Project Title: Postdoctoral research in Coral pathogen isolation

Principal Investigators:

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Background:

Florida's coral reefs are currently experiencing a multi-year disease-related mortality event that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of the Florida Reef Tract, and south into the Florida Keys. The best available information indicates that the disease outbreak is continuing to spread southwest through the Florida Keys.

An initial investigation into the transmission, infectiousness and differential host specificity of the disease outbreak was completed in 2017. Preliminary results have demonstrated that the currently investigated disease is transmissible from diseased *M. cavernosa* or *O. faveolata* fragments to healthy *M. cavernosa* or *O. faveolata* fragments, indicating the presence of an infectious agent. Additionally, disease progression can be slowed or halted by treatment with antibiotics, suggesting the infectious agent is bacterial. Additionally, multiple bacteria suspected to be pathogenic to coral were isolated from diseased samples from the transmission experiments during the 2017 study.

Project Goals and Objectives:

The purpose of this project is to isolate potentially pathogenic microorganisms and systematically determine if they can elicit disease signs in healthy corals. Identifying an etiological agent and its range of susceptible hosts is a major step for disease research, yet few coral pathogens have been identified to date. While the cause of the disease outbreak is currently unknown, isolating the potential pathogen(s) is a critical step to inform future management to remediate the disease outbreak.

The specific aims of this work include:

Task 1) Bacterial isolation and virulence screening

1. Testing of virulence of suspected bacterial pathogens isolated in 2017 that originate from diseased *M. cavernosa* from Broward county.
2. Screening for putative bacterial pathogens from diseased corals collected from the Keys.

Task 2) Controlled infection experiments with corals from Broward county and the Keys

The outcomes of this project will be incorporated into an on-going coral disease response effort that seeks to improve understanding about the scale and severity of the Florida Reef Tract

coral disease outbreak, identify primary and secondary causes, identify management actions to remediate disease impacts, restore affected resources and, ultimately, prevent future outbreaks.

Results:

Identification of suspected bacterial pathogens

From the preliminary experiments performed during Summer 2017, there were six bacterial isolates that displayed some level of virulence against *O. faveolata* and, to a much lesser extent, *M. cavernosa*. The six isolates, Mc-T4#15, Of-T6#17, Of-T6#21, Of-T7#21, Mc-T4#42, Mc-T4#56, were identified by sequencing their 16S rRNA gene and subsequently by whole-genome sequencing by Julie Meyer's laboratory at the University of Florida. The completes genomes of five of the six putative pathogens have been sequenced, with the remaining one to be tentatively completed.

The isolates identities are listed in Table 1. The isolates Of-T6#17, Of-T6#21 and Of-T7#21 are strains of *Vibrio coralliilyticus* which is supported by immunological assays developed by Claudia Häse's lab at OSU to detect this bacterium (data not shown). Strains of *V. coralliilyticus* are known coral pathogens that cause bleaching and/or tissue loss diseases in multiple Indo-Pacific coral species. The isolate Mc-T4#15 belongs to the genus *Alteromonas*. A species of *Alteromonas* has been shown to cause disease in *Acropora millepora* while a species from the closely-related genus, *Pseudoalteromonas*, has been demonstrated to cause disease in *Montipora capitata*. The isolate Mc-T4#42 and #56 are species of *Leisingera*, which is part of the Rhodobacteriaceae family that also contains the genera *Rhodobacter*, *Roseobacter*, *Roseovarius*, and *Phaeobacter*, which have been associated with or implicated in disease of a range of different marine organisms.

Table 1. The identities of the suspected coral pathogens isolated in 2017.

