"Conduct additional research for aquatic wildlife parasites and diseases and the impacts of biotoxins on fish and wildlife resources."
"Synthesize and consolidate understanding, and identify gaps in understanding, of marine flora/fauna diseases, pathogens, and biotoxin impacts on fish and wildlife resources."

This project addresses the Marine Implementation Goal "To improve coral reef restoration and conserve marine SGCN through planning and research" by filling information gaps to improve coral reef restoration and conservation efforts (Objective 2a).

Explain need for project

The unprecedented coral disease outbreak and high incidence of mortality during 2014–2017 in the Florida Reef Tract has highlighted the need for diverse transdisciplinary approaches to address the problem. Investigations are hampered by limited capacity to rapidly and accurately diagnose the etiological agent(s) of what appears to be several different diseases affecting more than 20 SGCN scleractinian corals species. In the absence of a definitive diagnosis, a characterized etiology, and an understanding of environmental drivers, management efforts are hindered to potentially control the spread of the disease(s) and to treat, mitigate or manage affected corals.

Project objectives

1) Identify and characterize putative RLOs/CLOs and other putative bacterial pathogens from Acropora spp. and from target SGCN coral species affected by the current disease
(2) Determine potential pathogens in at least three of those affected SGCN coral species compared to apparently healthy colonies at the same site
(3) Conduct transmission experiments to determine where the signature of the bacterial community changes from a disease community to a healthy community within a diseased coral

Benefits to Florida's Species of Greatest Conservation Need and habitats

Virtually all SGCN corals and associated SGCN fauna are threatened by the ongoing disease outbreak, which is causing coral mortality on a broad scale. The pathogen(s) responsible for the disease, the method of transmission, and the environmental factors that are contributing to the outbreak are currently unknown. Only by addressing these data gaps will managers have any chance of implementing management strategies to mitigate the impact of this disease outbreak on Florida's coral reefs.

Project approach and methodology

1. Identify and characterize RLO/CLOs and other putative bacterial pathogens from Acropora spp. and target coral species affected by current disease outbreak (e.g. Montastraea, Siderastrea, Colpophyllia) from healthy and diseased samples and affected and control areas. Continue diagnostics on archived samples and on field samples to be collected during 2018-2020. Utilize additional diagnostics e.g. special stains, FISH (fluorescent in-situ hybridization), laser capture microdissection (LCM) to target potential pathogens. New molecular probes for specific pathogens can be developed based on the microbiological analyzes to be conducted.
2. Conduct broad systematic analyses (molecular, histology, EM) for other potential pathogens across multiple affected coral species (plus healthy controls) and their structural compartments (e.g., mucus, tissues, skeleton with and without endolithic communities).
3. Coral samples will be collected along a transect from apparently healthy tissue into the tissue loss margin to determine where the internal tissue damage is located. Several measurements will be taken to quantify health state including: histology, TEM, and molecular analyses. Samples will be used to create a tissue homogenate of the sample type and to inoculate apparently healthy corals.

Save As Print Form

Florida Fish and Wildlife Conservation Commission
Florida's State Wildlife Grants Program
Application Form Instructions

The US Fish and Wildlife Service and the Florida Fish and Wildlife Conservation Commission appreciate your interest in State Wildlife Grants funding. State Wildlife Grants provides a funding opportunity for individuals, organizations and institutions to participate in the on-going process of data collection, conservation, management and restoration to benefit the species of greatest conservation need and address the threats identified in Florida's State Wildlife Action Plan. For additional information, consult Florida's State Wildlife Grants Program Guidelines.

The application form serves as a cover page and summarizes your proposed project. Applicants whose projects are selected for additional consideration will be asked to submit a proposed budget and scope of work with additional details. Application forms not submitted using this form may be deemed ineligible for funding. All sections of the application form are limited to the space provided.

Partners: Partners are integral to the implementation of Florida's State Wildlife Action Plan. List the names and affiliations of those contributing funding, supplies, equipment use, coordination, review or other support. To add a partner click "+" and to remove a partner click "-". This section also allows you to enter co-principal investigators by checking the box.

Authorizing Official: Typically the director of the applicant's agency or grant manager if different from the principal investigator. If Authorizing Official is not applicable, leave blank.

Project Duration: While projects may take less than three years to complete, projects of greater than three years' duration may not be considered for funding. Consult the grant announcement or your Florida's Wildlife Legacy Initiative contact for additional guidance concerning expected start dates for projects.

Target Species: List which of the Species of Greatest Conservation Need will benefit from this project. Consult Florida's **State Wildlife Action Plan** for the list of species. Species may be listed at any taxonomic level.

Target Habitats: List which of the habitats will benefit from this project. Consult Florida's **State Wildlife Action Plan** for the list of habitats.

Project Location: Identify the counties in which work will occur, as well as more explicit description of location(s) if space allows. If the project will occur in all counties, enter statewide as the location.

Match Source: Identify sources of match. Match cannot include federal funds. Consult the grant announcement or your Florida's Wildlife Legacy Initiative contact for additional guidance concerning match and the proportion required.

Relevance: Explain this project's relevance and significance to the management and conservation of Florida's Species of Greatest Conservation Need and habitats as described in Florida's State Wildlife Action Plan. Also describe how this project will meet one or more of **Florida's Wildlife Legacy Initiative Implementation Goals**.

Need: Provide justification for undertaking the project, such as a problem to be solved or opportunity to be used. Also consider the implications if this project is not funded, or funded at a later time.

Objectives: Describe what will be accomplished during the project. Objectives must be specific and measurable, and should identify knowledge to be gained and products created as a result of this project.

Benefits: Describe the benefits to Florida's Species of Greatest Conservation Need and habitats as a result of this project. Consider impacts both from this project and later, as a result of this project.

Approach and Methodology: For each objective, summarize specific procedures and any data analyses that will be used and the roles of key project personnel.

If you have additional questions about this form or State Wildlife Grants, please contact Florida's Wildlife Legacy Initiative.

When you have completed the form, please e-mail it to your State Wildlife Grants contact. You may also save or print a copy for your records; this form is enabled for saving in Adobe Reader.

Public Description: The unprecedented disease(s) outbreak and high incidence of mortality in over 20 scleractinian coral species during 2014−2017 in the Florida Reef Tract (FRT) has highlighted the need for diverse transdisciplinary approaches to address the problem. These tissue loss diseases have spread rapidly from the upper FRT towards the middle Florida Keys and appear to be continuing unabated (prior to Hurricane Irma). Few coral species are unaffected. In the absence of a definitive diagnosis, a characterized etiology (cause of disease), and an understanding of environmental drivers, management efforts are hindered to potentially control the diseases(s) spread, and to treat, mitigate, or manage affected corals. This project aims to identify cause(s) by intensive field sampling of three target species, characterizing the disease, utilizing diverse diagnostics to identify suspect pathogens and determine tissue damage caused, and conducting laboratory experimental transmission studies to recreate the disease.

Introduction: In recent decades, coral reef ecosystems are increasingly threatened by natural and anthropogenic environmental stressors, resulting in, or leading to, diverse diseases, with subsequent mortality, recruitment failure, and poor growth of corals (Carpenter et al. 2008, Muller et al. 2008, Pollock et al. 2014a). Compounded sub-optimal health conditions have led to longer to no recovery periods, associated declines or shifts in coral assemblages, and increasing dominance by macroalgal or cyanobacterial communities (Eakin et al. 2010, de Bakker et al. 2017, Neal et al. 2017). Since 1987, six mass coral bleaching (loss of symbiotic zooxanthellae) events have affected the entire FRT, with moderate incidents occurring every year since 2006 (barring 2013), thus reflecting an increasing global trend exacerbated by thermal stress (Manzello 2015). The FRT has also experienced an increasing prevalence of acute or chronic coral diseases that grossly manifest as colored bands, blotches, discolorations, abnormalities, and/or rapid tissue loss (RTL) (e.g., black band [BBD], white band [WBD], white patch [WPD], dark spot syndrome [DDS], or white plague [WP]) (Kuta & Richardson 1996, Richardson et al. 1998, Patterson et al. 2002, Peters 2015, Bruckner 2016). Once on a small scale, these sporadic diseases resulted in localized but recoverable population losses. However, more recently, partial or complete mortality and declines of the federally-threatened acroporids, *Acropora cervicornis* and *A. palmata*, were associated with WBD, WPD, and RTL (Aronson & Precht 2001, Patterson et al. 2002).The demise of the acroporids and recognition that disease-related morbidity or mortality is a major driver of population losses has added to the concern about the future stability and health of Florida's coral reef ecosystem (Williams & Miller 2012), and highlighted the previously underestimated role of disease (Miller et al. 2014). More disturbingly, since 2014, an unprecedented outbreak of several diseases affecting more than 20 SGCN scleractinian species has spread south throughout the upper half of the FRT with resultant sustained and wide-scale mortalities of multiple SGCN species (Precht et al. 2016, FDEP, FWC, unpub. data).

Despite the key role of healthy organisms in coral reef ecosystems, knowledge about most coral disease etiologies is still limited (Bourne et al. 2009, Sokolow 2009). Diseases known for decades may not be completely understood or etiologies once considered definitive have not been reconfirmed or have required reevaluation (Sunagawa et al. 2009, Muller & van Woesik 2012, Sutherland et al. 2016). In a few cases, bacterial pathogens have a primary role, while in other diseases, for example, BBD, a multi-complex suite of pathogenic microorganisms and environmental factors are involved (Rosenberg & Kushmaro 2011, Ushijima et al. 2014). Disease presentations can appear grossly similar in various species, but they may mask the role of multiple or different pathogens, or unexplored cryptic pathogens (e.g., viruses) (Sutherland et al. 2016, Sweet & Bythell 2017).

This proposal addresses the Marine Implementation Goal, "to improve coral reef restoration and conserve marine SGCN through planning and research" (objective 2a). Before coral SGCN species can be effectively protected and disease threats controlled, priority should be directed towards understanding the cause of disease. Thus far, during this ongoing outbreak, the manifestation of disease in SGCN scleractinian coral species both in the field and in closed aquaria systems (following the introduction of wild corals from disease-endemic areas), is suggestive of a waterborne pathogen (Val Paul, Smithsonian Institution, pers. comm.). Efforts to identify the causative agent(s) are challenged by several factors. Without characterization of the disease(s), including associated pathologies, in multiple coral species, it is unknown if: (1) one or several pathogens are involved, (2) this is a complex disease syndrome (that may change over

time from an initial cause), (3) there are novel or introduced primary pathogens, or (4) pathogenicity is being expressed in organisms normally present and virulence triggered by as yet undefined factors. Climatic change, prolonged El Niño years, and bleaching events have been considered as precursors to this disease outbreak (Precht et al. 2016), but other critical co-occurring abiotic factors may also be highly significant (Miller et al. 2016). It is also unknown if factors or agents other than pathogenic microorganisms are involved or if multiple synergistic factors are required for the disease(s) to manifest.

