Studies of the Ecology and Microbiology of Florida's Coral Tissue Loss Diseases

Final Report

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Introduction

Florida's coral reefs are currently experiencing a multi-year disease-related mortality event that has resulted in massive die-offs in multiple coral species. 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. This disease, termed Stony Coral Tissue Loss Disease (SCTLD), was first observed near Virginia Key in late 2014, and has since spread to the northernmost extent of the Florida Reef Tract, and south throughout much of the Florida Keys.

An initial investigation into the transmission, infectiousness and differential host specificity of SCLTD was completed in 2017-2018. Preliminary results have demonstrated that the currently investigated disease is transmissible from diseased *Montastraea cavernosa* fragments to healthy *M. cavernosa* and *O. faveolata* fragments, and from diseased *Colpophyllia natans* fragments to healthy *M. cavernosa* and *Meandrina meandrites* fragments, indicating the presence of an infectious agent. Additionally, disease progression can be slowed or halted by treatment with antibiotics for several species of corals, suggesting the infectious agent(s) are bacterial.

The overall goals of this project are to: 1) isolate potentially pathogenic microorganisms and systematically determine if they can elicit disease signs in healthy corals; 2) examine potential changes in virulence at different locations through time; 3) determine the ecology of the tissue loss disease affecting *Siderastrea siderea*; and 4) isolate potentially probiotic bacteria from target species of affected corals and test their ability to prevent infection of healthy corals.

Task 1. To identify the pathogen(s) responsible for SCTLD

Screening for culturable pathogens

The isolation of a culturable pathogen would be essential for the development of targeted treatments and diagnostic tools. Therefore, bacterial isolates are being cultured from infected corals used in transmission experiments conducted in filtered seawater (FSW). Diseased corals from the field were placed into contact with healthy corals and, if disease developed, bacteria would be cultured from the newly infected fragment. This method ensures that transmission occurs and greatly reduces background and the overwhelming number of opportunistic microorganisms commonly associated with diseased corals in the field. Culturable isolates were screened in batches of 5 isolates using healthy coral kept at the Smithsonian Marine Station (SMS) that was the same species as the infected fragment used in the initial transmission experiment (Table 1)(1, 2). Small 2 x 3 cm coral fragments in FSW were used for the virulence screens.

Table 1. Number of isolates screened for virulence

Transmission (diseased sp. → healthy sp.)	**# isolates screened**
M. cavernosa → *O. faveolata*	100
M. cavernosa → *M. cavernosa*	85
M. meandrites → *M. cavernosa*	25
C. natans → *M. cavernosa*	165
S. siderea → *S. siderea*	25
Total isolates screened to date	**400**

Though there have yet to be any definitive pathogens identified, multiple isolates have demonstrated the potential to elicit tissue loss and/or bleaching after being inoculated onto healthy coral fragments (Table 2). As of now, it is unclear if any of these isolates are primary pathogens, opportunistic colonizers, or potential secondary pathogens.

The isolates from diseased corals (Table 2) could be opportunistic colonizers or secondary pathogens, which could explain the inconsistent results with these isolates. A primary, difficult to culture, pathogen may be required to initiate disease, while secondary pathogens can progress tissue loss at a faster rate and/or cause bleaching. Alternatively, it may require a consortium of bacterial pathogens to initiate disease. Diseases like yellow or black band disease require a consortium of different microorganisms (3–5), while the coral pathogen *Pseudoalteromonas piratica*, responsible for acute tissue loss, is unable to infect healthy coral (*Montipora capitata*) effectively unless it is affected by a pre-existing infection, chronic *Montipora* white syndrome (6). The simplest explanation for these results is that the pathogen responsible for SCTLD is difficult to culture, which is an accepted limitation for a majority of environmental bacteria. Therefore, culture-independent approaches (see below) will be undertaken to focus our culturing efforts.

Table 2. Potentially pathogenic isolates from diseased corals

Isolate	Closest match (based on 16S rRNA gene)	Bacterial family	Isolated from (transmission type)	Individual virulence screens (diseased/total coral fragments) **
OfT6-17	*V. coralliilyticus**	Vibrionaceae	MCAV → OFAV	3/4 *M. cavernosa* 1/10 *O. faveolata*
OfT6-21	*V. coralliilyticus**	Vibrionaceae	MCAV → OFAV	1/5 *M. cavernosa* 8/10 *O. faveolata* 0/8 *O. faveolata*† 2/3 *M. meandrites*† 1/7 *M. cavernosa* †
OfT7-21	*V. coralliilyticus**	Vibrionaceae	MCAV → OFAV	0/6 *M. cavernosa* 4/12 *O. faveolata*
McT4-15	*Alteromonas* sp.	Alteromonadaceae	MCAV → MCAV	1/10 *M. cavernosa* 2/10 *O. faveolata*
McT4-42	*Leisingera* sp.	Rhodobacteraceae	MCAV → MCAV	2/2 *M. cavernosa* 2/2 *O. faveolata*
McT4-56	*Leisingera* sp.*	Rhodobacteraceae	MCAV → MCAV	2/2 *M. cavernosa* 2/2 *O. faveolata* 0/8 *O. faveolata* † 0/7 *M. cavernosa* † 2/3 *M. meandrites* (bleaching)†
MmMcT2-2	*Alteromonas* sp.*	Alteromonadaceae	MMEAN → MCAV	2/3 *M. meandrites* 0/2 *M. cavernosa* 0/2 *O. faveolata*
MmMcT2-4	*V. coralliilyticus**	Vibrionaceae	MMEAN → MCAV	1/3 *M. meandrites* 0/2 *M. cavernosa* 0/2 *O. faveolata*
MmMcT2-5	*Alteromonas* sp.*	Alteromonadaceae	MMEAN → MCAV	2/3 *M. meandrites* 0/2 *M. cavernosa* 0/2 *O. faveolata*
CNT1-3	*Thalassobius* sp.*	Rhodobacteraceae	CNAT → MCAV	3/6 *C. natans* 1/6 *C. natans*†
CNT1-13L	*Alteromonas* sp.*	Alteromonadaceae	CNAT → MCAV	3/6 *C. natans* 1/6 *C. natans*†
CNT1-13S	*Silicibacter* sp.*	Rhodobacteraceae	CNAT → MCAV	1/6 *C. natans* 0/6 *C. natans*†
CNT1-28	*Alteromonas* sp.*	Alteromonadaceae	CNAT → MCAV	2/6 *C. natans* (bleaching) 0/6 *C. natans*†

*Based on whole-genome sequencing
**Virulence screens with individual isolates (subsequent to cocktail screens)
†Controlled infection experiments with corresponding control fragments

Culture-independent approach to pathogen identification

A majority of bacteria are not easily culturable, so culture-independent methods are being implemented to identify potential pathogens. The resolution obtained from the Illumina sequencing platform, around 300 bp of the 16S rRNA gene, is not enough to accurately identify many bacteria beyond the level of family. Furthermore, species demarcation using the entire 16S

rRNA gene has been shown to be ineffective for multiple marine bacteria, including members of the families Rhodobacteraceae, Alteromonadaceae, and Vibrionaceae (6–8), which seem to be associated with corals displaying signs of SCTLD (Meyer et al., preprint available at https://www.biorxiv.org/content/10.1101/626408v1). Therefore, a metagenomic-based approach must be taken that sequences all the DNA present. Prior research has shown that bacteria are important for SCTLD progression because antibiotics can arrest or significantly slow disease progression. However, an underlying viral component is still possible, although the viral infection would probably not necessarily result in visible disease signs but would be detectable with metagenomic sequencing. Furthermore, metagenomic sequencing does not have an initial amplification step for the 16S rRNA gene, which can introduce additional bias to the analysis. If necessary, commercial kits are available to amplify the microbial DNA from samples while selectively removing coral DNA.

For our metagenomic sequencing samples, healthy fragments of *M. cavernosa* were infected through physical contact with diseased *M. cavernosa* collected from the field. These transmissions (*n*=5) were conducted in FSW using closed systems to reduce the microbial background. The original diseased fragment, infected transmission fragment, and a healthy fragment from the same colony as the infected transmission fragment were frozen for sequencing. Four of the five transmissions were successful, and all of the fragments were sequenced. Analysis of the metagenomic data is currently underway in the laboratory of Dr. Julie Meyer at the University of Florida.

Disease progression variability and detection of *V. coralliilyticus*

It was previously observed with diseased *M. cavernosa* collected from the Ft. Lauderdale area used in transmission studies that disease progression would sometimes arrest after corals were kept in captivity with FSW. A similar incidence was observed with genotypes of diseased *M.*

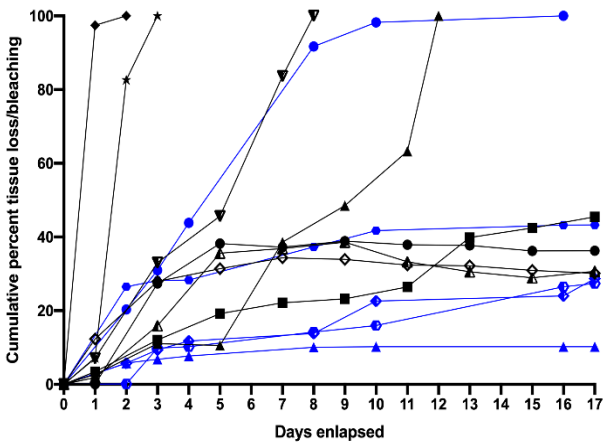

Figure 1. Disease progression with *M. cavernosa*. The cumulative percent tissue loss/beaching was determined by measuring the visible area of healthy tissue using ImageJ at the start of the experiment (t=0) then calculating the percent of tissue loss/beaching from the healthy tissue measurements from subsequent days photographed. Genotypes from Ft. Lauderdale are indicated by blue lines while black represents the FL Keys. No remaining healthy tissue equates to 100% percent tissue loss or bleaching. Lesions with tissue loss are indicated by "TL" while bleaching is indicated by "BLC" in the legend.

cavernosa collected from Ft. Lauderdale and the Florida Keys. The genotypes were trimmed using a rock saw and disease fragments were kept in 5 L of FSW with partial water changes every other day and monitored daily for disease progression using photographs and image analysis software (ImageJ); this analysis is represented for some of these genotypes in Figure 1. Diseased *M. cavernosa* fragments from 19 colonies collected around Ft. Lauderdale and 28 from the Florida Keys were analyzed. Of these diseased corals, lesion progression arrested for 26% (*n*=19) of the corals from Ft. Lauderdale and 32% (*n*=28) of the corals from the Florida Keys in FSW in laboratory aquaria. Interestingly, the proportion of corals to completely lose all of their tissue within 21 days was similar for both regions, 42% and 46% for Ft. Lauderdale and the Florida Keys, respectively. Though, it should be noted that the size of the fragment and initial amount of tissue is not taken into consideration for the proportion of those with complete tissue loss, but fragments are typically cut to a relatively similar size and amount of living tissue. However, there may be some differences in the rate of tissue loss between these two regions (described further below). There was no correlation between the presence of bleaching or tissue loss alone and disease progression with any genotype.