Isolate	Closest match	Genome sequenced?	Isolated from
Mc-T4#15	*Alteromonas* sp.	Yes	A *M. cavernosa* fragment that developed tissue loss after being in put into physical contact with a diseased *M. cavernosa* fragment collect from the Fort Lauderdale area.
Of-T6#17	*V. coralliilyticus*	Yes	A *O. faveolata* fragment with tissue loss after being in put into physical contact with a diseased *M. cavernosa* fragment collect from the Fort Lauderdale area.
Of-T6#21	*V. coralliilyticus*	Yes	From the same fragment as Of-T6 #17.
Of-T7#21	*V. coralliilyticus*	Yes	A different *O. faveolata* fragment put into contact with the same coral fragment as Of-T6#21
Mc-T4#42	*Leisingera* sp.	In progress	From the same diseased fragment as Mc-T4 #15.
Mc-T4#56	*Leisingera* sp.	Yes	From the same diseased fragment as Mc-T4 #15.

Testing isolates from 2017 for virulence against available corals

M. cavernosa are adept at sloughing bacterial cultures inoculated into the tank water

Infection experiments were carried out using a block design, in which for every block of treatments all the coral fragments originated from the same colony to control for intraspecific variation. Corals were treated with a negative bacterial control (a non-virulent *Pseudoalteromonas* sp. strain Mc-H1#7 from healthy *M. cavernosa*), or one of the putative pathogens Of-T6#21, Mc-T4#15, and Mc-T4#56. All bacterial strains were inoculated to a final concentration of approximately 10^8 CFU/ml of tank water by pipetting the culture directly onto the submerged fragment. None of the seven different *M. cavernosa* genotypes displayed any disease signs during the 15-day experiments. This was anticipated from field observations, the relatively low disease transmission rates in laboratory aquaria, and low infection rates during the 2017 experiments. Most of the infections occurring during the pathogens screens in 2017 were conducted with *O. faveolata*, which is comparatively more susceptible to this disease, but healthy specimens are less available. It was observed that when *M. cavernosa* were inoculated with the suspected pathogens, there appeared to be a rapid (within 30 min of inoculation) muco-ciliary response that seemed to agglutinate and slough the bacterial inoculums (Fig. 1). Anecdotally, this was not observed after inoculation with the control bacterium Mc-H1#7. This suggests that these putative pathogens may be unable to effectively colonize healthy *M. cavernosa* under laboratory conditions, which may be the reason for their relative resistance to this disease during transmission experiments. However, this demonstrates that using this standard inoculation protocol, *M. cavernosa* is not a suitable infection model for this work.

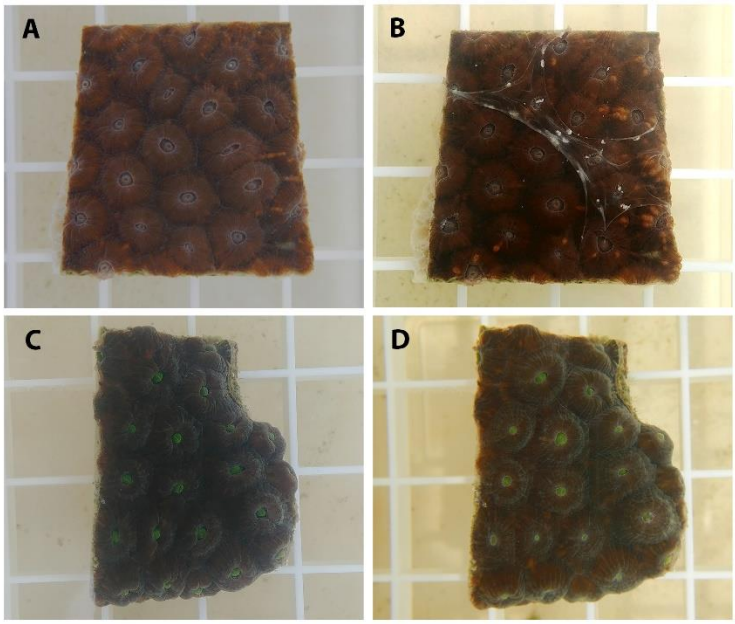

Figure 1. *Montastrea cavernosa* sloughing bacterial inoculums. A) *M. cavernosa* fragment before inoculation, and B) the same fragment 30 min post-inoculation with *V. coralliilyticus* strain OF-T6#21. C) Fragment before inoculation, and D) the same fragment post-inoculation with the control bacterium *Pseudoalteromonas* sp. Mc-H1#7. The squares in the grating represents 1 cm^2.