Initial analyses of diseased SGCN coral species (e.g., *Montastraea cavernosa, Diploria labyrinthiformis* and *Colpophyllia natans*) collected from the FRT in 2015−2016 have shown the consistent presence of unidentified putative intracellular coccoid-like bacteria (rickettsia or chlamydia-like organisms [RLOs/CLOs]) (Fig. 1) that could potentially be involved in the WP-like disease (one of the primary diseases reported). RLOs/CLOs are also present in apparently healthy specimens, but no quantification has yet been done. The putative RLOs/CLOs superficially appear similar to those reported, but as yet still uncharacterized, from endangered *A. cervicornis*, which were postulated to have played a role in the demise of the elkhorn acroporid reefs in the FRT (Miller et al. 2014, Fish & Wildlife Research Institute [FWRI] 2003 acroporid tissue loss study, Fig. 2). Laboratory experiments showed high relative abundances of Rickettsiales bacteria within homogenates created from diseased corals. The RLO was also present in the healthy homogenates, which did not cause tissue loss, although at much lower levels (Fig. 3, Muller et al. unpubl.). RLOs/CLOs have been documented in corals (Casas et al. 2004, Vega Thurber et al. 2009, Miller et al. 2014), but little is known about their potential pathogenicity, although they are significant pathogens of higher vertebrates (Corsaro & Venditti 2004) and other aquatic invertebrates (Crosson et al. 2014, Gollas-Galván et al. 2014). It is unknown what role, if any, these putative RLOs/CLOs may play in the ongoing disease outbreak and if they are identical species in all affected corals. Characterization and identification of the putative RLOs/CLOs is needed, with comparisons across affected coral species; their levels compared to levels in healthy corals and unaffected areas within the same species; their associated pathology (if any) determined; and transmission experiments done to assess their potential pathogenicity and virulence.

Endolithic algae and fungi are commonly present in the skeleton of most samples thus far examined (qualitative observations only), and are occasionally observed penetrating the basal body wall of the coral tissue. It is unknown if potential community shifts or dominance by several species might be significant, and a trigger for internal lesion development. Endoliths are a normal microflora component, but following bleaching incidents, they can increase in biomass and potentially affect skeletal integrity (Peters 1984, Fine et al. 2006). Endolithic fungi and bacteria are also potential pathogens (Bentis et al. 2000, Ainsworth et al. 2015, Marcelino et al. 2017). In *M. cavernosa*, preliminary pathological evaluations of polyps and coenenchyme at the tissue loss margin show a bleaching response, degenerate zooxanthellae in the gastrodermal cells, and damage to the surrounding tissue (Fig. 4). Compared to tissues taken from an apparently healthy reference specimen (Fig. 5), internal lesions appear necrotic (almost like liquefactive necrosis or tissue lysis) as if some putative toxin or lytic pathogen was involved (Fig. 4). The appearance of internal deep lesions (Fig. 6) and gastrodermal lesions (Fig. 4) compared to surface epidermal lesions, and the evident absence of surface body wall lesions (in healthy tissue away from the leading edge of the distinct, often bleached, disease margin) (Fig. 7) might suggest an internal pathogenesis or that pathological processes are occurring from the inside of the coral to the outside. One hypothetical scenario could be a cascade of bleaching, upward phototactic growth response of endolithic algae, reduced skeletal calcification (from symbiotic zooxanthellae loss), structural stress, microbial infections, associated pathology at the skeletal-coral tissue interface, tissue necrosis, and subsequent surface lesions, then tissue loss. Putative bacteria have been observed by transmission electron microscopy (TEM) (Fig. 8), but it is unknown if these organisms are primary or secondary. Surficial exposed skeletal infestations by the ciliates, *Halofolliculina*, considered to play a role in skeletal eroding disease, and often co-occurring with WBD (Cróquer et al. 2006, Verde et al. 2016), have been documented here (Fig. 9), but are considered to be secondary.

Disease diagnosis can be hampered by a lack of baseline information on the normal microbial flora of healthy corals and how this may vary in different species and during disease events. The microbiome is recognized as a diverse but essential community of organisms associated with normal, healthy corals (Bourne et al. 2009, Ainsworth et al. 2010). Potential imbalances or dominance by certain pathogens or

introductions of new pathogens may change with environmental stress or upon the initiation of disease. While apparently healthy and diseased corals may or may not show comparative shifts in microbial communities (Sunagawa et al. 2009, Cardenas et al. 2012, Pollock et al. 2014b, Roder et al. 2014, Pollock et al. 2017), these data alone do not discern which specific microbes (or suites of microbes) cause pathological tissue changes, or are secondary opportunists in compromised tissues, nor do they determine the microbial location in or on the host tissue in relation to active tissue loss or pathogenesis. A multi-faceted diagnostic approach is needed to identify and characterize potential pathogens in different coral compartments and microscale niche habitats, to assess shifts in microbial communities, and to describe histopathological changes (Sussman et al. 2008, Sweet et al. 2011, Pollock et al. 2017).

Discerning disease processes is challenging when multiple organisms (e.g., pathogenic, opportunistic, and symbiotic) are present (and change over time) in lesioned tissues and it is uncertain if the etiology involves a primary pathogen or a consortium of organisms. It is possible that the active disease is subsurface or distant from the grossly observable lesion margin (as hypothesized above) and pathogens could be missed in sampling. Experimental approaches can be designed along a coral colony transect from healthy to diseased tissue to identify the microbial community where active tissue loss is occurring. Then healthy corals can be inoculated with putative pathogens or microbial homogenates (to recreate disease experimentally, and if the putative pathogens are re-isolated in culture or molecularly identified then Henle-Koch's postulates will be fulfilled) (Work et al. 2008). Treatment options can be explored that mimic the use of a lesion occlusion method ("firebreak"), a strategy to control disease spread (Aeby et al. 2015).

Objectives: (1) Identify and characterize putative RLOs/CLOs and other putative bacterial pathogens from *Acropora* spp. and from target SGCN coral species affected by the current disease, (2) Determine potential pathogens in at least three of those affected SGCN coral species compared to apparently healthy colonies at the same site, (3) Conduct transmission experiments to determine where the signature of the bacterial community changes from a disease community to a healthy community within a diseased coral.

Methods: To conduct obj. 1−3, diseased and healthy colonies of three target SGCN species (*Montastraea cavernosa*, *Orbicella faveolata*, and *Siderastrea siderea*) will be surveyed in Yr 1 (and as needed in Yr 2) from the FRT, utilizing ongoing monitoring efforts by the FWRI Coral Reef Evaluation and Monitoring Program (CREMP), or as necessary through dedicated collection trips to obtain samples to meet these objectives. Species will be selected as deemed appropriate for prioritizing next steps in the disease investigation at project startup. As feasible, five active disease sites will be surveyed and assessments made on the health of multiple colonies and species (all georeferenced). If additional species are noted to be diseased (e.g., *Meandrina meandrites*, *Colpophyllia natans*) then every effort will be made to sample these species as well (Yrs. 1−3), as covered by existing permits. An additional healthy reference site, at least 30 miles south of the known active disease boundary (to be reassessed at the time of sampling), will be surveyed in Yr 1 to obtain three apparently healthy samples from each of the three target SGCN species.

At least five diseased colonies and three apparently healthy colonies (i.e., reference sample) of each species will be targeted per site. Gross macroscopic photographs of diseased and healthy colonies will be taken. For obj. 1−2, two one-inch (2.5 cm) diameter cored samples of diseased and unaffected apparently healthy areas (15−20 cm from the lesion border) on the same colony will be targeted. For obj. 3, five one-inch (2.5 cm) diameter cored samples of diseased and unaffected apparently healthy areas on the same colony will be targeted along a 20 cm-transect (Fig. 10). All coral samples will be collected following standard FWRI SOPs. Samples for the diagnostic analyses and experiments will be collected in parallel in sterile containers and then rapidly refrigerated, frozen (with liquid nitrogen), or placed in Z-Fix Concentrate (1 part): seawater (4 parts) or seawater-formalin solutions for light microscopy (LM), or Trump's fixative or glutaraldehyde solution for TEM, and transported to FWRI-FWC for analysis and experimentation. To assess the microbial communities of the different coral compartments (mucus, tissue, skeleton) a further set of samples will be taken. Additional coral plugs will be collected from a sub-set of diseased and unaffected apparently healthy corals and will be stored in separate sterile containers. These will be transported back to the laboratory for extraction of microbial communities from specific compartments (obj. 1−2). Samples

will be archived or prepared for complete diagnostics (e.g., histopathology, microbiology, molecular analyses, and TEM) at the FWRI (FWC, St. Petersburg), or prepared for a co-PI facility (Mote Marine Laboratory [MML] and George Mason University [GMU]). Histopathology and TEM will be done at FWRI, molecular microbiology and transmission experiments at MML, and histopathology and fluorescent in situ hybridization (FISH) and laser capture microdissection (LCM) (see obj. 1) at GMU. Representative tissue samples will be archived at -80°C. Histological slides will be shipped to GMU and evaluated.

(1). RLO/CLOs and other putative bacterial pathogens from *Acropora* spp. and target SGCN coral species will be identified and characterized from healthy and diseased corals in affected and control areas. During 2015−2017, ~100 diseased, unaffected, and apparently healthy coral reference samples from 10 species were archived and are in the process of being completed and evaluated for histology. Additional samples collected during this project will also be processed for histology and TEM as needed. Slides will be evaluated after utilizing special histological stains targeting DNA (thionin, Giemsa, Macchiavello) to demonstrate the presence of RLOs/CLOs and Gram-negative bacteria (Gram stain). Samples with RLOs/CLOs tentatively present at high visible biomass by LM (Miller et al. 2014) will be selected for further work up using TEM. Archived histological samples of *Acropora* with RLOs/CLOs from a disease outbreak in 2003 are available at FWRI and others at GMU for reexamination. Paraffin blocks at GMU can be recut and stained with special stains and/or potentially, DNA of suspect bacteria visualized in the tissue sections can be extracted using LCM at GMU, then extracted for polymerase chain reaction (PCR) and sent to MML for subsequent sequencing. Representative diseased, unaffected, and healthy samples will also be analyzed blind using FISH to target RLOs/CLOs and other potential pathogens as warranted (for obj. 2). By using molecular probes (sequences specific for identifying most bacteria [EUB-I] and rickettsia/chlamydia [EUB-II], the nature of the microorganisms found in the coral tissue or mucus can be identified (Ainsworth et al. 2006, Wada et al. 2016). New probes for specific pathogens can be developed based on the microbiological analyzes to be conducted (see obj. 2).