The intraspecific variability and cessation of disease progression should be taken into account when designing experiments. Disease progression stopped without any antibiotic or probiotic treatment while being kept under identical conditions, which has the potential to skew results. Additionally, there are instances like genotype McD-2 where disease progression appeared to stop or slow significantly from days 5 – 9 but started again by day 11 (Figure 1). As mentioned above, this difference in disease presentation could be attributed to 1) differential host responses to infection, 2) different pathogens present, 3) secondary infections that are contributing to the progression of disease, or 4) a combination of these scenarios.

For the genotypes collected from the Florida Keys, an immunoassay specific to *V. coralliilyticus* was used to detect the presence of this known opportunistic pathogen (Figure 2). The *Vibriosis VcpA RapidTest* (http://www.mabdx.com/) was created for the shellfish industry to detect the toxic protein, VcpA, produced by pathogenic strains of *V. coralliilyticus*. Previous studies have demonstrated purified VcpA to be toxic to shellfish and coral by causing rapid tissue damage.

Figure 2. Vibriosis VcpA RapidTest. The immunological assay tests for the presence of the toxin, VcpA, secreted by virulent strains of *V. coralliilyticus*. A positive test is indicated by two lines, while a negative test is one. The assay is also semi-quantitative with more VcpA (and theoretically more bacteria) indicated by a darker line.

This assay has an advantage over DNA sequencing because toxin production correlates to live bacteria, and previous research indicates that *V. coralliilyticus* will only produce easily detectable levels of this toxin when actively contributing to tissue loss on coral and not when it is in seawater or at sub-infectious levels on a coral surface (Ushijima and Häse, *in preparation*). Approximately 10% and 21% of the diseased corals from Ft. Lauderdale and the Florida Keys, respectively, tested positive for the *V. coralliilyticus* toxin VcpA. Intriguingly, regardless of region of origin, the lesions positive for VcpA appeared to progress faster than VcpA-negative lesions (Figure 3). These results could suggest that *V. coralliilyticus* may have been causing a secondary infection on these corals; colonizing the already-present lesions and releasing their toxins. Secondary infections in addition to the SCTLD pathogen could explain the variability of disease progression and presentation observed.

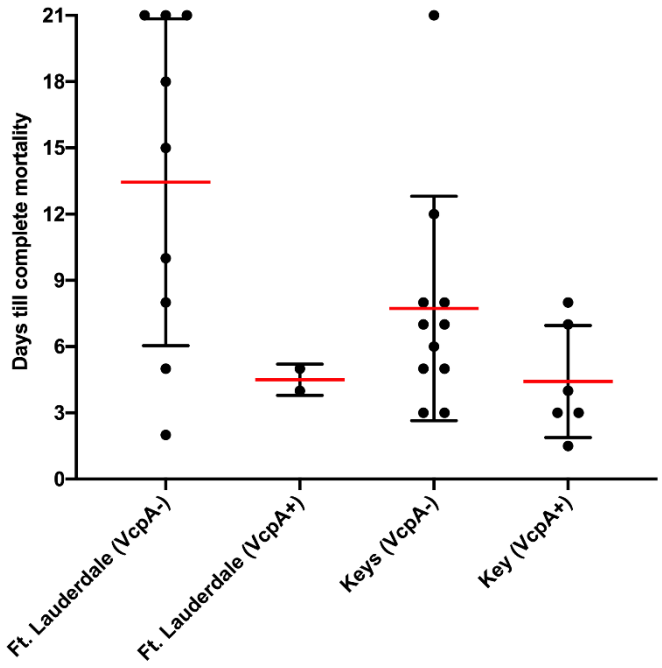

Figure 3. Comparison of survival for diseased *M. cavernosa* and the presence of *V. coralliilyticus*. The days until complete mortality for diseased *M. cavernosa* with progressive lesions. Fragments that survived for the entire 21-day observation period were assigned a value of 21 days for this graph. The red bar represents the mean, while the black bars are ±1 standard deviation.

Additionally, the potential presence of a sizable population of *V. coralliilyticus* on some diseased corals should be taken into consideration because, first, regardless if they are the primary pathogen, they can still be toxic to coral and cause opportunistic infections under certain conditions (Ushijima and Häse, *in preparation*). Second, this bacterial species is highly-resistant to beta-lactam antibiotics like amoxicillin (Figure 4), which is being used in field treatments, and can produce one or two different beta-lactamases (depending on the strain) that actively destroy beta-lactams (Ushijima and Videau, *unpublished data*). Third, in shellfish hatcheries with semi-recirculating seawater (similar to some land-based coral nurseries), the *V. coralliilyticus* levels can be >50x higher than the surrounding environment and will be the dominant *Vibrio* species (9). This may be partially due to *V. coralliilyticus* being able to rapidly and effectively kill off competing bacteria in a contact-dependent manner (Guillemette et al., *in review*). Plans have

been proposed to expand our collaboration with Dr. Michael Marusich (mAbDx, Inc.) to continue screening incoming diseased coral using these assays for their levels of *V. coralliilyticus* to determine if the trend between the presence of this pathogen with increased lesion progression and a difficulty to treat with probiotics continues with a larger sample size. If true, we have the advantage of already having a valuable diagnostic tool and can focus on developing targeted treatments for *V. coralliilyticus* to supplement existing treatment efforts.

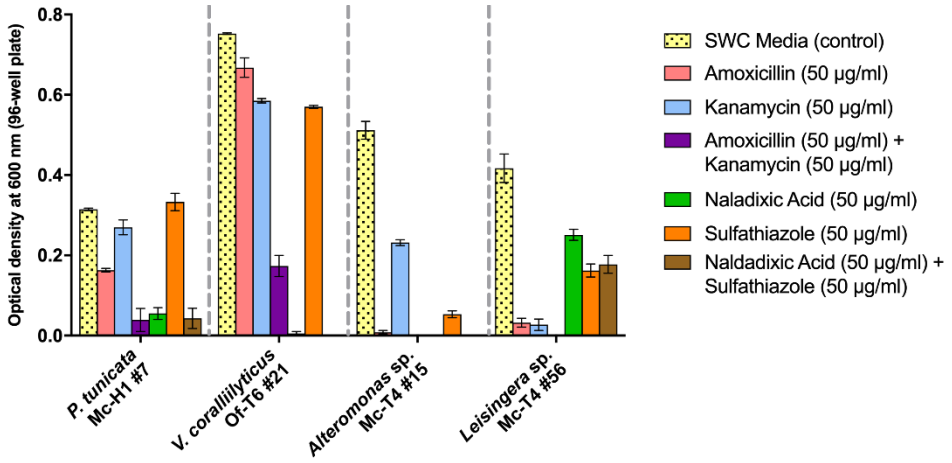

Figure 4. Antibiotic susceptibilities of the coral-associated microbes. Liquid culture of each bacterium was used to inoculate 96-well plates containing growth media with known quantities of antibiotics. The plate was incubated for 24 h at 28°C then culture density (strain growth) was measured.

Task 2. To Assess Virulence as the Disease Moves South

As the disease has expanded down the reef tract into the Florida Keys, it appears there has been an increase in the number of species affected and on the rate of tissue loss on some coral species as compared to earlier reports from northern sites. This suggests that the pathogen could be evolving increased virulence through time. To examine this, we are comparing the disease dynamics in a more resistant species (*M. cavernosa*) in the Ft. Lauderdale region where the disease passed through in 2014-2015 with *M. cavernosa* in the Florida Keys where the disease is just emerging. In each region, tagged colonies were followed through time to examine virulence, and manipulative studies were conducted to compare rates of transmission.

Comparing the rates of mortality between tagged colonies of *M. cavernosa* in the Keys vs. Fort Lauderdale region

Seventeen colonies of *M. cavernosa* with subacute to acute tissue loss disease and two healthy colonies (total = 19 colonies) were tagged on Nov 7, 2018 at Looe Key. Colonies were mapped out on the reef, photographed and coral lesions described. Colonies were resurveyed and photographed on Feb. 18, 2019 and April 12, 2019. At the Ft. Lauderdale site, we tagged 20 *M. cavernosa* with tissue loss disease in July 2017 and have been following them through time. For the comparison with the Keys corals, we used the tagged Ft. Lauderdale colonies that were still alive (17 colonies) and surveyed them during a similar time period between Oct. 25, 2018 and May 9, 2019. Fourteen colonies had disease lesions and 3 colonies did not at the start of this study (total=17 colonies). Surveys on Ft. Lauderdale colonies were conducted on Oct. 25, 2018, Dec. 18, 2018, March 29, 2019 and May 9, 2019. Disease state at the start of the studies was described as focal bleaching without recent tissue loss, subacute (1-5cm) or acute (>5cm) tissue loss and lesions with or without a distinct bleached border. Disease prevalence and colony mortality were followed through time.