An injection of a mixture of the V. coralliilyticus and Leisingera sp. into the gastral cavity of M. cavernosa results in tissue lysis

Histological examinations of diseased *M. cavernosa* from the field by Jan Landsberg and colleagues revealed tissue damage within the inner layers of tissue that is believed to progress outwards towards the epidermis. This suggests that the etiological agent(s) for this disease may be entering the coral tissue and disseminating internally. Observations of disease progression in the laboratory support this hypothesis. Therefore, infection experiments with the suspected pathogens Of-T6#21, Mc-T4#15, and Mc-T4#56 were repeated with three genotypes of *M. cavernosa* except that a sterile 1cc syringe fitted with a 25G needle was used to inject approximately 10^8 CFU of each strain directly into the gastrovascular cavity of a single coral polyp in the middle of each fragment. It should be noted, that this was approximately 10^8 cells total and not the 10^8 CFU/ml concentration used in the previous experiments. As controls, autoclaved seawater or 10^8 CFU of the control bacterium Mc-H1#7 was injected in the same manner. An additional treatment was also included, a 1:1 mixture of Of-T6#21 and Mc-T4#56 (the final concentration of this treatment was adjusted to ~10^8 CFU).

At 18 h post-inoculation, one of the three genotypes injected with Of-T6#21 developed tissue loss (Fig. 2 A-C). Exposed skeletal processes could be observed protruding from the coenosarc tissue. Tissue loss progressed for the next three days before arresting, and the fragment started to regrow tissue over the exposed skeleton four days post-inoculation for the duration of the 14-day experiment. Two of the three genotypes injected with the mixture of Of-T6#21 and Mc-T4#56 developed acute tissue loss and progressed for the next three days before arresting four days post-inoculation (Fig. 2 D-F). Both fragments began to regrow tissue over the exposed coral skeleton five days post-inoculation.

A corresponding set of the same three *M. cavernosa* genotypes was pre-treated with a mixture of the broad-spectrum antibiotics kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml) for two days prior to injection to disrupt the native microflora. The coral microflora is hypothesized to protect their host from bacterial infections, especially for Pacific coral species against the pathogen *V. coralliilyticus* (Ushijima, unpublished data). However, these fragments did not appear to be any more susceptible to infection than the non-treated fragments. None of the fragments injected with Of-T6#21 or Mc-T4#56 individually developed tissue loss. Two of the three genotypes infected with the Of-T6#21 and Mc-T4#56 mixture developed tissue loss overnight, like the non-antibiotic treated fragments. Additionally, the lesions progressed for 2-3 days before starting to heal over starting on 3 days post-inoculation. In either experiment, none of the fragments injected with sterile seawater or the control bacterium Mc-H1#7 developed any disease signs during the 14-day experiment (Fig. 2 G-I). In all, this suggests that the microflora present on these coral fragments does not seem to influence susceptibility to these suspected pathogens.

These results demonstrate that for infection of *M. cavernosa* the suspected pathogens may need to enter the gastrovascular cavity of the coral for infection. This is consistent with

histological results that suggest that disease lesions begin with the inner tissue layers first. Furthermore, a synergistic effect may be occurring between the *V. coralliilyticus* and *Leisingera* sp. to cause disease. However, the *M. cavernosa* used seemed to be able to fight off the infection and eventually begin to heal over the lesions beginning in a matter of days. More replicates using additional genotypes of *M. cavernosa* are planned to confirm these results.