(2). Conduct broad systematic analyses (molecular, histology, TEM) for other potential pathogens across multiple affected coral species (plus apparently healthy reference samples). As histological slides are evaluated for RLOs/CLOs, any other potential pathogens of will be recorded along with pathological findings. Samples with microscopic organisms of interest can be further processed for TEM as warranted. If possible, skeletal compartment samples will be separated from other tissues prior to decalcification to assess biota. For field collected samples for microbiology, the cores will be first centrifuged in 50-mL plastic centrifuge tubes to collect the surface mucus layer. The tissue will then be air brushed off into sterile sealable bags. The resulting skeleton will be divided into skeleton with no visible endolithic organisms and skeleton with endolithic organisms. All four compartments, for each of the colonies sampled (up to three per species), will be stored in 100% ethanol and will be treated as follows: total DNA will be extracted from each sample using the MoBio Powersoil DNA isolation kit with an extended bead-beating time of one hour (MoBio Inc., Carlsbad, CA). The bacterial community of each sample will be analyzed using 16S rDNA Illumina sequencing on the MiSeq platform. Amplification of the 16S rRNA gene will be conducted using 515 forward and 806 reverse primer set. Sequence data will be processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). The rarefied percent composition of bacterial groups from each sample will be analyzed at the operational taxonomic unit (OTU) level using a permutation multivariate analysis of variance (PERMANOVA) using the 'vegan' package of the statistical program R (Oksanon et al. 2017, R core team 2017). A similarity percentages (SIMPER) analysis will provide the percent dissimilarity between the disease and healthy samples caused by each bacterial OTU. The top contributor to dissimilarity will be tested for differences among sites using a Kruskal-Wallis test. Bacterial OTU data will then be processed through non-metric multidimensional scaling (nMDS), which applies the rank orders of data to represent the position of communities in multidimensional space using a reduced number of dimensions. The nMDS results will then be plotted in two-dimensional ordination space. The dominant (>3%) classes of bacteria will also be compared between the apparently healthy and disease samples using frequentist statistics.

(3). Conduct transmission experiments to determine where the signature of the bacterial community changes from a disease community to a healthy community within a diseased coral. Disease

studies often show that tissue near the edge of tissue mortality contains a bacterial community different from apparently healthy corals. However, apparently healthy tissue on a coral with active disease mortality also, at times, has the same bacterial community as apparently healthy corals. These data suggest that tissue infection is isolated, rather than systemic, and that isolating diseased tissue from apparently healthy tissue may be a method of triage to prevent total colony loss. The location of transition between apparently healthy and diseased bacterial communities is unknown. At least two target SGCN species (*O. faveolata* and *S. siderea*) samples will be collected along a transect from apparently healthy tissue into the tissue loss margin (Fig. 10) to determine where the internal tissue damage is located. Several measurements of these collections will be taken to quantify health state including: histology, TEM, and molecular analyses. To test for infectivity, mucus and tissue samples will be collected (see methods above) along the tissue loss margin into apparently healthy tissue for laboratory infectivity experiments. These samples will be used to create a tissue homogenate of the sample type and inoculate apparently healthy corals at MML's International Center for Coral Reef Research and Restoration (IC2R3). It is hypothesized that the samples taken from infected corals, which contain bacterial communities similar to completely (apparently) healthy colonies, will not infect experimental corals whereas those samples with the diseased coral-bacterial community signature will induce disease (Fig. 11).

***Schedule**:*

	FY18-19				**FY19-20**				**FY20-21**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Purchase supplies (obj. 1-)	x	x			x	x			x	x		
Collect field samples (obj. 1-3)	x	x			x	x			x	x		
Work up field samples (obj. 1-3)	x	x			x	x			x	x		
Identify/characterize RLOs/CLOs (obj.1)	x	x	x	x	x	x	x	x				
Identify potential pathogens (obj. 2)	x	x	x	x	x	x	x	x	x	x		
Conduct transmission experiments (obj. 3)	x	x			x	x						
Data collection, entry, review, database (obj. 1-3)	x	x	x	x	x	x	x	x	x	x	x	
Annual report/final report				x				x				x

Biographical Sketches:

Dr. A. Alonso Aguirre (Co-Principal Investigator) is Chair and Professor in the Department of Environmental Science and Policy at GMU, Fairfax, Virginia, where he heads a program of collaborative research that focuses on the ecology of wildlife disease and the links to human health and conservation of biodiversity. He also chairs the university Institutional Animal Care and Use Committee. He has worked for the past three decades in over 23 countries focusing on integrative research, transdisciplinarity, professional leadership training and capacity building. He served as the Executive Director of the Smithsonian-Mason School of Conservation. Previously he was Senior Vice President at EcoHealth Alliance (formerly known as Wildlife Trust) in New York, also holding different appointments at the Consortium for Conservation Medicine, the Center for Environmental Research and Conservation at Columbia University and the Center for Conservation Medicine at Tufts University Cummings School of Veterinary Medicine. Dr. Aguirre received his MS, PhD, and DVM degrees from Colorado State University. Dr. Aguirre cofounded the emerging discipline of conservation medicine and is senior editor of two seminal books. Dr. Aguirre has advised governments of several countries in the Americas, Southeast Asia and Western Europe and briefed the U.S. and Mexican Congresses. He has received numerous awards including the Colorado State University Warner College of Natural Resources Distinguished Alumnus Award, the Harry Jalanka Memorial Medal from Finland for outstanding contributions to wildlife medicine and the Conservation Award of the Year from the Mexico State Commission of Natural Parks and Wildlife for his role in conserving protected areas for monarch butterflies. He recently was appointed to the Board on Life Sciences of the National Academy of Sciences. His new book "*Tropical Conservation: Perspectives on Local and Global Priorities*" was released in 2016. He has published over 160 peer-reviewed articles. He cofounded the Journal *EcoHealth* and the International Association of Ecology and Health. He is also associate editor of the *Journal of Wildlife Diseases* and the *European Journal of Wildlife Management*.

Clark Gray is a Research Associate with the Fish and Wildlife Health (FWH) group at FWC/FWRI where he has been employed since 2011. He received his B.S. in Biology (2000) from Appalachian State University and M.S. in Marine Biology (2007) from the University of North Carolina Wilmington, where he studied the neural ultrastructure of box jellyfish and employed various electron microscopy techniques. Papers include: Gray, G.C., Martin, V.J., Satterlie, R.A., 2009. Ultrastructure of Retinal Synapses in Cubozoans. *Biol Bull*. 217: 35-49; Satterlie, R.A., Thomas, K.S., Gray, G.C., 2005. Muscle organization of the cubozoan jellyfish *Tripedalia cystophora*, Conant 1897. *Biol Bull*. 209: 154-163.

Lindsay Huebner (Co-principal Investigator) is a Marine Research Assistant with the Coral Program at FWC/FWRI where she has been employed since 2014. She received her B.S. in Biological Sciences (2008) from the University of Notre Dame and M.S. in Biological Sciences – Marine Biology (2010) from Auburn University, where she studied the community ecology of cnidarian symbioses with fish and shrimp. At FWRI she co-leads a coral and octocoral-recruitment project and has conducted coral disease prevalence surveys and tissue sample collection. Papers include: Huebner LK, Dailey B, Titus BM, Khalaf M, Chadwick NE. 2012. Host preference and habitat segregation among Red Sea anemonefish: effects of sea anemone traits and fish life stages. *Mar Ecol Prog Ser* 464, 1-15; Huebner LK, Chadwick NE. 2012. Reef fishes use sea anemones as visual cues for cleaning interactions with shrimp. *J Exp Mar Biol Ecol* 416–417, 237–242; Huebner LK, Chadwick NE. 2012. Patterns of cleaning behaviour on coral reef fish by the anemone shrimp *Ancylomenes pedersoni*. *J Mar Biol Assoc UK* 92(7), 1557-1562.

Dr. Yasunari Kiryu (Co-principal Investigator) has been an Associate Research Scientist in the FWH program at FWC-FWRI, St. Petersburg, since 2003. He holds a B.S (1989) in Fisheries from Hokkaido University, Hokkaido, Japan; an M.S. (1992) in Fisheries from Auburn University, Auburn, Alabama; and a Ph.D. (1999) in Fisheries Resources from the University of Idaho, Moscow, Idaho. After completion of his Ph.D., he worked as a Postdoctoral Research Associate (1999–2002) at the Virginia Institute of Marine Science, Gloucester Point, VA, and as an Assistant Research Scientist (2002–2003) at the USGS, National Fish Health Research Laboratory, Kearneysville, WV, focusing on a fungal disease (causative agent, *Aphanomyces invadans*) of Atlantic menhaden in Chesapeake Bay at both institutes. Research projects at FWRI have included diseases of corals, crustaceans, mollusks, and a variety of fish

and amphibian species. With Dr. Landsberg and staff in FWC's DMFM he reviews SAL applications and has been involved in developing coral health criteria for the permitting process. He has published ~ 15 peer-reviewed papers on aquatic animal health. Recent relevant papers include: Kiryu Y, Landsberg JH, Peters EC, Tichenor E, Burleson C, Perry N. 2015. Pathological effects of cyanobacteria on sea fans in southeast Florida. *J. Invert. Pathol*. 129:13–27; Kiryu Y, Behringer DC, Landsberg JH, Petty BD. 2009. Microsporidiosis in the Caribbean spiny lobster *Panulirus argus* from southeast Florida, USA. *Dis. Aquat. Org.* 84:237–242; Sosa, ER, JH Landsberg, Y Kiryu, CM Stephenson, TT Cody, AK Dukeman, HP Wolfe, MW Vandersea & RW Litaker. 2007. Pathogenicity studies with the fungi *Aphanomyces invadans*, *Achlya bisexualis*, and *Phialemonium dimorphosporum*: induction of skin ulcers in striped mullet. *J. Aquat. Anim. Health* 19:41–48; Kiryu, Y, JD Shields, WK Vogelbein, H Kator, VS Blazer. 2003. Infectivity and pathogenicity of the oomycete *Aphanomyces invadans* in Atlantic menhaden *Brevoortia tyrannus*. *Dis. Aquat. Org.* 54:135–146; Kiryu, Y and CM Moffitt 2002. Models of comparative acute toxicity of injectable erythromycin in four salmonids species. *Aquaculture* 211:29–41.