Results

Lesions differ among regions

At Looe Key, of the 17 diseased *M. cavernosa* at the start of the study, 9 (53%) had acute lesions, 8 (47%) had subacute lesions and 3 of the 17 (17.6%) colonies had distinct bleached zones along lesions. In contrast, at the Ft. Lauderdale site 10 of the 17 (58.8%) diseased *M. cavernosa* started the study period in Oct. 2018 with bleached lesions with no recent tissue loss (disease re-emerging), 23.5% had no active lesions, and three colonies had subacute tissue loss lesions (17.6%) of which 2 colonies had bleached zones along lesions. As a comparison, in July 2017 these same tagged colonies at Ft. Lauderdale presented 10% with acute lesions and 90% with subacute lesions with 70% of the colonies having a bleached lesion.

Disease prevalence and mortality differ among regions

Disease prevalence on tagged *M. cavernosa* at Looe Key was high throughout the study (>80%), but prevalence declined through time in colonies at the Fort Lauderdale site (Fig 5).

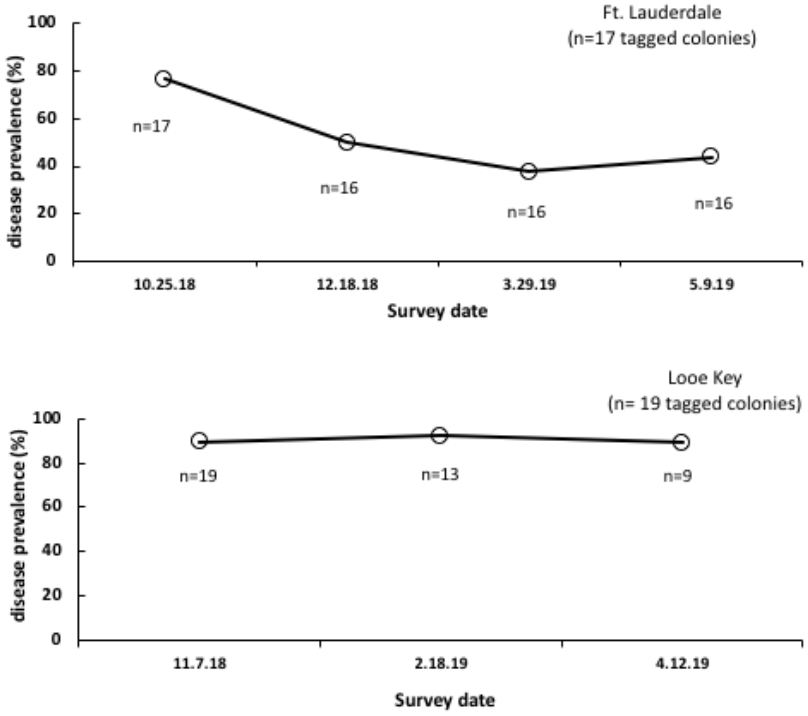

Figure 5. Disease prevalence through time on tagged colonies at Fort Lauderdale (top graph) compared to Looe Key (bottom graph) during similar survey dates.

Colonies at Looe Key had more active lesions at the start of the study (tissue loss) as compared to the tagged *M. cavernosa* at Ft. Lauderdale (bleached borders with no recent tissue loss) and also had a higher overall average amount of tissue loss on colonies (Fig. 6), and a higher case fatality rate (Fig. 7).

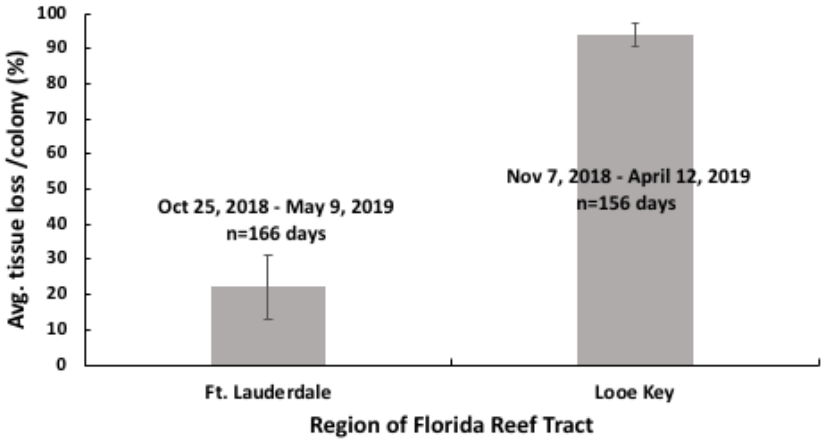

Figure 6. Differences in rate of tissue loss in tagged *M. cavernosa* at Ft. Lauderdale (n=17) vs. Looe Key (n=19).

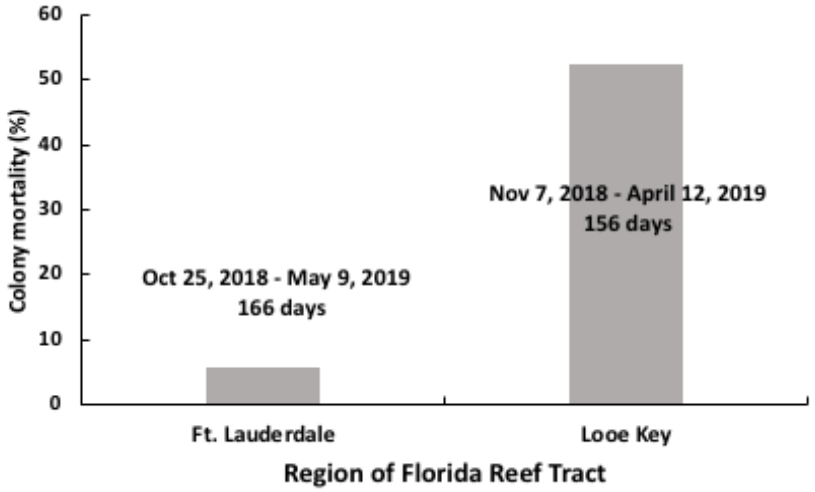

Figure 7. Differences in case mortality rate of diseased *M. cavernosa* at Ft. Lauderdale (n=17) vs. Looe Key (n=19).

Comparing the rates of transmission between Keys and Fort Lauderdale *M. cavernosa*

Experiments were conducted under static conditions using a block design in which three aquaria (Keys, Fort Lauderdale and control) were used, with the Keys and Fort Lauderdale aquaria each containing a fragment of diseased *M. cavernosa* from its respective region and the control aquaria housing a healthy fragment (aggression control) of *M. cavernosa* from the Key West Nursery (originally collected at Safe Harbor Light). Another healthy fragment was cut into three pieces and each piece was placed in direct contact with the *M. cavernosa* fragments (diseased or healthy) in each aquarium. The use of coral fragments from the same colony for transmission

studies controls for intraspecific differences in disease susceptibility, while using a healthy *M. cavernosa* in place of the diseased *M. cavernosa* in the control aquaria accounts for any intraspecific aggression (tissue loss from competition between coral fragments). Photographs of all fragments were taken at day 0 and daily thereafter, and at the same time all fragments were examined daily for signs of tissue loss. Water quality was maintained through partial water changes daily, and each aquarium had a bubbler to create water motion. Aquaria were held under natural light and summer temperatures (28-29 ^{o}C).

Results

Transmission rates differ between regions

We collected 11 fragments of diseased *M. cavernosa* from the Keys (24.54770°N, 81.45696°W), all of which had subacute tissue loss lesions, and none had bleached zones along the lesions. We collected 11 fragments of diseased *M. cavernosa* from the Ft. Lauderdale reefs (26.14975°N, 80.09597°W), all of which had subacute tissue loss lesions and 36.4% had distinct bleached zones along the lesions. The diseased *M. cavernosa* from the Ft. Lauderdale reefs had a 45.5% transmission success after an average of 3.8 days (range=2-7 days). Three additional healthy test corals developed a bleached zone at the point of contact but did not develop into tissue loss during the experiment (maximum duration of 14 days). Diseased *M. cavernosa* from the Keys reefs had a 27.3% transmission success after an average of 6 days (range=3-9 days) (Fig. 8). They also had an additional three healthy test corals develop a bleached zone at the point of contact that did not develop into tissue loss during the experiment (maximum duration of 14 days).