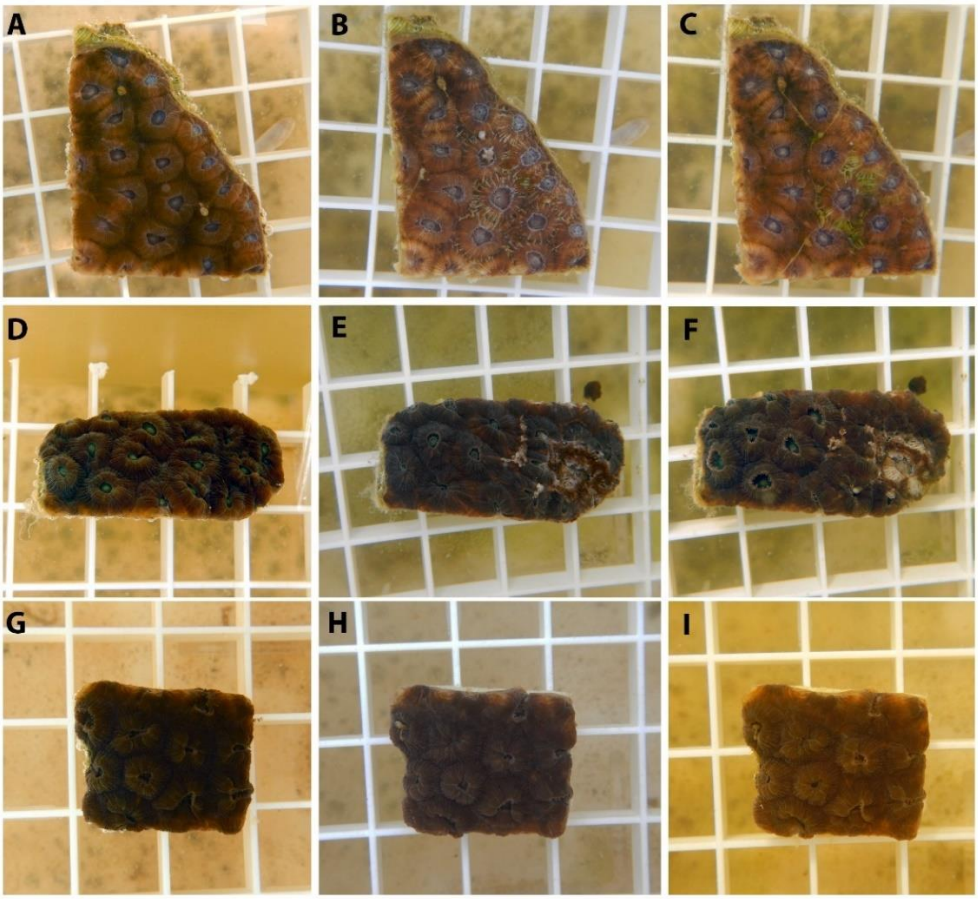

Figure 2. *Montastrea cavernosa* fragments with bacterial inoculums injected directly into gastrovascular cavity. A) A fragment before injection with *V. coralliilyticus* strain Of-T6#21, B) the same fragment approximately 18 h post-injection, and C) three days post-injection. D) A fragment before injection with a 1:1 mixture of Of-T6#21 and *Leisingera* sp. strain Mc-T4#56, E) the same fragment approximately 18 h post-injection, and F) three days post-injection. G) A control fragment pre-injection with *Pseudoalteromonas* sp. strain Mc-H1#7, H) 18 h post-injection, and I) three days post-injection. The squares in the grating represents 1 cm^2.