Dr. Jan H. Landsberg (Principal Investigator) is a Research Scientist in the FWH group at FWC-FWRI, St. Petersburg, where she has been employed since 1989. She received a Ph.D. in Zoology from London University, England in 1981. From 1982−1987, she worked at the Fish Disease Laboratory, Israel. In 1988 she conducted aquatic animal health research at the College of Veterinary Medicine at North Carolina State University in Raleigh. Dr. Landsberg supervises statewide aquatic health research and develops and implements research projects to resolve aquatic health issues. With Dr. Kiryu and staff in FWC's DMFM she reviews SAL applications and has been involved in developing coral health criteria for the permitting process. Research projects at FWRI have included sea grass, coral reef, shellfish, fish, aquatic bird, sea turtle, amphibian, and manatee diseases and mortalities, and investigating the role of microalgal biotoxins in aquatic animal disease and mortality events. She has published 80 peer-reviewed papers on aquatic animal health/harmful algal bloom impacts, and has identified 16 new species of protozoa (animal parasites or dinoflagellates). She is currently co-PI on the FDEP grant, "Investigation of the Coral Disease Outbreak Affecting Scleractinian Coral Species along the Florida Reef Tract, $34,066). Dr. Landsberg has managed ~ 20 multi-year, multi-collaborator grants obtained from state, federal, and foundation funds. She was project director on two SWG grants: 1) Disease Surveillance in Selected Species of Greatest Conservation Need – Bats and Amphibians (2010-2013), 2) The Potential Role of Harmful Algal Blooms in Bird Mortalities (2006–2009). Relevant publications include: Landsberg, J. H. 1995. Tropical reef fish disease outbreaks and mass mortalities in Florida: what is the role of dietary biological toxins? *Dis. Aquat. Org*. 22:83-100; Landsberg JH 2002. The effects of harmful algal blooms on aquatic organisms. *Rev. Fish. Sci.* 10:113-390; Sosa ER, Landsberg JH, Kiryu Y, Stephenson CM, Cody TT, Dukeman AK, Wolfe HP, Vandersea MW, Litaker RW. 2007. Pathogenicity studies with the fungi *Aphanomyces invadans*, *Achyla bisexualis*, and *Phialemonium dimorphosporum*: induction of skin ulcers in striped mullet. *J. Aquat. Anim. Health* 19:41–48; Landsberg JH, Van Dolah F, Doucette G 2005. Marine and estuarine harmful algal blooms: impacts on human and animal health. *In* Oceans and Health: Pathogens in the Marine Environment, Belkin S, Colwell R. (eds.), pp. 165-215, Springer, New York; Kiryu Y, Behringer DC, Landsberg JH, Petty BD. 2009. Microsporidiosis in the Caribbean spiny lobster *Panulirus argus* from southeast Florida, USA. *Dis. Aquat. Org.* 84:237–242; Kiryu Y, Landsberg JH, Peters EC, Tichenor E, Burleson C, Perry N. 2015. Pathological effects of cyanobacteria on sea fans in southeast Florida. *J. Invert. Pathol*. 129:13–27.

Dr. Erinn Muller (Co-principal Investigator) is a Staff Scientist and the Coral Health and Disease Program Manager at MML Sarasota, FL, where she has been an employee since 2012. Dr. Muller has studied coral diseases for the last 14 years in many places throughout the world including the US Virgin Islands, Puerto Rico, the FL Keys, and as far away as Indonesia and Saudi Arabia. She has published 20 peer-reviewed publications and two book chapters on the subject of coral health and disease and the coral microbiome. Dr. Muller's research is currently funded by the National Science Foundation, the Environmental Protection Agency (two current awards), the National Park Service, NOAA's Coral Reef Conservation Program, Florida Fish and Wildlife Conservation Commission, the Florida Protect Our Reefs License Plate and through philanthropy. She received the prestigious Young Scientist of the Year Award from the International Society for Reef Studies in 2015; only one recipient is recognized worldwide each

year. Relevant publications include: Muller EM, Leporacci NM, Macartney KJ, Shea AG, Crane RE, Hall ER, Ritchie KB (2017). Low pH reduces the virulence of black band disease on *Orbicella faveolata*. *PLoS ONE* 12(6): e0178869; Muller EM, Fine M, Ritchie K (2016). The resilient microbiome of inter and sub-tidal anemone species under increasing pCO^2. ***Sci. Rep.*** 6, 37387; doi: 10.1038/srep37387; Muller EM, van Woesik R (2014) Genetic susceptibility, colony size, and water temperature drive white-pox disease on the coral *Acropora palmata*. *PLoS ONE* 9(11): e110759. doi:10.1371/journal.pone.0110759; Muller EM, van Woesik R. (2012) Caribbean coral diseases: primary transmission or secondary infection? *Global Change Biology* 18:3529-3535

Noretta Perry is a Biological Scientist II with the FWH group at FWC/FWRI where she has been employed since 1987. She received her B.S. in Biology (1983) and M.S. in Zoology (1986) from the University of South Florida, and currently supervises the Histology lab at FWRI, St. Petersburg. Relevant papers: Kiryu Y, Landsberg JH, Peters EC, Tichenor E, Burleson C, Perry N. 2015. Pathological effects of cyanobacteria on sea fans in southeast Florida. ***J. Invert. Pathol***. 129:13–27; Landsberg, J. H., Vermeer, G. K., Richards, S. A. and Perry, N. 1991. Control of the parasitic copepod *Caligus elongatus* on pond-reared red drum. ***J. Aquat. Animal Health***. 3:206-209.

Dr. Esther C. Peters (Co-Principal Investigator) is a Term Associate Professor in the Department of Environmental Science and Policy at GMU, a position she has held since August 2008. She started her academic career as an adjunct professor at GMU in 1999 while also working in environmental consulting at Tetra Tech, Inc., and she is also an Adjunct Scientist with MML and an Adjunct Professor at Nova Southeastern University (NSU, Oceanographic Center). At GMU Dr. Peters teaches histology and histotechniques, is director of the Histology Laboratory, and supervises graduate and undergraduate students in diverse research projects; she is studying cell and tissue alterations as a bridge to understanding the molecular and microbiological aspects of disease processes on populations, communities, and ecosystems. She received a BS degree from Furman University in Biology, a MS degree in Marine Science from the University of South Florida, and PhD degree from the University of Rhode Island. She was a post-doctoral fellow and research associate at the National Museum of Natural History and then received a post-doctoral fellowship and later worked as the Invertebrate Pathologist in the Registry of Tumors in Lower Animals, funded by the National Cancer Institute. She has more than 35 years of experience in aquatic toxicology, pathobiology, project management, and quality assurance. Her expertise includes research on the effects of exposures to xenobiotics and other environmental stressors on a variety of invertebrates and fishes in both field and laboratory studies. She has performed extensive work on the comparative histopathology of invertebrates and fishes, particularly carcinogenesis, as well as the relationships between adverse environmental conditions and diseases caused by pathogens and parasites. She is an internationally recognized expert on coral reefs and diseases of coral reef organisms. She taught the 1-week course "Diseases of Corals and Other Reef Organisms" at MML's Tropical Research Laboratory for 14 summers, and has been teaching the graduate-level course, "Understanding Corals from the Inside Out: Coral Comparative Histopathology," each summer at NSU since 2013. She is a Fellow of the American Association for the Advancement of Science and a member of the International Society for Reef Studies and National Society for Histotechnology. She participates in the Coral Disease and Health Consortium, the Technical Advisory Committee of the Southeast Florida Coral Reef Initiative, and the Science Advisory Committee for the Coral Restoration Foundation. Dr. Peters is author or co-author of 11 book chapters, 36 peer-reviewed journal articles, 26 technical reports, 10 other papers, and 1 Web site. Relevant publications include: Mullen KM, Peters EC, Harvell CD. 2004. Coral resistance to disease. In *Coral Health and Disease*, ed. Rosenberg E, Loya Y, pp. 377-399. Springer-Verlag, Heidelberg, Germany; Vargas-Angel B, Peters EC, Kramarsky-Winter E, Gilliam DS, Dodge RE. 2007. Cellular reactions to sedimentation and temperature stress in the Caribbean coral *Montastraea cavernosa*. ***J. Invertebr. Pathol***. 95:140 145; Peters EC. 2013. Histological Examination of Coral Tissue Samples From St. Thomas, USVI, East End Reserve (STEER), Final Data Report. Submitted to S. Ian Hartwell, NOAA/National Status and Trends Program, Silver Spring, Maryland; Miller MW, Lohr KE, Cameron CM, Williams DE, Peters EC. 2014. Disease dynamics and potential mitigation among restored and wild staghorn coral, *Acropora cervicornis*. *PeerJ*

2:e541; Peters EC. 2015. Diseases of coral reef organisms. In *Coral Reefs in the Anthropocene*, ed. C. Birkeland, pp. 147-178. Springer Science+Business Media, Dordrecht, The Netherlands.

Rob Ruzicka (Co-Principal Investigator) is the Research Administrator for the FWC-FWRI Coral Reef Research group and has been the Principal Investigator for the Coral Reef Evaluation and Monitoring Program (CREMP) since joining FWRI in 2008. His responsibilities are both administrative and scientific and include the management of coral reef research grants and contracts, preparation of annual budgets and reports, and planning and coordination of monitoring and research activities. Mr. Ruzicka holds a B.S. in Biology from Hanover College and earned an M.S. in Biology from the University of Georgia Southern. He currently serves as a member of the Technical Advisory Committee for the Florida Keys National Marine Sanctuary and biological expert for the Gulf of Mexico Fisheries Management Council Coral Scientific and Statistical Committee. Mr. Ruzicka has been the first author or coauthor on seven manuscripts published in peer-reviewed journals such as *Plos One, Coral Reefs,* and *Marine Ecology Progress Series*. Prior to joining FWRI, Mr. Ruzicka worked for the Florida Department of Environmental Protection coral program as the Fishing and Diving Project Coordinator

Patrick Wilson is a Biological Scientist II with the Fish and Wildlife Health group at FWC/FWRI. He has been employed there since 2007. He graduated with a B.S. in Biology and Psychology in 1992 from Winona State University, ASCP certifications include an MB, HTL, and a QIHC qualification. Currently he works in the Histology lab at FWRI, St. Petersburg and at the Anatomic Pathology laboratory at Quest Diagnostics in Tampa. Patrick has extensive experience in immunohistochemistry and adaptation of special stains to aquatic animal tissues.