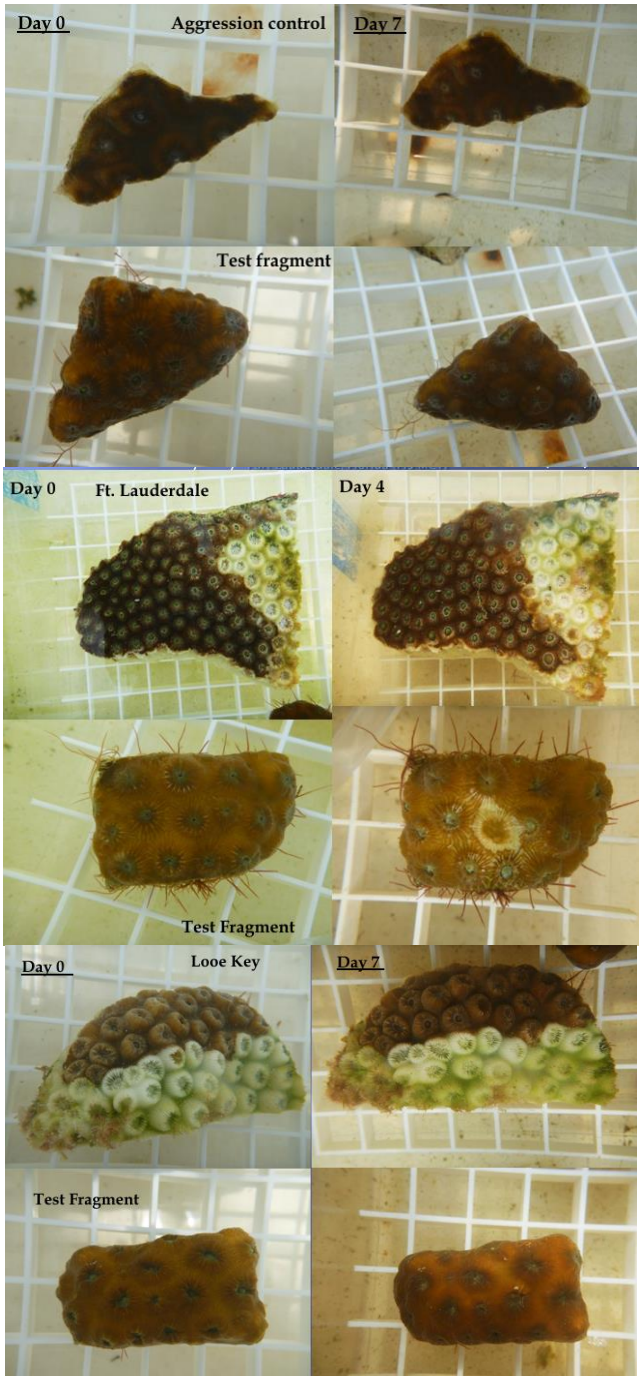

Figure 8. Examples from experiment comparing rate of transmission from disease *M. cavernosa* from Ft. Lauderdale vs. the Keys. Top panel shows control corals, middle panels shows successful transmission from a diseased fragment from Ft. Lauderdale and bottom panel shows no transmission from a diseased fragment from the Keys. Note that the disease lesion on this Ft. Lauderdale fragment progressed more rapidly than on this Keys fragment.

Conclusions

We compared the rate of mortality in tagged colonies of *M. cavernosa* with tissue loss disease between Looe Key and Ft. Lauderdale. The average amount of tissue loss per colony and the case fatality rate was much higher at Looe Key. There could be regional differences in environmental conditions, which are affecting pathogen-host dynamics, allowing the pathogen to have higher virulence at Looe Key. Alternatively, we did not begin monitoring the Ft. Lauderdale colonies until years after the event was first found in that region, and so it could be that the most susceptible colonies to SCTLD had already died at the Ft. Lauderdale site before we started our monitoring program and the remaining corals in the outbreak area differ in some component(s) of their defenses that allowed them to initially resist infection and once infected allow them to reduce disease virulence. We also found that the morphology of the disease lesions differed between regions with Looe Key having more lesions scored as acute and Ft. Lauderdale having more lesions scored as subacute and more recently (2018) bleached with no recent tissue loss. It is possible the different lesion morphologies represent different pathogens so we cannot rule out that multiple pathogens might be involved in the disease process including primary and secondary pathogens.

Since the mortality rate was higher in *M. cavernosa* at Looe Key, we hypothesized that the rate of transmission would also be higher when using diseased fragments from Looe Key vs. from Ft. Lauderdale for aquaria studies. However, when controlling for test coral genotypes, environment and mode of transmission (direct contact only) we found the rates of transmission were similar between regions suggesting that environmental co-factors may be influencing disease processes in the field. However, it also must be kept in mind that the processes that initiate an infection (transmission study) can differ from those that subsequently affect disease progression (mortality of tagged colonies in the field).

Task 3. Determining the Ecology of Tissue Loss Disease in Siderastrea siderea

For the current Florida disease outbreak of SCTLD, the disease signs vary among affected coral species with differences in rate of tissue loss (acute and sub-acute) and lesion occurrence (focal and multi-focal). There are also several coral species that are not affected by the disease (apparently disease resistant), and one coral species, *S. siderea*, displays unusual lesions with multi-focal bleached or purple spots. One question is whether *S. siderea* is affected by the same pathogen(s) as the other species but disease signs differ or if *S. siderea* has a different disease altogether. To answer this, we are conducting studies parallel to those ongoing with coral species having the usual tissue loss lesions associated with SCTLD (*M. meandrites, C. natans, M. cavernosa, O. faveolata)*. Tagged colonies are being followed through time to examine disease virulence, and manipulative transmission studies are testing whether the disease is transmissible and, if so, whether there were interspecific differences in susceptibility to disease. Finally, we are using treatments with antibiotics to determine whether bacteria are involved in the disease process (experiments in 2018 and 2019).

Tagged colonies

There are 8 *S. siderea* tagged at our site off Ft. Lauderdale (near FtL5), but to date they have not developed disease and no diseased *S. siderea* have been found at the site. Dr. Aeby is in contact with FWC to review pictures from the diseased *S. siderea* from their permanent plots in the Keys.

Transmission from diseased *S. siderea* to healthy *S. siderea* and *Orbicella faveolata*

Experiments were conducted using a block design of four aquaria. Within each block, there were two aquaria (experimental and control) used for each test species and two test species (*S. siderea* and *O.* f*aveolata*) were used in each trial for a total of 4 aquaria/block. In the experimental tanks, an infected fragment of *S. siderea* with a distinct lesion was placed in direct contact with a healthy fragment (direct transmission) and the other healthy fragment was placed ~10 cm away (waterborne transmission). In the control aquaria, the diseased fragment was replaced with a healthy fragment of *S. siderea* to control for lesions created by coral to coral aggressive interactions. Diseased fragments were cut in half and used for the comparative study between intra- and inter-specific rates of transmission, ensuring that each test species (*S. siderea* or *O.* f*aveolata*) was exposed to a similar level of infectiousness from the diseased coral. To discriminate between lesions caused by aggression versus a transmissible disease, any corals that developed lesions in the experimental or control tanks were removed from contact and observed for signs of lesion progression or recovery until the end of the experiment. Lesions that progressed following removal from contact were considered indicative of disease transmission. Lesions that failed to progress or healed were considered indicative of coral to coral aggression.

All aquaria were maintained in larger water tables with circulating freshwater adjusted with a cooling and heating system to maintain water temperatures between 28 to 29 °C. The experiment was conducted for a maximum of 21 days.

Results

Disease is infectious and transmission rates differ among species

When we exposed healthy *S. siderea* and *O. faveolata* to diseased *S. siderea* (bleached or discolored lesions) we found successful disease transmission (development of a lesion) occurred for both species upon direct contact and through the water column. Three of the 10 healthy *S. siderea* fragments (30%) touching a diseased *S. siderea* developed lesions after an average of 13.5 days and one of 10 non-touching *S. siderea* developed a lesion (10%) after 8 days. Figure 9 shows an example of successful transmission. Transmission to *O. faveolata* was more successful with 6 out of 10 (60%) of the touching fragments developing a lesion after an average of 7.5 days and 2 of 10 (20%) of the non-touching fragments developing disease signs after an average of 7.5 days. From this we conclude that the lesions on *S. siderea* are transmissible (contact and through the water) and there are differences among species in susceptibility.

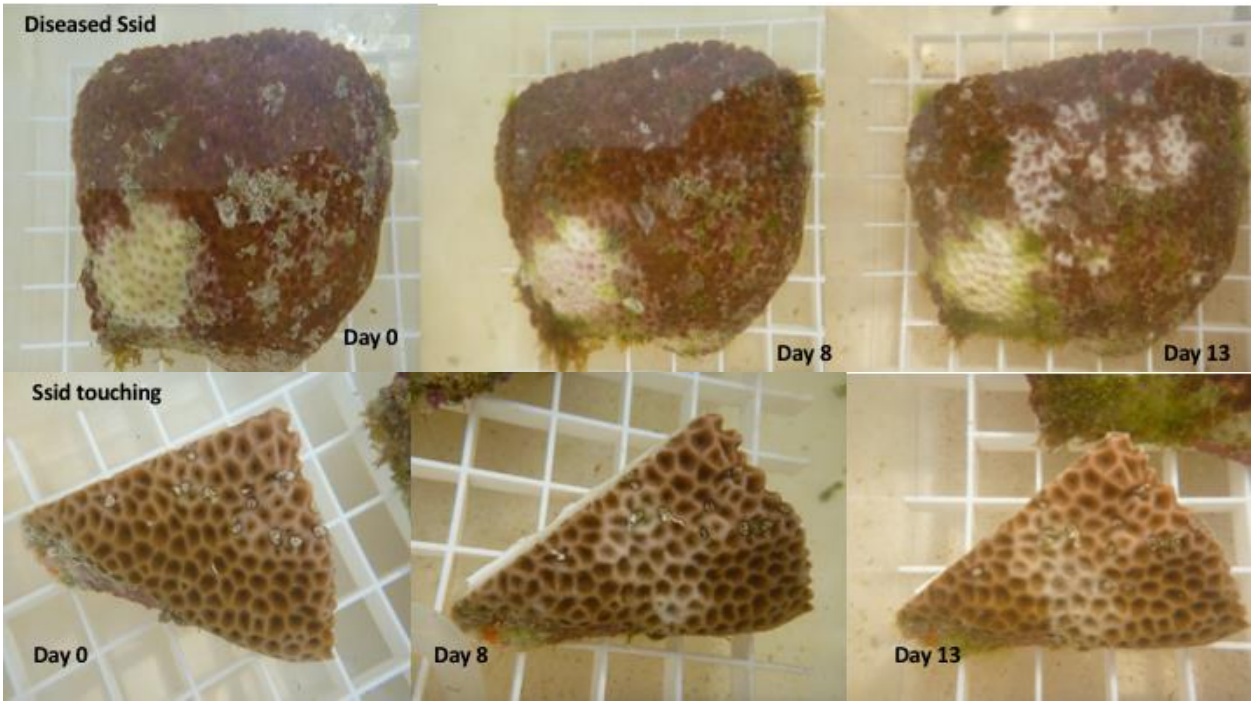

Figure 9. Examples of disease progression in *S. siderea* (top panel) and successful transmission (bottom panel). Contact between diseased and healthy fragments was stopped on day 8.