The isolates from 2017 may infect Meandrina meandrites

Three apparently healthy colonies of *M. meandrites* were collected from the Keys were utilized for infection experiments with the suspected pathogens *V. coralliilyticus* strain OF-T6#21, *Leisingera* sp. strain Mc-T4#56, or a 1:1 mixture of both these strains. Infection experiments where the bacterial cultures and inoculated into the tank water were set up as described above. One out of three *M. meandrites* fragments exposed to Mc-T4#56 started to

bleach 8 days post-exposure, which developed into tissue lysis 12 days post-exposure (Fig. 3 A,B,C). One out of three fragments exposed to Of-T6#21 developed tissue lysis 12 days post-exposure without any obvious signs of bleaching (Fig. 3 D,E,F). One out of three fragments exposed to the mixture of Of-T6#21 and Mc-T4#56 started bleaching with tissue loss 8 days post-inoculation. None of the fragments exposed to the control bacterium Mc-H1#7 displayed any obvious signs of disease. It is unclear if Mc-T4#56 always causes bleaching before tissue loss, if Of-T6#21 only causes tissue loss without bleaching, or if there are other variables affecting disease presentation. However, the existence of multiple pathogens could explain the slightly variable disease signs observed in the field, but more replication is needed.

Even with the small sample size, infection experiments with *M. meandrites* appear to be more feasible than those with *M. cavernosa*. Field observations suggest that *M. meandrites* may be one of the first susceptible coral species to become infected when this disease enters a new area, while *M. cavernosa* is one of the last remaining species in areas that have experienced an outbreak. Therefore, these results demonstrate that more *M. meandrites* specimens must be collected for continued work in verifying the virulence of suspected pathogens.

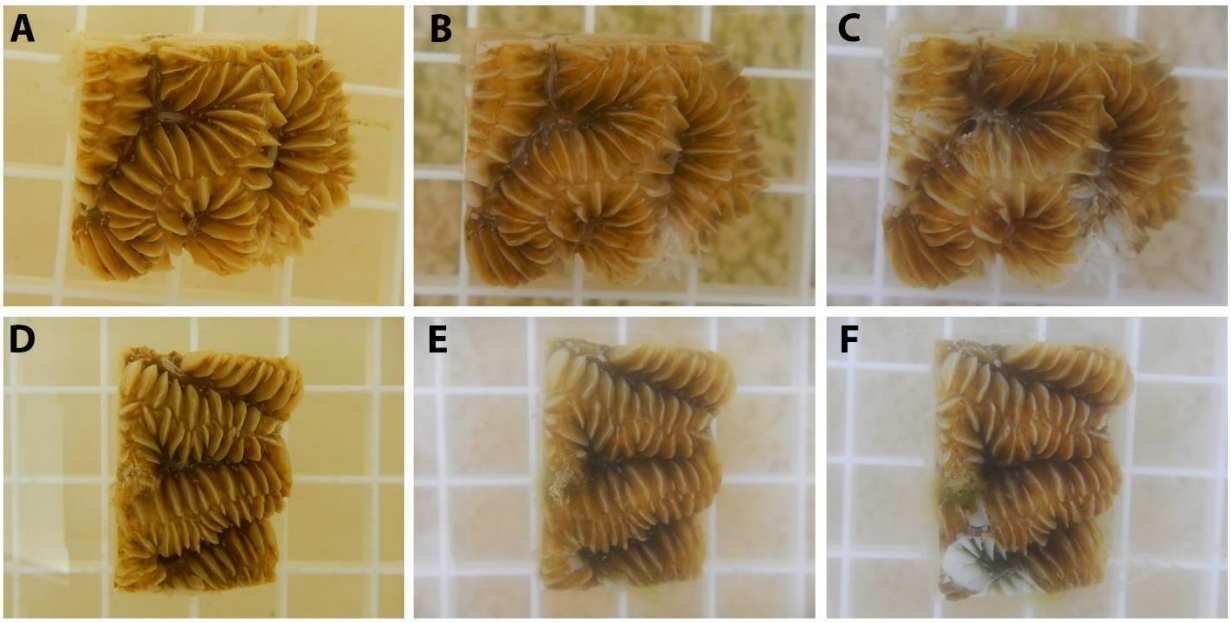

Figure 3. Healthy *Meandrina meandrites* fragments inoculated with the suspected pathogens Mc-T4#56 and Of-T6#21. A) A fragment pre-inoculation with Mc-T4-56, B) first obvious signs of bleaching eight days post-inoculation, and C) first signs of tissue loss 12 days post-inoculation. D) A fragment pre-inoculation with OF-T6#21, E) the first obvious signs of tissue loss 12 days post-inoculation, and F) the progression of tissue loss 15 days post-inoculation. The squares in the grating represents 1 cm^2.