Budget Narrative:

This research project will be conducted in the laboratory and field over a three-year period. To achieve the three objectives we require a multi-investigator, multi-institutional approach with experts in coral biology, coral health, aquatic animal disease and histopathology, histochemistry and molecular techniques, microbiology, molecular biology, disease diagnostics, and investigative disease response. PI Landsberg will coordinate the project, liaise with co-project investigators, coordinate meetings and reports, assist with coral sample diagnostics, slide reading and interpretation in consultation with Peters and Kiryu, and will oversee research staff Kiryu, Perry, Wilson, and Clark. Co-PI Kiryu will coordinate coral sample macroprocessing for histology, will read slides for histopathology with Peters and Landsberg, and will assist with TEM interpretation at FWRI St. Petersburg. FWC biologists Perry and Wilson will process coral tissues for histology, and FWC biologist Gray will process coral samples for TEM at FWRI St. Petersburg. Co-PIs Ruzicka and Huebner will coordinate field trips, coral sampling logistics and management, and transport of specimens to FWRI St. Petersburg. Co-PI Muller based at MML Sarasota will participate in coral sample collections for transmission experiments, will conduct diagnostic molecular and microbiological analyses and interpretation, will conduct transmission experiments (with a TBA technician), and will compile reports. Co-PI Peters based at GMU will conduct FISH and LCM and will evaluate slides for histopathology in consultation with Landsberg and Kiryu (see additional details below under contracts). All co-PIs will provide consultation input on all aspects of the project. A total of $305,000 is being requested (FWC = $93,573.74, MML = $136,503, and GMU = $74,923.26).

Match: Is rated at ~35% non-federal match of the total project cost. Salary match for Landsberg (FWC research scientist) is calculated at 16 weeks salary plus 26.16% fringe, and for Kiryu (FWC associate research scientist) at 16 weeks salary plus 28.82% fringe.

	Commission Division	State Fund	State Category	Amount of Match
FY 2018/2019	FWRI	MRCTF	010000	$54,116.27
FY 2019/2020	FWRI	MRCTF	010000	$54,116.27
FY 2020/2021	FWRI	MRCTF	010000	$54,116.27

Salaries and wages: Salary is requested for two part time OPS positions (with 42.0% fringe benefits): (1) to cover part-time OPS salary for research staffer Clark Gray based at FWRI St. Petersburg to conduct sample analyses for TEM, to provide diagnostic support and interpretation of TEM samples, to compile photographic images and reports, to conduct data collection and entry, and to provide assistance to project staff on interpretation of TEM imagery (rate at $ 20.00/hr, total 3-year request = $7,100); (2) to cover part time salary for OPS research staffer Patrick Wilson based at FWRI St. Petersburg to utilize special stains for histochemistry to be used in the interpretation of potential pathogens, to provide slides for FISH diagnostics, and to ship histological samples to external diagnostic facilities (at GMU), collect and enter data, assist with purchasing (rate at $15.00/hr, total 3-year request = $10,224). Salary (total = $11,644) is also requested for four OPS biological scientist positions (TBD) in FWRI CREMP (rate at $20.00/hr) for each of 80 hours, and a projected 15 hours of overtime each (rate at $30.00/hr) to conduct coral sampling.

Fringe benefits: These are calculated at 42% for FWC OPS staff.

Equipment: Equipment is not requested. FWC/FWRI in St. Petersburg has appropriate facilities to conduct coral tissue preparations, obtain and analyze diagnostic samples for histopathology, microbiology, electron microscopy; and equipment to hold and archive tissues. Equipment includes Biosafety cabinets, analytical and top loading balances, five refrigerators, four -20ºC freezers, two walk-in -20ºC freezers, five -80ºC freezers, desk top and high speed floor model refrigerated centrifuges; multiple photomicroscopes with epifluorescence, Polaroid capabilities, and digital camera systems; a Jeol JEM-1400 Transmission

Electron Microscope; sterile isolation hoods; incubators for microbiological cultures; multiple shaker/incubators, benchtop orbital shakers, autoclaves, laminar flow hoods; necropsy facilities; dissection microscopes; state-of-the-art histology facilities for both paraffin and plastic tissue processing (embedding, sectioning, and staining), microtomes, automated slide stainer, use of routine histological stains (e.g., hematoxylin and eosin [H&E], thionin) and development of special stains and immunohisto-chemistry; laboratory bench space, and field equipment, including boats, scuba, and gear for the collection, fixation, refrigeration and shipment of coral specimens.

The GMU Histology Laboratory is available for the preparation of coral tissue samples for histopathological examination by LM. The Tissue Processing lab has a large fume hood for handling fixed tissue samples, automated tissue processor, embedding center, computer and monitor, dissecting and brightfield compound microscopes, and sample (wet), paraffin block, and histoslide storage areas. The Slide Preparation lab, hash two microtome work stations, water baths, slide warmers, lab oven and vacuum oven, smaller fume hood and counter space for manually staining the tissue sections, chemical storage, and flammables cabinet. Additional microscopes with cameras and laser capture microdissection, and DNA sample preparation equipment are located elsewhere on the GMU campus.

At MML Sarasota, FL, in addition to office space, there is a fully equipped molecular laboratory with benchspace, laminar flow hood, vortex, microcentrifuges, pipettes, gel electrophoresis equipment, gel documentation equipment, and two thermocyclers for PCR. The Elizabeth Moore IC2R3, Summerland Key, FL is a state-of-the-art 26,000 ft^2 research facility with wet and dry lab space, lodging space, and is Gold LEED and a category 5 hurricane resistant structure. The outdoor wet lab space allows for control and monitoring of temperature (and other seawater parameters), as well as physiological analyses of corals and other organisms in flow-through raceway tables to large mesocosm systems. The dry lab space is equipped with refrigerator (4 ^oC), freezers (-20^oC and -80^oC), low temperature oven, incubator, vortex, stir plates, homogenizer, pH meters and controllers, heat blocks, peristaltic pumps, heating/cooling circulators, water baths, vacuum pumps, table-top centrifuge, refrigerated centrifuge, fume hood, loop sterilizer, tube shaker and rocker, transformer, filtration apparatus, compound fluorescent microscope with digital camera, stereo microscope, balance, PC computers, digital camera, light/depth/O2 meter, pH meter, multiplate reader, Nanodrop 2000 Spectrophotometer, 2 Mastercycle Pro Thermal Cyclers and a Real-Time PCR System (Roche LightCycler 480) as well as smaller electrophoresis and gel documentation equipment. IC2R3 also houses an expansive wet laboratory that includes aquaria of various sizes, plus outdoor and indoor open seawater raceways and wet tables. Flow-through raceways are available for experimentation; water supply is optional between nearshore inflow system and marine well water. This proposal will utilize the nearshore inflow system to ensure chemical similarities of water used in the experiments with reef water. Research vessels with SCUBA capabilities are available at Mote IC2R3.

Expense: Expense is requested to cover costs for conducting diagnostics and experimental transmission studies, for histopathology, molecular analyses, replenishing basic field and laboratory supplies, aquaria set up and maintenance, and for shipment of coral samples and slides. Expenses for various field and sample collection supplies (total $6,834.19) include Z-fixative, Trump's fixative, bleach, sharpies, sterile sample jars, whirl packs, ziplock bags, nitrile gloves, underwater paper, clipboards, tank air fills, and SCUBA supplies. Expenses for histology include various supplies and reagents for processing tissue samples including EDTA for decalcification, alcohols, xylenes, paraffin wax, and special stains. Expenses for molecular analyses include DNA extraction kits, consumables, and sequencing ($74.50 per sample). We anticipate several quarterly shipments of samples and are covering costs for shipping and for the purchase of slide boxes. Costs are estimated at $10.00/slide for routine stains and $12.00/slide for special stains, producing extra slides for consultation by Dr. Peters, and for slides for FISH and LCM. Costs are $10/sample for TEM.

Travel: Travel costs in the amount of $7,648 are requested for FWC-FWRI for the field collection of coral samples for histology, TEM, molecular analyses and other diagnostics. Sampling will be conducted by staff of the FWC-FWRI CREMP program. Travel costs cover vehicle transportation (estimated at $300),

lodging ($100/day), meals ($36/day), per diem ($80/day) and boat fuel ($50/day x 10 days) for 4 personnel for one 12-day trip (allowing for inclement weather and other potential delays). Routine phone and teleconference calls between PI's will be conducted to minimize costs required for meeting.

Consultant Services/Sub-Contracts: Mote Marine Laboratory (MML - $136,503). Tasks to be conducted at MML will address obj. 2 (Conduct broad systematic analyses [molecular, histology, TEM] for other potential pathogens across multiple affected coral species, plus apparently healthy reference samples); and obj. 3 (Conduct transmission experiments to determine where the signature of the bacterial community changes from a disease community to a healthy community within a diseased coral). Dr. Muller (salary requested at $37.10 per hour for Yrs. 1-3, fringe benefits at 35.75%) and a technician (TBA, salary requested at $15.0 per hour for Yrs. 1 and 2, fringe benefits at 35.75%) will be responsible for processing samples for molecular 16S quantification of the bacterial community, overseeing sequencing assays, and conducting statistical analyses and interpretation of molecular data. Dr. Muller will be responsible for conducting the transmission experiment at the MML IC2R3 on Summerland Key, FL. Here, she and a technician will conduct field work for sample and coral collections, set up and conduct the experiment to test for transmission of disease using homogenates. Disease infection rates, progression rates, and samples for molecular and histological work will be collected and analyzed. Dr. Muller will also conduct all data analysis, compilation, and report writing that is related to her scope of work. Travel costs to cover vehicle transportation (estimated at $3,320), lodging ($30/day) and per diem ($30/day) for two personnel for up to 21 days are requested for MML for field sampling to collect coral samples for the transmission experiments and parallel samples for histology and other diagnostics. Their budget will also support boat time for coral sampling in Yr 1 for 3 trips (estimated at $1000 x 3), bench fees in Yr 1 for three trips of up to 21 days for the transmission experiments at IC2R3 (estimated at $1,920), lab supplies (estimated at $9,200), and sequencing costs (estimated at $67,497). Boat rental fees include costs of boat time, fuel, and captain time and expertise.