Differences in transmissibility among lesion types

The lesions on diseased *S. siderea* differed in morphology, and we noticed variability in transmission success among lesions types. We scored the initial lesions on the diseased *S. siderea* as purple discoloration, purple discoloration with tissue loss, focal bleaching, or focal bleaching with tissue loss. We then calculated the transmission success among the different lesion types overall and for each test species (*S. siderea* and *O. faveolata*) and found that *S. siderea* with lesions that were bleaching with tissue loss had the highest transmission success for both test species (Fig. 10) but larger sample sizes are needed to adequately evaluate differences among lesion types.

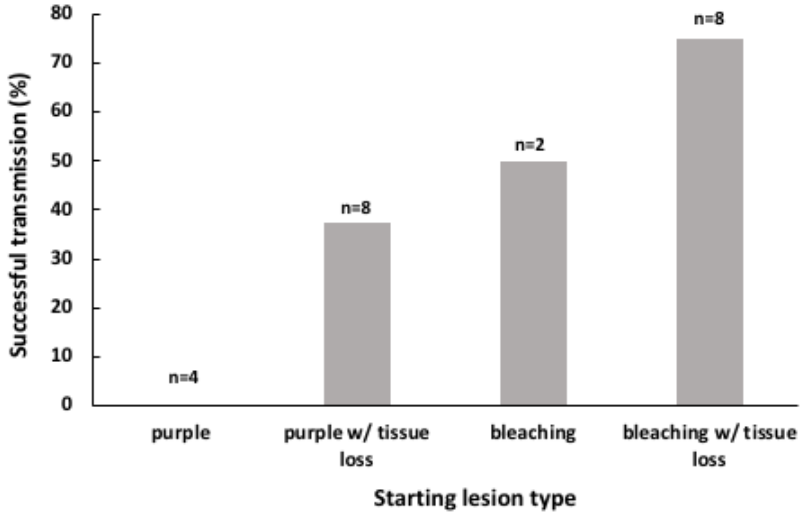

Figure 10. Differences in rates of transmission for *S. siderea* with different types of lesions. Transmission experiments with *S. siderea* and *O. faveolata* were combined for this analysis and so do not reflect independent samples, e.g. a single lesion was cut in half and used to test both test species simultaneously.

Conclusions

Successful transmission of *S. siderea* with tissue loss lesions suggest this is an infectious disease. Lesion morphology differed among colonies, as did transmission success among the lesion types. However, sample sizes are too small to adequately evaluate differences among lesion types so we recommend the experiment be repeated with adequate sample sizes. Further research is also needed to determine whether the underlying pathology, etiology and field virulence differs among lesion types.

Transmission from diseased *C. natans* to healthy *S. siderea*

To help determine whether the lesions observed in *S. siderea* reflect the same disease as other species, we conducted aquaria studies to directly examine susceptibility of *S. siderea* to acute tissue loss disease. Experiments were conducted using two aquaria per set (experimental and control). In the experimental tanks, an infected fragment of *C. natans* with an acute lesion was

placed in direct contact with a healthy fragment of *S. siderea* (direct transmission), and another healthy fragment of *S. siderea* was placed ~10 cm away (waterborne transmission). In the control aquaria, the diseased fragment was replaced with a healthy fragment of *C. natans* to control for lesions created by coral to coral aggressive interactions. Healthy test corals were cut into four pieces ensuring the same test genotype in all aquaria sets. To discriminate between lesions caused by aggression versus a transmissible disease, any corals that developed lesions in the experimental or control tanks were removed from contact and observed for signs of lesion progression or recovery until the end of the experiment. Lesions that progressed following removal from contact were considered indicative of disease transmission. Lesions that failed to progress or healed were considered indicative of coral to coral aggression. All aquaria were maintained in larger water tables with circulating freshwater adjusted with a cooling and heating system to maintain water temperatures between 28 to 29 °C. The experiment was conducted for a maximum of 10 days. One set of aquaria were run in November 2018 (n=3) and a second set in June 2019 (n=2) for a total of five experimental sets.

Results

S. siderea is vulnerable to infection from diseased C. natans

When healthy *S. siderea* were exposed to *C. natans* with tissue loss disease, all 5 touching *S. siderea* (100%) developed tissue loss lesions after an average of 8.4 days (range 6-10 days) and 4 of the 5 (80%) non-touching *S. siderea* developed tissue loss lesions after an average of 7 days (range 6-8 days). Lesions in *S. siderea* fragments touching *C. natans* progressed after contact was discontinued. In control aquaria, healthy *S. siderea* touching healthy *C. natans* also developed tissue loss lesions but all lesions began to heal once contact was terminated.

Comparison with other coral species previously tested with diseased C. natans

Previous experiments with two test species (*M. cavernosa* and *M. meandrites*) showed 100% transmission for both species for fragments directly touching the *C. natan* lesions (10 out of 10) and no significant difference in contact transmission between the two species. *M. cavernosa* fragments developed tissue loss lesions after an average of 3.8 days (range 3-5 days) and *M. meandrites* developed lesions after an average of 3.4 days (range 3-5 days). Lesions in both species progressed after contact was discontinued. The non-contact fragments in each species also developed tissue loss lesions in 6 of the 10 fragments of *M. cavernosa* after an average 4.7 days (range 4-5 days) and in 10 of 10 fragments in *M. meandrites* after an average of 4.3 days (range 3-6 days).

Conclusions

S. siderea is susceptible to *C. natans* tissue loss disease but did not catch the disease as rapidly as either *M. cavernosa* or *M. meandrites.*

Therapeutic diagnosis with antibiotic treatment 2018

A therapeutic diagnosis approach using antibiotics was taken to test for the possible role of bacteria in disease progression with 11 pairs of diseased *S. siderea.* For each experimental pair, a coral fragment with a disease lesion was cut in half with a rock saw so that each fragment had a relatively equal area of the disease lesion. One fragment from each pair was left untreated as a control, while the experimental fragments were treated with antibiotics resuspended in the aquarium water. Each fragment was individually housed in an aquarium with FSW and a bubbler for water motion. All aquaria were maintained in larger water tables with circulating freshwater adjusted with a cooling and heating system to maintain water temperatures between 28 to 29 °C. Coral fragments were photographed daily and partial water changes were conducted every other day to maintain water quality. A complete water change was conducted weekly. For the experimental fragments, water was replaced with FSW pre-mixed with a combinational treatment of amoxicillin (50 μg/ml of tank water) and kanamycin (50 μg/ml of tank water). Experiments were maintained for a maximum of 4 weeks.

Results

For the 11 pairs of diseased *S. siderea*, we observed 3 types of responses in the lesions: 1) lesions progressed with increased bleaching or tissue loss, 2) lesions healed with re-pigmentation of bleached patches or 3) no change in lesions (Fig. 11). Of these responses, we had 6 pairs (54.5%) of fragments (control and treatment) that had the same and approximately equal lesion response during the experiment, e.g. antibiotic treatment did not change the course of the lesion. Of these 6 pairs, in 3 pairs both fragments showed signs of healing, in 2 pairs both fragments showed equal progression and in one pair neither fragment showed changes in lesions. In 3 other pairs, the treated fragment had lesion progression while the control fragment healed. In one other pair, the treated fragment had slower progression as compared to the control and in the final pair, the treated fragment progressed whereas the control showed no change. A treatment regime of amoxicillin and kanamycin, dosed every other day, was not affective at stopping lesion progression within *S. siderea*.

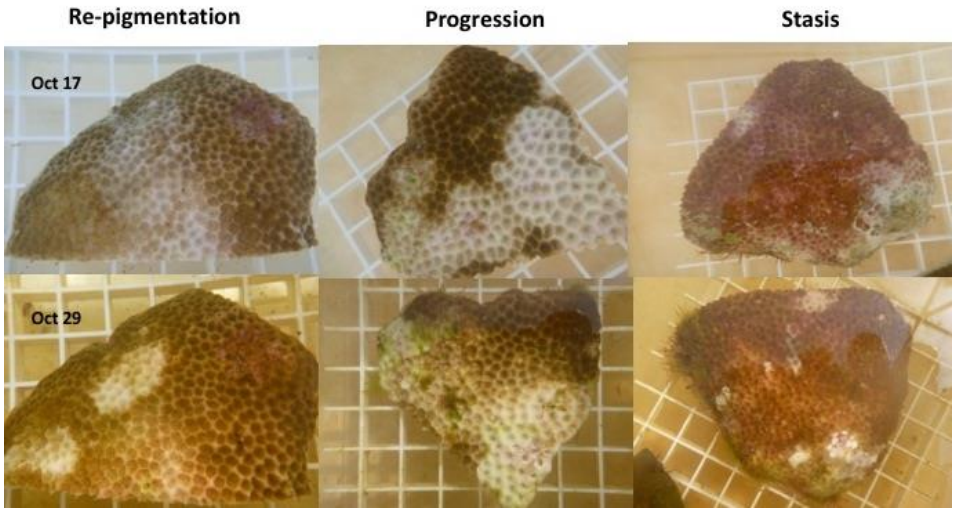

Figure 11. Examples of *S. siderea* lesions through time in aquaria studies. Top row is coral fragments at the start of the experiment (Oct 17, 2018) and bottom row is the same coral fragments showing differences in lesion progression after 12 days.

Therapeutic diagnosis with antibiotic treatment 2019

The experiment was repeated in June 2019 using the same methods except that corals were dosed daily with antibiotics and the trial was ran for 10 days. This allowed our results to be directly compared with prior antibiotic trials with other coral species exhibiting disease. Seven pairs of *S. siderea* were tested.