Screening diseased *Meandrina meandrites* from the Keys for disease agents

Therapeutic diagnosis suggests infection is caused by a bacterial agent(s)

Multiple diseased *M. meandrites* colonies were collected from Looe Key for continued work to identify the etiological agent(s) responsible for this disease outbreak. However, first, therapeutic diagnosis using antibiotics was used to determine if bacterial agents are involved with disease progression. For each experimental block, a larger fragment with a disease lesion was cut up so that each smaller fragment had a portion of the lesion. As a control, one fragment was not treated with antibiotics, while the others were treated with a mixture of amoxicillin (50 µg/ml of tank water) and kanamycin (50 µg/ml of tank water).

All the *M. meandrites* colonies had controls with disease lesions that progressed throughout the 10-day experiment, while disease progression for the experimental fragments was completely halted by the amoxicillin/kanamycin treatment (Fig. 4). Cessation of lesion progression (n=3) suggests that the infectious agent(s) is bacterial, however, more trials are planned.

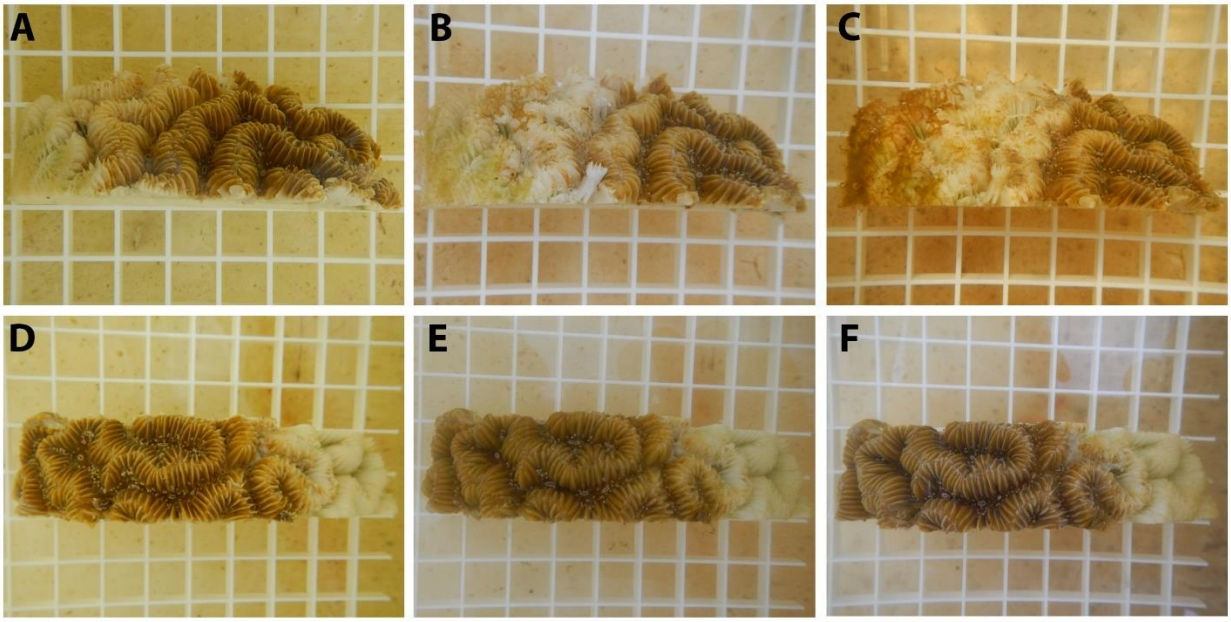

Figure 4. Therapeutic diagnosis with diseased *M. meandrites* from the Keys. A) the non-treated control at the start of the experiment, B) disease progression after 3 days, C) disease progression after 8 days. D) a corresponding fragment before treatment with amoxicillin and kanamycin, E) the same fragment after 3 days, F) after 8 days. The squares in the grating represents 1 cm^2.