George Mason University (GMU - $74,923.26). The tasks to be conducted by GMU will address obj. 1 (Identify and characterize putative RLOs/CLOs and other putative bacterial pathogens from *Acropora* spp. and from target coral species affected by the current disease); obj. 2 (Determine potential pathogens in at least three of those affected coral species compared to healthy controls); and Obj. 3 (Conduct transmission experiments to determine where the signature of the bacterial community changes from a disease community to a healthy community within a diseased coral). Dr. Peters ($26,081.83 over years 1−3) will examine selected histoslides of apparently healthy and diseased coral samples using LM, conferring with Landsberg and Kiryu on these and the results of TEM performed at FWRI. She will oversee the work of one graduate student during Year 1, who will perform FISH on up to 50 selected samples (3 slides each) from 2016–2017 samples and new samples with EUB-1, EUB-II, and nonsense probes to determine bacterial categories with fluorescence microscopy and compare results with H&E and Giemsa-stained sections with LM (report prepared for master's project). She will oversee the work of another graduate student who will perform LCM on up to 25 selected samples (*Acropora* and 3 or more other species of corals) to target specific categories of suspect bacterial pathogens (based on cell type or tissue infected, morphology and size of bacterial cells in microcolonies or clusters), then extract the bacterial DNA, perform PCR, clean up, etc., and send the extracts to MML for sequencing (as part of the student's master's thesis; $10,233.60 for the graduate students over years 1−2). Dr. Aguirre will provide general oversight and facilitation of research and reporting for this project ($6,226.19 over years 1−3). $6,750 is also requested to purchase materials and supplies for the analyses above.

Indirect Costs: For budgeting purposes, FWC uses an estimated indirect cost on salaries and wages of 15%. If approved, FWC will only bill the actual NICRA for each fiscal year. The negotiated IDC for MML and GMU respectively is 50% of salaries and wages and 52% MTDC respectively (see attached NICRA information).

Literature Cited:

Aeby GS, Work TM, Runyon CM, Shore-Maggio A, Ushijima B, Videau P, Beurmann S, Callahan SM (2015) First record of black band disease in the Hawaiian archipelago: response, outbreak status, virulence, and a method of treatment. *PloS One* 10:e0120853

Ainsworth TD, Fine M, Blackall LL, Hoegh-Guldberg O (2006) Fluorescence in situ hybridization and spectral imaging of coral-associated bacterial communities. *Appl Environ Microbiol* 72:3016−3020

Ainsworth TD, Krause L, Bridge T, Torda G, Raina J-B, Zakrzewski M, Gates RD, Padilla-Gamiño JL, Spalding HL, Smith C (2015) The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. *ISME J* 9:2261−2274

Ainsworth TD, Thurber RV, Gates RD (2010) The future of coral reefs: a microbial perspective. *Trends Ecol Evol* 25:233−240

Aronson RB, Precht WF (2001) White-band disease and the changing face of Caribbean coral reefs. *Hydrobiologia* 460:25−38

Bentis CJ, Kaufman L, Golubic S (2000) Endolithic fungi in reef-building corals (Order: Scleractinia) are common, cosmopolitan, and potentially pathogenic. *Biol Bull* 198:254−260

Bourne DG, Garren M, Work TM, Rosenberg E, Smith GW, Harvell CD (2009) Microbial disease and the coral holobiont. *Trends Microbiol* 17:554−562

Bruckner AW (2016) White syndromes of western Atlantic reef-building corals. In: Woodley CM, Downs CA, Bruckner AW, Porter JW, Galloway SB (eds) *Diseases of Coral*. John Wiley & Sons, Inc, p 316−332

Cardenas A, Rodriguez RL, Pizarro V, Cadavid LF, Arevalo-Ferro C (2012) Shifts in bacterial communities of two caribbean reef-building coral species affected by white plague disease. *ISME J* 6:502−512

Carpenter KE, Abrar M, Aeby G, Aronson RB, Banks S, Bruckner A, Chiriboga A, Cortes J, Delbeek JC, Devantier L, Edgar GJ, Edwards AJ, Fenner D, Guzman HM, Hoeksema BW, Hodgson G, Johan O, Licuanan WY, Livingstone SR, Lovell ER, Moore JA, Obura DO, Ochavillo D, Polidoro BA, Precht WF, Quibilan MC, Reboton C, Richards ZT, Rogers AD, Sanciangco J, Sheppard A, Sheppard C, Smith J, Stuart S, Turak E, Veron JE, Wallace C, Weil E, Wood E (2008) One-third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* 321:560−563

Casas V, Kline DI, Wegley L, Yu Y, Breitbart M, Rohwer F (2004) Widespread association of a *Rickettsiales*-like bacterium with reef-building corals. *Environ Microbiol* 6:1137−1148

Corsaro D, Venditti D (2004) Emerging chlamydial infections. *Crit Rev Microbiol* 30:75−106

Cróquer A, Bastidas C, Lipscomb D, Rodríguez-Martínez R, Jordan-Dahlgren E, Guzmán HM (2006) First report of folliculinid ciliates affecting Caribbean scleractinian corals. *Coral Reefs* 25:187−191

Crosson LM, Wight N, Van Blaricom GR, Kiryu I, Moore JD, Friedman CS (2014) Abalone withering syndrome: distribution, impacts, current diagnostic methods and new findings. *Dis Aquat Org* 108:261−270

de Bakker DM, van Duyl FC, Bak RPM, Nugues MM, Nieuwland G, Meesters EH (2017) 40 years of benthic community change on the Caribbean reefs of Curaçao and Bonaire: the rise of slimy cyanobacterial mats. *Coral Reefs*:1-13

Eakin CM, Morgan JA, Heron SF, Smith TB, Liu G, Alvarez-Filip L, Baca B, Bartels E, Bastidas C, Bouchon C, Brandt M, Bruckner AW, Bunkley-Williams L, Cameron A, Causey BD, Chiappone M, Christensen TR, Crabbe MJ, Day O, de la Guardia E, Diaz-Pulido G, DiResta D, Gil-Agudelo DL, Gilliam DS, Ginsburg RN, Gore S, Guzman HM, Hendee JC, Hernandez-Delgado EA, Husain E, Jeffrey CF, Jones RJ, Jordan-Dahlgren E, Kaufman LS, Kline DI, Kramer PA, Lang JC, Lirman D, Mallela J, Manfrino C, Marechal JP, Marks K, Mihaly J, Miller WJ, Mueller EM, Muller EM, Orozco Toro CA, Oxenford HA, Ponce-Taylor D, Quinn N, Ritchie KB, Rodriguez S, Ramirez AR, Romano S, Samhouri JF, Sanchez JA, Schmahl GP, Shank BV, Skirving WJ, Steiner SC, Villamizar E, Walsh SM, Walter C, Weil E, Williams EH, Roberson KW, Yusuf Y (2010)

Caribbean corals in crisis: record thermal stress, bleaching, and mortality in 2005. *PloS One* 5:e13969

Fine M, Roff G, Ainsworth TD, Hoegh-Guldberg O (2006) Phototrophic microendoliths bloom during coral "white syndrome." *Coral Reefs* 25:577−581

Gollas-Galván T, Avila-Villa LA, Martínez-Porchas M, Hernandez-Lopez J (2014) Rickettsia-like organisms from cultured aquatic organisms, with emphasis on necrotizing hepatopancreatitis bacterium affecting penaeid shrimp: an overview on an emergent concern. *Rev Aquacult* 6:256−269

Kuta KG, Richardson LL (1996) Abundance and distribution of black band disease on coral reefs in the northern Florida Keys. *Coral Reefs* 15:219−223

Manzello DP (2015) Rapid recent warming of coral reefs in the Florida Keys. *Scientific reports* 5:16762

Marcelino VR, Morrow KM, van Oppen MJH, Bourne DG, Verbruggen H (2017) Diversity and stability of coral endolithic microbial communities at a naturally high pCO2 reef. *Mol Ecol* 2017: 1−14

Miller MW, Karazsia J, Groves CE, Griffin S, Moore T, Wilber P, Gregg K (2016) Detecting sedimentation impacts to coral reefs resulting from dredging the Port of Miami, Florida USA. *PeerJ* 4:e2711

Miller MW, Lohr KE, Cameron CM, Williams DE, Peters EC (2014) Disease dynamics and potential mitigation among restored and wild staghorn coral, *Acropora cervicornis*. *PeerJ* 2:e541

Muller EM, Rogers CS, Spitzack AS, van Woesik R (2008) Bleaching increases likelihood of disease on *Acropora palmata* (Lamarck) in Hawksnest Bay, St John, US Virgin Islands. *Coral Reefs* 27:191−195

Muller EM, van Woesik R (2012) Caribbean coral diseases: primary transmission or secondary infection? *Global Change Biol* 18:3529−3535

Neal BP, Khen A, Treibitz T, Beijbom O, O'Connor GT, Coffroth MA, Knowlton N, Kriegman D, Mitchell BG, Kline DI (2017) Caribbean massive corals not recovering from repeated thermal stress events during 2005-2013. *Ecol Evol* 7:1339−1353

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GI, Solymo P, Henry M, Stevens H, Szoecs E, Wagner H (2017). vegan: *Community Ecology Package*. R package version 2.4-4. https://CRAN.R-project.org/package=vegan

Patterson KL, Porter JW, Ritchie KB, Polson SW, Mueller E, Peters EC, Santavy DL, Smith GW (2002) The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, *Acropora palmata*. *Proc Natl Acad Sci USA* 99:8725−8730

Peters EC (1984) A survey of cellular reactions to environmental stress and disease in Caribbean scleractinian corals. *Helgol Meeresunters* 37:113−137

Peters EC (2015) Diseases of Coral Reef Organisms. In: Birkeland C (ed) *Coral Reefs in the Anthropocene*. Springer Netherlands, Dordrecht, p 147−178

Pollock FJ, Lamb JB, Field SN, Heron SF, Schaffelke B, Shedrawi G, Bourne DG, Willis BL (2014a) Sediment and turbidity associated with offshore dredging increase coral disease prevalence on nearby reefs. *PloS One* 9:e102498

Pollock FJ, Naohisa W, Torda G, Willis BL, Bourne DG (2017) White syndrome-affected corals have a distinct microbiome at disease lesion fronts. *Appl Environ Microbiol* 83:1−15