Results

For the second trial of antibiotic treatment of diseased *S. siderea* we found a similar response as in the first trial. Some lesions progressed, others healed, and a few stayed the same. In two pairs the disease progressed in both fragments in each pair, in one pair they both started healing and in four pairs the response differed between fragments within the pairs. Again, there was no consistent pattern in response to antibiotics and so we conclude that diseased *S. siderea* do not respond to antibiotic treatment with amoxicillin and kanamycin in the same manner as other diseased coral species.

Task 4. To Develop Effective Probiotics to Treat and Prevent SCTLD

The extensive coral mortality associated with the current outbreak calls for the development of treatments to reduce mortalities and disease transmission among field populations or rescued captive coral. One option is to use probiotics, beneficial microorganisms that can directly kill bacterial pathogens or prevent them from colonizing hosts. We now have a library of inhibitory bacterial isolates from five different coral species, some of which were isolated from coral fragments seeming more resistant to the current tissue loss disease, which can prevent the growth of known or putative coral pathogens. We would like to determine: 1) if any of these inhibitory isolates can stop lesion progression with diseased coral from the field or can prevent or reduce disease transmission to healthy pre-treated coral, and 2) what would be the optimal treatment protocol for each potential probiotic. The development of a probiotic treatment could be beneficial for keeping captive coral populations for restoration healthy, treating rescued coral from the field, and for mitigation efforts for the ongoing disease outbreak.

Characterization of a library of inhibitory isolates from healthy coral

We are currently investigating the use of beneficial microorganisms, probiotics, which can be used to protect healthy or treat diseased captive corals. A library of over 600 isolates displaying varying levels of antibacterial activity has been cultured from various healthy coral fragments that were seemingly more resistant to SCTLD. Each "inhibitory" isolate has been screened for their ability to inhibit the "target" bacteria McT4-15, McT4-56, and OfT6-21 that were cultured from diseased corals, which belong to the bacterial families Alteromonadaceae, Rhodobacteraceae, and Vibrionaceae, respectively. Inhibition screens were conducted by spotting a liquid culture of the isolate to be tested for inhibitory activity onto a plate with the target strain spread over the surface. The radius of the zone of inhibition that occurs around each inhibitory strain was measured after incubation for 24 h (Figure 12). Our library is organized into "sets" that are isolates originating from the same coral fragment with the idea that isolates from the same fragment will be less antagonistic toward each other (see multi-probiotic treatment section below) and that probiotics will work best (i.e., colonize better) with the coral species they originated from.

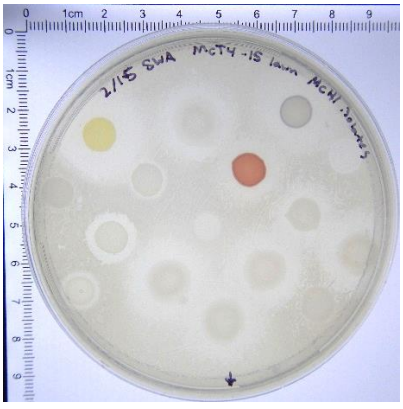

Figure 12. Plate-based inhibition screen. Example of a screen for inhibitory isolates that inhibit the growth of *Alteromonas* sp. McT4-15. Each inhibitory isolate is tested in triplicate against multiple target bacteria and the zones of inhibition are measured.

Potential probiotic strain McH1-7

A majority of the inhibitory isolates were selected based upon inhibitory activity and colony morphology to identify the inhibitory compounds produced by each bacterium. As of now, a primary inhibitory compound produced by one of these strains, *Pseudoalteromonas* sp. strain McH1-7, (Figure 12) has been isolated in our laboratory and identified as the antibiotic korormicin A (10) (Figure 13). This antibiotic specifically inhibits the activity of the important respiratory protein NADH-quinone reductase (NQR), making it specific to Gram-negative marine/halophilic bacteria (10, 11). Further analysis of the genome identified the genes in McH1-7 produce two other known antibacterial compounds, marinocine (12) and 2,3,4,5-tetrabromopyrrole (TBP) (13, 14). The production of at least three different antibacterial compounds make McH1-7 an attractive candidate for use as a probiotic because potential pathogens would be less likely to develop resistance to all three compounds. Additionally, it is also possible that McH1-7 produces a fourth (or more) inhibitory compound and analysis of the chemical extractions are currently underway.

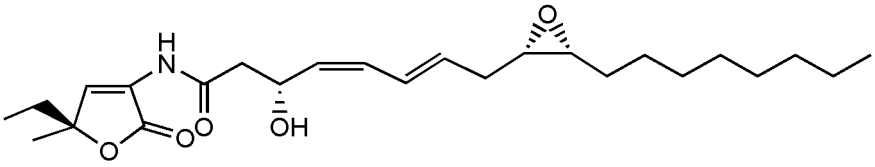

Figure 13. Korormicin A. Structure of the antibiotic korormicin; produced by the putative probiotic McH1-7.

Probiotic strain McH1-7 can slow or stop SCTLD progression

When cultures of McH1-7 were inoculated onto *M. cavernosa* fragments from Ft. Lauderdale with signs of SCTLD, disease progression in 9/10 treated fragments was significantly slowed or stopped compared to the untreated control fragments (Figure 14; Table 3). The cumulative tissue loss of each control and treated fragment was tracked daily with photographs, measured with ImageJ, and then plotted over time (Figure 15A-D). The preliminary results with diseased *M. cavernosa* from Ft. Lauderdale (*n*=10) suggest that treatment with McH1-7 can slow or stop SCTLD progression. Furthermore, McH1-7 did not elicit any obvious negative side effects when inoculated onto healthy *M. cavernosa* (*n*=7), *M. meandrites* (*n*=3), *C. natans* (n=2), and *O.*

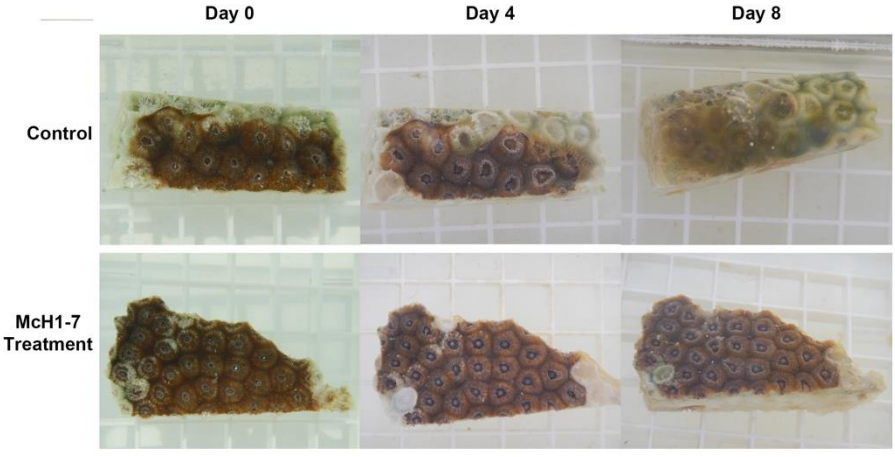

Figure 14. Diseased *M. cavernosa* fragment treated with the putative probiotic McH1-7. Both diseased fragments are from the same disease lesion. The colony, McD-11 FtL, was collected around the Ft. Lauderdale area. Each square in the grid under the coral is 1x1 cm.

faveolata (*n*=6), because it was routinely used as our negative bacterial control for infection experiments. Therefore, the non-toxigenic and potential probiotic effects of McH1-7 strongly suggest it is a potential candidate for an effective treatment for coral.

Treatment experiments were repeated with diseased *M. cavernosa* from the Florida Keys (n=14). Diseased corals were equally split into three fragments. For each replicate, one fragment was left untreated (control), one fragment was treated with McH1-7, and the third fragment was treated with a mutagenized version of McH1-7 that does not have an observable inhibitory activity or produce the antibiotic korormycin A (designated strain M389-53 or "mutant") if enough fragments were available. Overall, the disease lesions on corals from the Florida Keys were seemingly more active than the corals from Ft. Lauderdale, but McH1-7 was able to stop disease progression on one genotype and slowed tissue loss on the other four genotypes (Table 3, Figure 15E-H).

Table 3 presents results from only the experimental sets with control fragments (no treatment) that had progressive lesions. A total of 12 genotypes from Ft. Lauderdale and 14 genotypes from the Florida Keys were tested, but only 10 and 5 genotypes, respectively, from each region had controls with progressive lesions (Table 3). For pairs where disease in the control coral arrested, they were removed from the experiment and analysis. In general, disease progression was less with the fragments treated with McH1-7 compared to the controls. Interestingly the non-inhibitory mutant did not affect disease progression, suggesting that the production of antibacterial compounds is the mechanism by which this probiotic benefits coral. The production of antibiotics has been speculated to be the mechanism used by beneficial constituents of the coral microflora to protect their host, but these results represent the first experimental evidence to try to demonstrate this.

One important discovery made was that the coral lesions that were difficult to treat with McH1-7 were positive for the presence of the VcpA toxin specific to *V. coralliilyticus*. As described above, the disease lesions positive for *V. coralliilyticus* tend to be more aggressive infections, which could play a role in their difficulty to treat in the laboratory. Additionally, this pathogen is known to possess a variety of antibiotic resistance and its own antibacterial mechanisms. We have cultured multiple strains of this pathogen from our disease corals and have a rapid and effective diagnostic tool to screen for *V. coralliilyticus*. This gives us an advantage because we will continue to develop probiotic treatments that are specifically more effective against and resistant to *V. coralliilyticus* while we will continue our collaboration with Dr. Michael Marusich (mAbDx, Inc.) on continued monitoring for *V. coralliilyticus*.