Transmission of disease from M. meandrites to M. cavernosa and the isolation of putative pathogens

Transmission experiments were performed between diseased *M. meandrites* fragments from the Keys and healthy *M. cavernosa* (n=4) to ensure the disease lesions were contagious and to increase the success of isolating putative pathogens. In short, healthy *M. cavernosa* fragments were placed in direct physical contact with the lesions on diseased *M. meandrites* colonies and

monitored for transmission (i.e. tissue loss or bleaching). One of the *M. cavernosa* fragments developed tissue loss after two days in contact with a diseased *M. meandrites* colony. The *M. cavernosa* fragment was left in contact with the disease lesion for another two days to ensure tissue loss was progressing. After relocating the fragment to a separate aquarium with FSW tissue loss progressed over the next 24 h so a sample was taken of mucus and diseased tissue and plated onto growth media (SWC agar). Isolates were streaked for purification and immediately cryopreserved.

Groups of five isolates were screened against healthy fragments of *M. meandrites* with a final bacterial concentration of approximately 10^8 CFU/ml of tank water. Three genotypes of *M. meandrites* were screened with each group of isolates. Two of the three genotypes exposed to one group of isolates (cocktail #1) started to bleach 2 and 3 days post-inoculation, which developed into tissue loss 5 and 7 days post-inoculation (Fig. 5 A-F). The "cocktail #1" isolates were also tested on three genotypes of healthy *M. cavernosa* and one of the genotypes started losing tissue by 2 days post-inoculation (Fig 5 G-I).