Pollock FJ, Wood-Charlson EM, van Oppen MJH, Bourne DG, Willis BL, Weynberg KD (2014b) Abundance and morphology of virus-like particles associated with the coral *Acropora hyacinthus* differ between healthy and white syndrome-infected states. *Mar Ecol Prog Ser* 510:39−43

Precht WF, Gintert BE, Robbart ML, Fura R, van Woesik R (2016) Unprecedented disease-related coral mortality in Southeastern Florida. *Scientific reports* 6:31374

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Richardson LL, Goldberg WM, Carlton RG, Halas JC (1998) Coral disease outbreak in the Florida Keys: Plague Type II. *Rev Biol Trop* 46:187−198

Roder C, Arif C, Daniels C, Weil E, Voolstra CR (2014) Bacterial profiling of white plague disease across corals and oceans indicates a conserved and distinct disease microbiome. *Mol Ecol* 23:965−974

Rosenberg E, Kushmaro A (2011) Microbial diseases of corals: pathology and ecology. In: Dubinsky Z, Stambler N (eds) *Coral Reefs: An Ecosystem in Transition*. Springer, Netherlands, p 451−464

Sokolow S (2009) Effects of a changing climate on the dynamics of coral infectious disease: a review of the evidence. *Dis Aquat Org* 87:5−18

Sunagawa S, DeSantis TZ, Piceno YM, Brodie EL, DeSalvo MK, Voolstra CR, Weil E, Andersen GL, Medina M (2009) Bacterial diversity and white plague disease-associated community changes in the Caribbean coral *Montastraea faveolata*. *ISME J* 3:512−521

Sussman M, Willis BL, Victor S, Bourne DG (2008) Coral pathogens identified for White Syndrome (WS) epizootics in the Indo-Pacific. *PloS One* 3:e2393

Sutherland KP, Berry B, Park A, Kemp DW, Kemp KM, Lipp EK, Porter JW (2016) Shifting white pox aetiologies affecting *Acropora palmata* in the Florida Keys, 1994–2014. *Phil Trans Roy Soc B: Biol Sci* 371: 20150205

Sweet M, Bythell J (2017) The role of viruses in coral health and disease. *J Invertebr Pathol* 147:136−144

Sweet MJ, Croquer A, Bythell JC (2011) Bacterial assemblages differ between compartments within the coral holobiont. *Coral Reefs* 30:39−52

Ushijima B, Videau P, Burger AH, Shore-Maggio A, Runyon CM, Sudek M, Aeby GS, Callahan SM (2014) *Vibrio coralliilyticus* strain OCN008 is an etiological agent of acute *Montipora* white syndrome. *Appl Environ Microbiol* 80:2102−2109

Vega Thurber R, Willner-Hall D, Rodriguez-Mueller B, Desnues C, Edwards RA, Angly F, Dinsdale E, Kelly L, Rohwer F (2009) Metagenomic analysis of stressed coral holobionts. *Environ Microbiol* 11:2148−2163

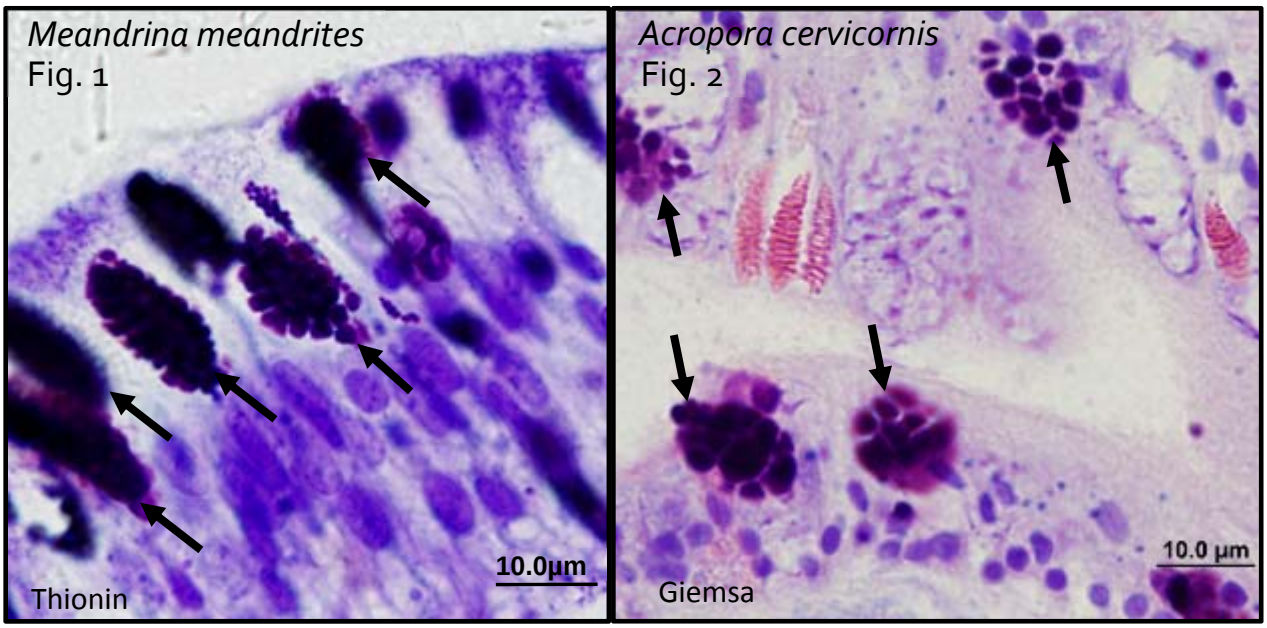
Verde A, Bastidas C, Croquer A (2016) Tissue mortality by Caribbean ciliate infection and white band disease in three reef-building coral species. *PeerJ* 4:e2196

Wada N, Pollock FJ, Willis BL, Ainsworth T, Mano N, Bourne DG (2016) In situ visualization of bacterial populations in coral tissues: pitfalls and solutions. *PeerJ* 4:e2424

Williams DE, Miller MW (2012) Attributing mortality among drivers of population decline in *Acropora palmata* in the Florida Keys (USA). *Coral Reefs* 31:369−382

Work TM, Richardson LL, Reynolds TL, Willis BL (2008) Biomedical and veterinary science can increase our understanding of coral disease. *J Exp Mar Biol Ecol* 362:63-70

Appendix Figures:



Figs. 1 & 2. Histological sections showing putative RLOs/CLOs (arrows) infecting mucocytes of the epidermis of *Meandrina meandrites* and *A. cervicornis* in diseased corals from the FRT (years 2015 and 2003 respectively).

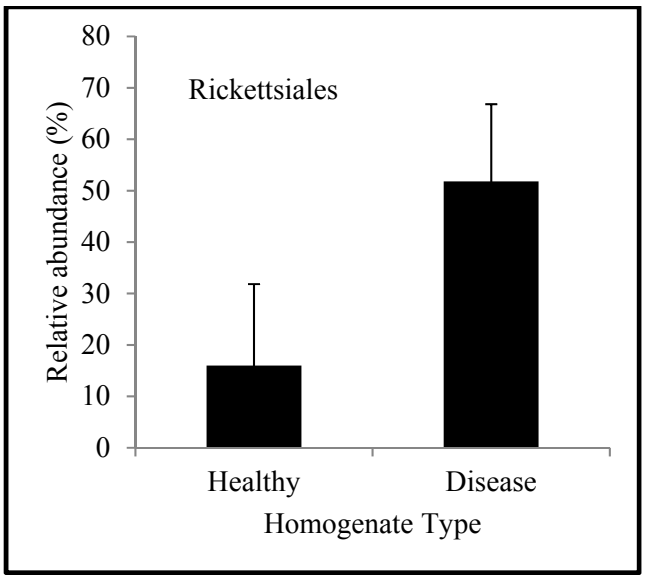
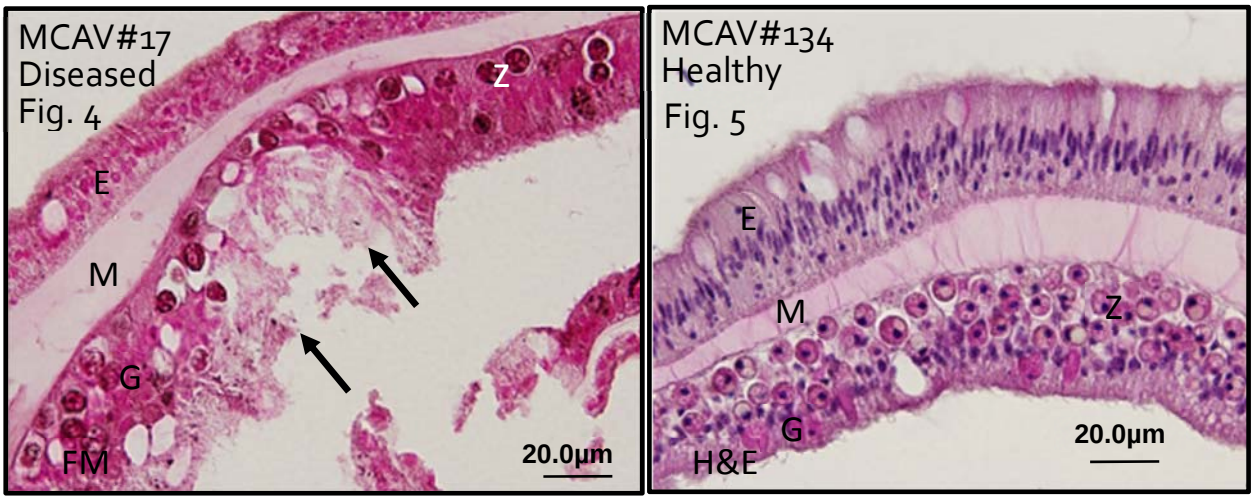


Fig. 3. The average relative abundance of bacteria within the order Rickettsiales from healthy and disease tissue homogenates. All samples are included within the graph (n = 4 per treatment). Error bars represent standard error of the mean.



Figs. 4 & 5. Histological comparison of diseased and healthy surface body wall (comprised of three layers, E = epidermis, M = mesoglea, G = gastrodermis) of *M. cavernosa* (2016) showing gastrodermal lesion (arrows) and loss of zooxanthellae (Z). FM = Fontana Masson stain, H&E = hematoxylin & eosin stain.