Table 3. Results from McH1-7 treatment of diseased *M. cavernosa*

Ft. Lauderdale corals				
Colony ID	**FSW control**	**Mutant control**	**McH1-7 Treatment**	**VcpA assay**
FtL-9	Progressed over 21 d	N/D	Stopped	**N/D**
FtL-D	Progressed over 21 d	N/D	Stopped	**N/D**
FtL-18	Progressed over 21 d	N/D	Stopped	**N/D**
FtL-C	Died -18 d	N/D	Stopped	**N/D**
McD-11 FtL*	Died -8 d	N/D	Stopped	-
McD-12 FtL*	Progressed over 21 d	Progressed over 21 d	Slowed*	-
McD-14 FtL*	Progressed over 21 d	Progressed over 21 d	Stopped	-
McD-15 FtL*	Progressed over 21 d	Progressed over 21 d	Slowed*	-
McD-16 FtL*	Died -8 d	Died -11 d	Stopped 9 d → Progress	+
McD-17 FtL*	Died -1 d	N/D	Died -2 d	+
Ft. Lauderdale Totals	**6/10 progressed >21 d 4/10 complete mortality**	**3/4 progressed >21 d 1/4 complete mortality**	**6/10 stopped 3/10 slowed 1/10 no effect**	**2/6 positive**
FL Keys corals				
Colony ID	**FSW control**	**Mutant control**	**McH1-7 Treatment**	**VcpA assay**
McD-2	Died -18 d	Died -5 d	Stopped	-
McD-4	Died -8 d	N/D	Died -18 d	+
McD-7	Died -1 d	Died -1 d	Died -4 d	+
McD-8	Died -3 d	Died -3 d	Died -7 d	+
McD-21	Died -3 d	Died -4 d	Died -7 d	+
FL Keys Totals	**5/5 complete mortality**	**4/4 complete mortality**	**1/5 stopped 4/5 slowed but died**	**4/5 positive**

*Slowed in comparison to the control coral based upon daily tissue measurements; they did not die during the experiment. N/D = not done.

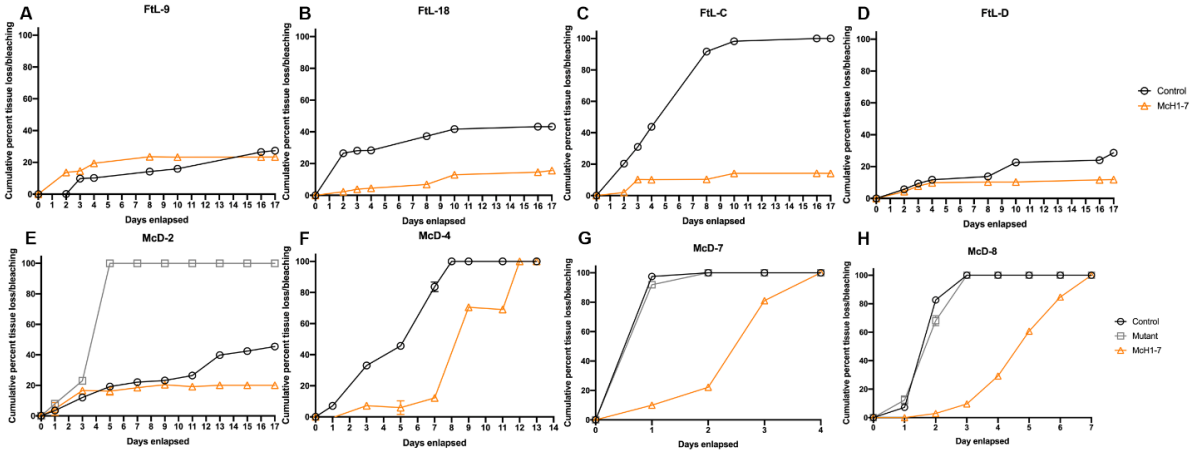

Figure 15. Cumulative tissue loss/bleaching over time for individual fragments used in the probiotic trials. A – D) Genotypes of diseased *M. cavernosa* collected from the Ft. Lauderdale area with black circles representing the non-treated controls and orange triangles representing the diseased fragments treated with McH1-7. E – H) Genotypes of diseased *M. cavernosa* collected from the Keys with black circles representing the non-treated controls, orange triangles representing the diseased fragments treated with McH1-7, and gray squares representing fragments treated with a non-inhibitory mutant derived from McH1-7. For each replicate all fragments originate from the same disease colony/disease lesion.

Protection from disease transmission with McH1-7 treatment

In addition to testing if our probiotic treatments can arrest SCTLD progression, we have started investigating if healthy fragments treated with probiotics are more resistant to disease transmission. Each replicate consisted of three tanks: (1) a diseased fragment in contact (secured with fishing line) with a healthy fragment (disease control), (2) a diseased fragment in contact with a healthy fragment treated for 48 h with the probiotic McH1-7 (experimental), and (3) a healthy fragment in contact with another healthy fragment treated with McH1-7 not from the same colony (healthy control) (Figure 16). The last tank is to control for any intraspecific antagonism. Treatments with probiotics were done in separate tanks so once corals were placed into contact with one another, no probiotic cultures were added to the tank water for the duration of the experiment (21 days). Within each replicate set of three tanks, all diseased fragments were from the same lesion and healthy fragments (treated and untreated) were from the same colony. Fragments were placed side-by-side and not flipped onto one another like previously described transmission experiments by Aeby *et al.* in order to replicate what could occur in a nursery or with probiotic-treated restoration corals placed back into the field. Only the experimental sets with control fragments (no treatment) that had progressive lesions are presented. A total of 4 genotypes from Ft. Lauderdale and 6 genotypes from the Florida Keys were tested and 4 genotypes from each region had controls with progressive lesions (Table 4).

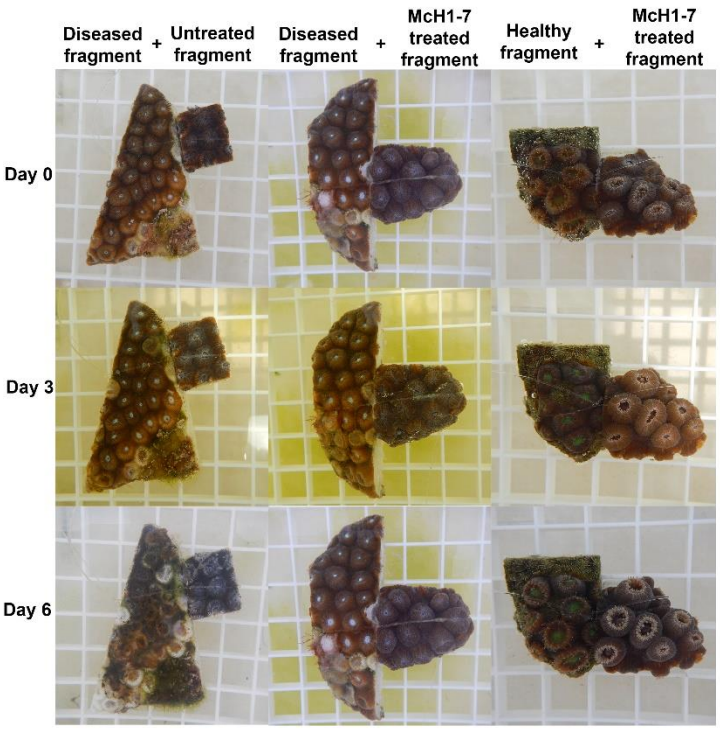

Figure 16. Disease transmission protection with probiotics. Disease progression of diseased *M. cavernosa* McD-12 over a 6-day period. The first column is diseased fragment in contact with the untreated healthy fragment (disease control). The second column is a disease fragment in contact with a healthy fragment pre-treated with the probiotic McH1-7 (experimental). The last column is a healthy fragment in contact with another healthy fragment from a different colony pre-treated with the probiotic McH1-7 (healthy control). No tissue was left on the diseased fragment in contact with the untreated coral after 6 days, while the disease lesion on the fragment in contact with the pre-treated fragment did not progress during the 21-day experiment.

No disease transmission was observed between the diseased control genotypes from Ft. Lauderdale (n=4) while 50% of the disease controls from the Florida Keys transmitted (n=4) (Table 4). None of the pre-treated fragments in contact with diseased coral contracted disease

during the 21-day experiment. Amazingly, all the lesions on diseased fragments that where in contact with corals pre-treated with McH1-7 stopped or had slowed progression compared to their counterparts in contact with untreated corals, which all died completely or continued to progress (*n*=4 for each region) (Table 4). This suggests that the beneficial effects of the probiotic treatment are transferrable from one coral to another coral. This has implications for the utility of probiotics in the field, where disease treatment could potentially be paired with restoration efforts with coral outplants pre-treated with probiotics acting as delivery mechanism.

Table 4. Results from experiments on the effect of McH1-7 on SCTLD transmission.