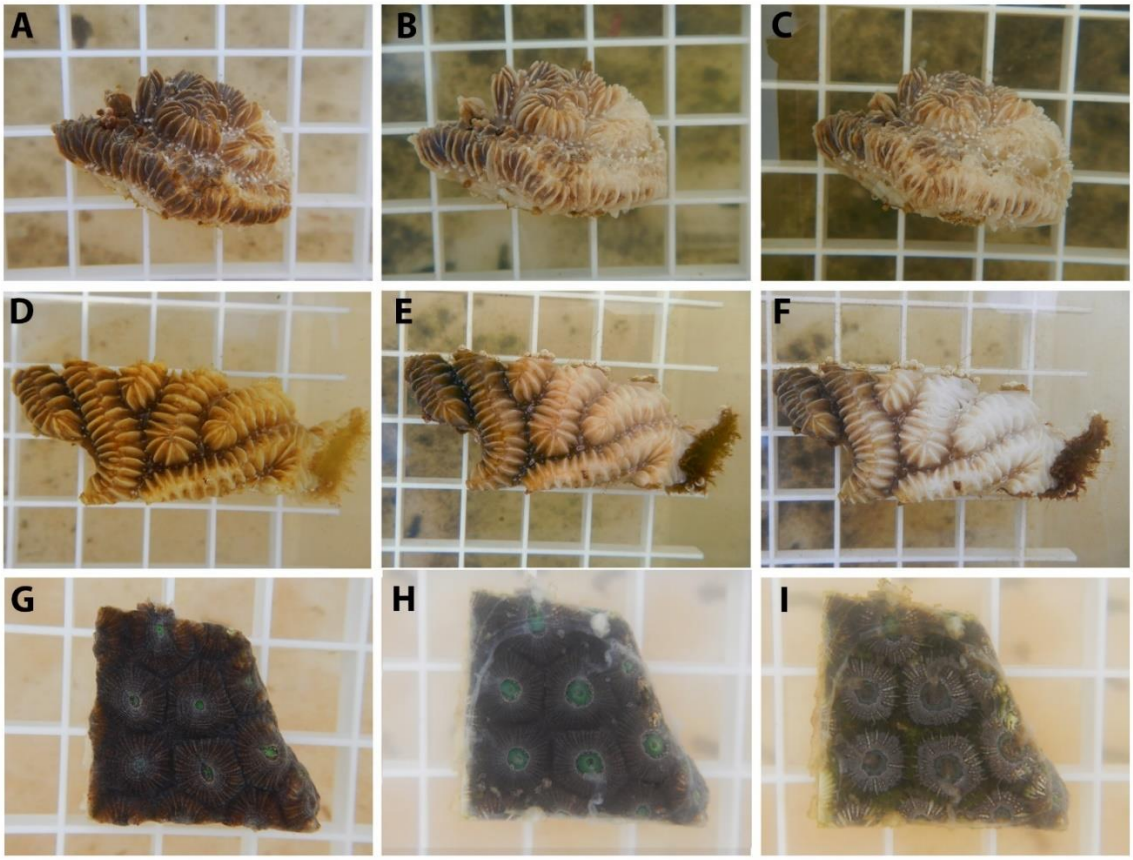

Figure 5. Diseased coral fragments after inoculation with the isolate group "cocktail #1" during a screen for putative pathogens using samples collected from the Keys. A) a healthy *M. meandrites* fragment pre-inoculation, B) the same fragment with extensive bleaching 2 days post-inoculation, and C) the first signs of tissue loss 5 days post-inoculation. D) a healthy *M. meandrites* fragment pre-inoculation, E) the same fragment with extensive bleaching 3 days post-inoculation, and F) the first signs of tissue loss 7 days post-inoculation. G) a healthy *M. cavernosa* fragment pre-inoculation, H) the same fragment with tissue loss around the edges 2 days post-inoculation, and I) extensive tissue loss 4 days post-inoculation.

The isolates from cocktail #1 are currently being tested individually on healthy *M. meandrites* fragments. The re-test of cocktail #1 has resulted in extensive bleaching and tissue lysis with two out of the three fragments exposed to it by 2 days post-inoculation. The results with the individual isolates are pending. All five isolates in cocktail #1 have been sent for 16S rRNA gene sequencing to preliminarily identify them. If these isolates from diseased corals collected from the Keys are similar to the 2017 isolates from the Fort Lauderdale area, then it suggests that the same etiological agent(s) are responsible for the disease in both areas. Additional healthy *M. meandrites* genotypes will be required for the continuation of these experiments.

Conclusions

Though these results require more replication to make any conclusive statements, they do suggest that, first, *M. cavernosa* is not a suitable specimen for current or future virulence studies. However, the ability to remove particles from its surface and the possible link to *M. cavernosa* being resistance to this disease suggests that the pathogen(s) may be spreading on fomites/vectors in the water column. This should be kept in mind for coral kept in captivity, where filtering the incoming water could prevent infection of any healthy corals. Second, the bacterial pathogen(s) responsible for this disease enter the coral tissue. If these pathogens can penetrate the coral tissue, then treatments, especially topical treatments, must be able to penetrate or diffuse into inner tissue layers or cavities to effectively target any pathogenic bacteria. Treatments that simply cleanse the surface of diseased coral may not be effective at treating this infection. Third, there may be multiple bacterial pathogens that induce disease. If there are multiple coral pathogens, then that must be taken into consideration when tracking the spread of this disease. Epidemiological studies or pathogens must consider that there may be multiple pathogens that utilize different modes of transmission. Fourth, the diseased *M. meandrites* in the Keys may be succumbing to a bacterial infection. Though the pathogen(s) has not yet been identified, it appears that the coral from the Keys, at least *M. meandrites*, could be succumbing to a bacterial infection. This suggests that mitigation efforts that treat for pathogenic bacteria could be continued for these diseased corals. More experiments are required to further tests these running hypotheses.