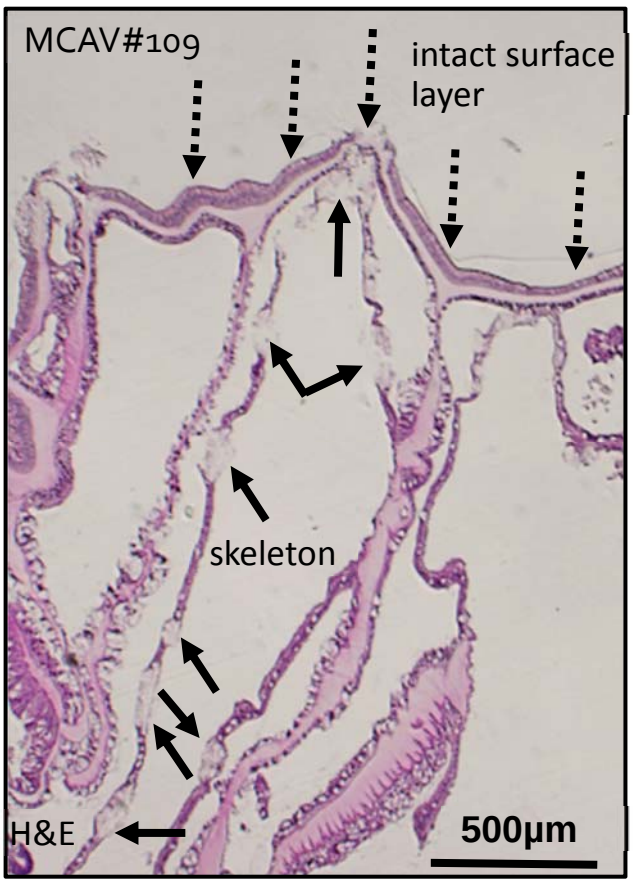


Fig. 6. Histological section of *M. cavernosa* showing deeper lesions in the basal body wall adjacent to the skeleton (closed arrows) compared to healthy surface (dashed arrows).

Fig. 7. Field macrophotograph of diseased *M. cavernosa* showing a white band of tissue loss and exposed skeleton (bottom, closed arrows) and a white blotch area of active early disease (middle, dashed arrows).

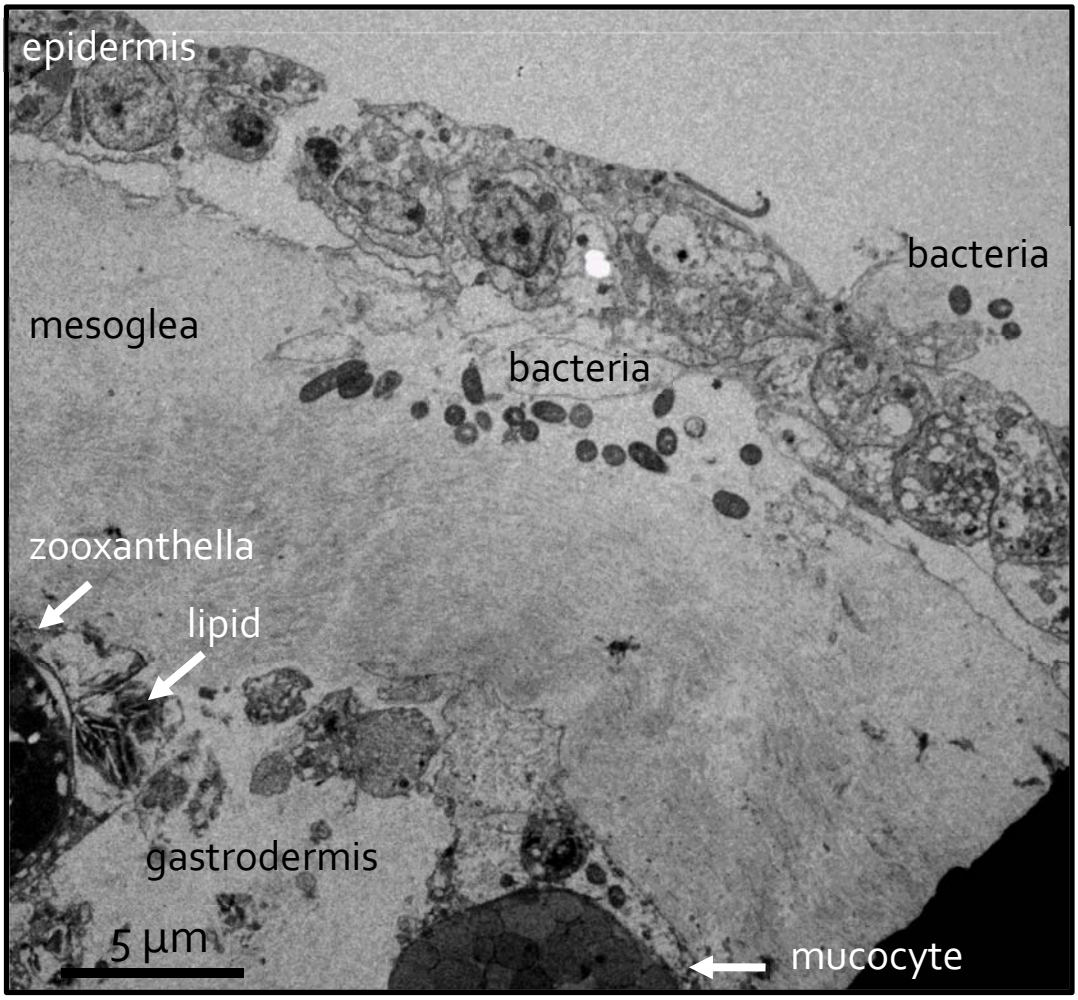


Fig. 8. TEM micrograph of *M. cavernosa* showing bacterial aggregates and presumptive associated tissue damage in the body wall between the epidermal and mesogleal layers.

Fig. 9. Macrophotograph of *M. cavernosa* polyps showing tissue loss and bare skeleton (right polyp) colonized by ciliates, *Halofolliculina* (black arrows).

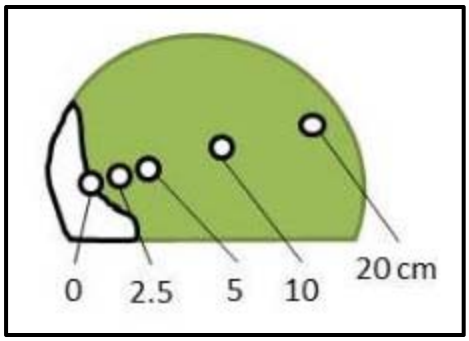


Fig. 10. Image showing sample collection scheme of transect from the progressing disease front until 20 cm into apparently healthy tissue. The image shows a massive coral colony that is infected with a white-plague like disease (white area) progressing into the apparently healthy tissue (green area). The black circles represent the location of coral cores taken for determining the transition from a disease bacterial community to an apparently healthy bacterial community.

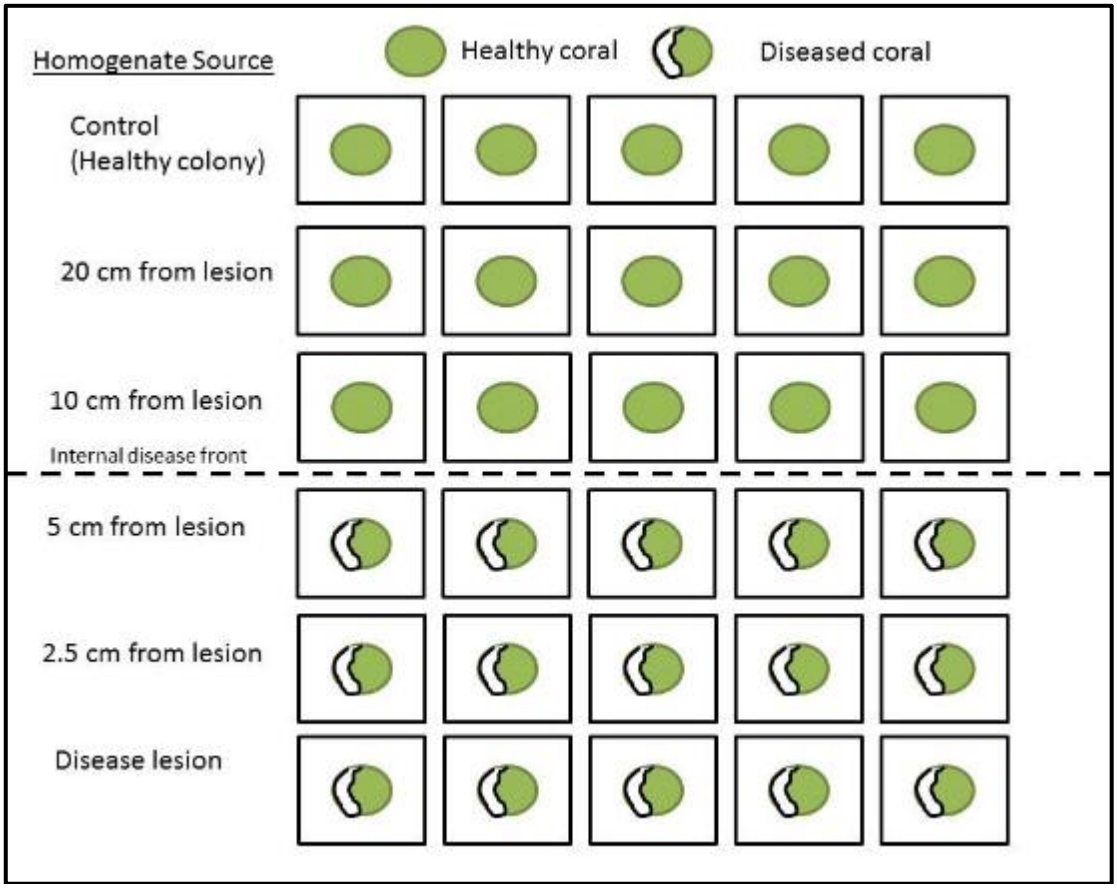


Fig. 11. Schematic diagram showing the experimental design for the transmission experiment. Five total homogenates will be made from a single diseased coral colony. Each homogenate will be created from samples taken along different distances from the progressing disease margin. An additional control sample will be taken from an apparently healthy colony of the same species. Those homogenates will then be used to inoculate contained tanks, each holding a single apparently healthy coral colony. The infectivity rate will help to determine the location where the diseased bacterial community transitions to a healthy bacterial community within an infected colony. Here, the results would suggest that the disease to healthy bacterial community happens between 5 and 10 cm away from the lesion.