Ft. Lauderdale corals						
Colony ID	**Diseased frag + non-treated healthy frag**	**Non-treated healthy frag**	**Diseased frag + treated healthy frag**	**Treated healthy frag**	**Healthy control pair**	**VcpA assay**
McD-4 FtL	Progressed over 21 d	No transmission	Stopped -7 d	No transmission	No damage/disease	-
McD-5 FtL	Died -2 d	No transmission	Stopped -7 d	No transmission	No damage/disease	-
McD-8 FtL	Died -14 d	No transmission	Stopped -4 d	No transmission	No damage/disease	-
McD-9 FtL	Died -17 d	No transmission	Slowed	No transmission	No damage/disease	-
Ft. Lauderdale Totals	**1/4 progressed >21 d 3/4 complete mortality**	**0/4 transmitted**	**3/4 stopped 1/4 slowed**	**0/4 transmitted**	**0/4 had disease or damage**	**0/4 positive**
FL Keys corals						
Colony ID	**Diseased frag + non-treated healthy frag**	**Non-treated healthy frag**	**Diseased frag + treated healthy frag**	**Treated healthy frag**	**Healthy control pair**	**VcpA assay**
McD-12	Died -7 d	Disease transmitted	Stopped -6 d	No transmission	No damage/disease	-
McD-13	Died -15 d	No transmission	Stopped -2 d	No transmission	No damage/disease	-
McD-14	Died -4 d	Disease transmitted	Slowed	No transmission	No damage/disease	-
McD-17	Died -6 d	No transmission	Slowed	No transmission	No damage/disease	-
FL Keys Totals	**4/4 complete mortality**	**2/4 transmitted**	**2/4 slowed 2/4 stopped**	**0/4 transmitted**	**0/4 had disease or damage**	**0/4 positive**

Assessment of isolates to create probiotic mixtures

In the natural environment a single probiotic strain is unlikely to be what is conferring protection to its coral host, rather, it would be a collective group or community of microorganisms. Therefore, treatment with multiple probiotic strains is likely to be more effective than single-strain treatments. Therefore, we are evaluating the level of antagonism between our inhibitory isolates (*via* the plate-based assays described above) and their inhibitory activity against the same sets of target microorganisms. The goal is to identify groups of potential probiotics that have a low-level of antagonism between themselves and individually inhibit different microorganisms to increase their collective inhibitory range. Characterization is currently underway. An example of this process is represented in Figure 17, which shows the results from

one of our sets (McH1). For example, a potential pairing is between isolate SMS1, McH1-7 and McH1-42, which have relatively low levels of antagonism among them. Additionally, McH1-7 has higher levels of activity against isolates McT4-15 and Of-T6-21 (Alteromonadaceae and Vibrionaceae, respectively) compared to SMS1, which has higher activity against McT4-56 (Rhodobacteraceae). Conversely, McH1-42 appears to have a moderate level of inhibitory activity against all three target strains McT4-15, McT4-56, and Of-T6-21.

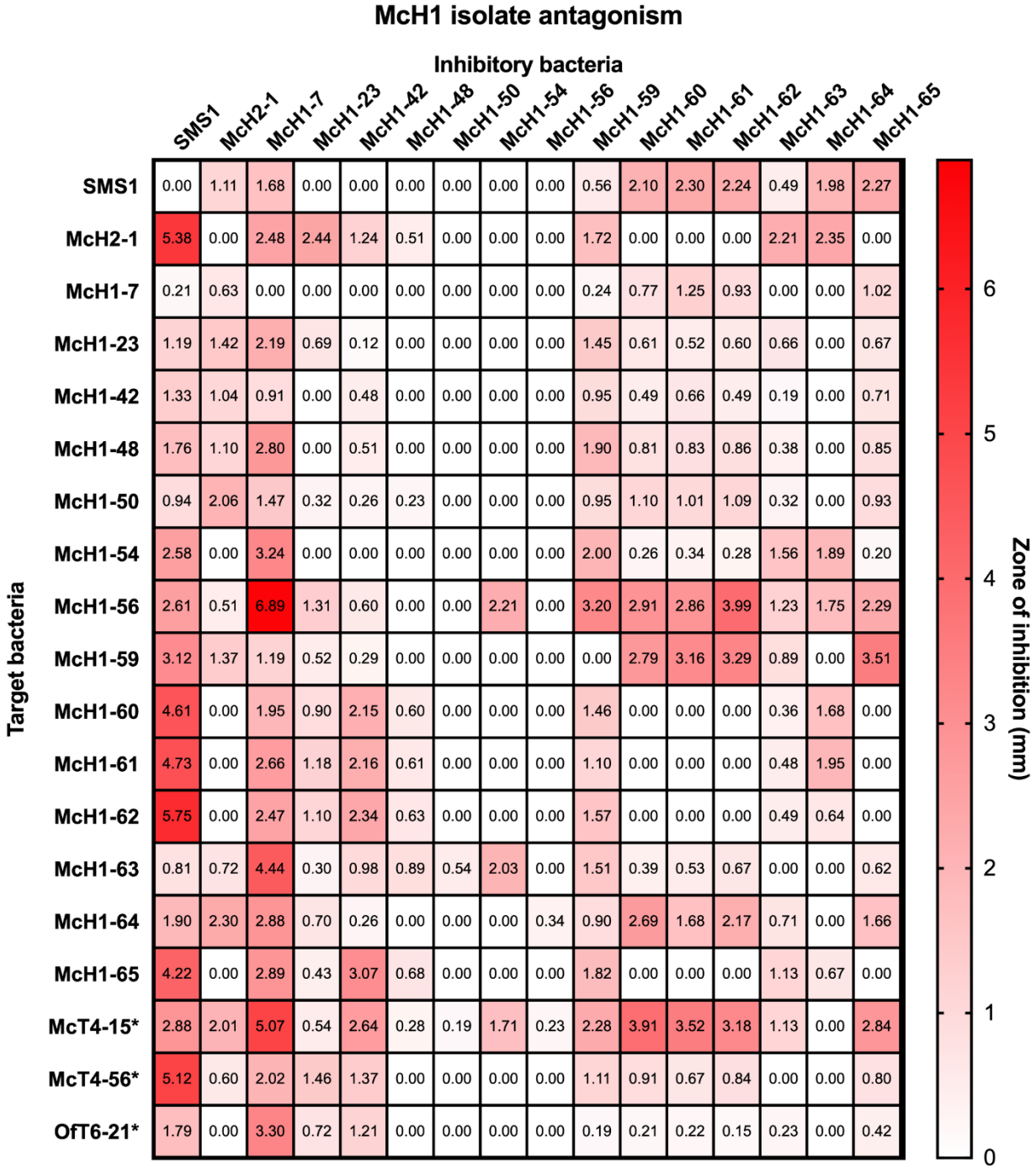

Figure 17. Levels of antagonism between the inhibitory isolates from set McH1 and their activity against isolates from diseased coral. The table is organized with the potentially inhibited bacteria on the y-axis and the inhibitor bacteria listed on the x-axis. Each isolate was also screened against the isolates McT4-14, McT4-56, and OfT6-21 that are from diseased corals (that are from the bacterial families Alteromonadaceae, Rhodobacteraceae, and Vibrionaceae). The values within each square represent the radius of the zone of inhibition in mm.

Results Summary and Future Directions

Task 1: To Identify the Pathogen(s).

- Roughly 400 isolates cultured from various diseased corals infected through laboratory transmission experiments have been screened for virulence.
- A majority of the initial positive hits (initiated disease) were from the bacterial families Rhodobacteraceae, Alteromonadaceae, and Vibrionaceae.
- Tests with individual isolates so far have been inconsistent and have yet to indicate a primary pathogen – suggesting they may be opportunistic colonizers or secondary pathogens.
- Some corals may be succumbing to secondary infections by the pathogenic bacterium *Vibrio coralliilyticus*, which has been detected on diseased *M. cavernosa* from Ft. Lauderdale (2/19 colonies were positive) and the Florida Keys (6/28 colonies were positive).
- The diseased *M. cavernosa* colonies that tested positive for the *Vibrio coralliilyticus* toxin (using a special immunoassay developed for this pathogen) had comparatively faster disease progression than colonies negative for the toxin.
- Continued efforts are being pursued to investigate the correlation between the presence of *V. coralliilyticus* and faster lesion progression.
- Another major focus will be the corals with faster lesion progression that are negative for *V. coralliilyticus* to determine if these coals are succumbing to a different secondary infection and what is the causative agent. By identifying the secondary pathogens first, it may make it easier identify a primary cause of SCTLD.
- A culture-independent metagenomics approach is also being taken to identify potential patterns between healthy and diseased corals to focus our efforts (in collaboration with Dr. Julie Meyer at Univ. of Florida).

Task 2. To Assess Virulence as the Disease Moves South

- 19 colonies (17 diseased and 2 healthy) of *M. cavernosa* with SCTLD were tagged at Looe Key in Nov 2018 and monitored through April 2019
- Monitoring of *M. cavernosa* colonies at Ft. Lauderdale was continued throughout the same time period as Looe Key colonies (13 diseased and 4 healthy).

Lesions on diseased colonies differed among regions

- In the Keys, 53% colonies had acute lesions, 47% had subacute lesions, and 17.6% of the colonies had bleached lesion edges. In October 2018, 58.8% of the colonies at the Ft. Lauderdale had bleached lesions with no recent tissue loss (disease re-emerging), and

17.6% had subacute tissue loss lesions of which 2 colonies had bleached zones along lesions.

Disease prevalence and mortality differed among regions

- In the Keys, disease prevalence in Nov. 2018 was 89.5% and remained high through April 2019. At Ft. Lauderdale, disease prevalence started in Oct 2018 at 76.5% and declined to 43.8% by May 2019.
- Case fatality rate was 52.6% for the Keys and 7.1% for colonies at the Ft. Lauderdale site.
- Average loss of live tissue from colonies was 94.1% in the Keys and 22.3% in Ft. Lauderdale.

Transmission rates differ between regions

- The rates of direct transmission between healthy *M. cavernosa* and diseased *M. cavernosa* from the Keys and Fort Lauderdale were compared in aquaria studies.
- Diseased *M. cavernosa* from the Ft. Lauderdale reefs had a 45.5% transmission success after an average of 3.8 days (range=2-7 days).
- Diseased *M. cavernosa* from the Keys reefs had a 27.3% transmission success after an average of 6 days (range=3-9 days).
- The processes affecting disease initiation (primary pathogen + environment) and disease progression (primary pathogen + possible secondary pathogens + environment) can vary. Disease initiation (transmission) was similar among regions but disease progression in the field (tagged colonies) was faster and more virulent in the Keys.
- The aquaria studies suggest that the infectivity of the pathogen (ability to initiate disease) is similar between regions and so there is no evidence of the evolution of reduced virulence through time at Fort Lauderdale. Rather, there appears to be environmental co-factors and/or differences in susceptibility of the corals in different regions affecting the degree of colony mortality in the field.

Task 3. Determining the Ecology of Tissue Loss Disease in *Siderastrea siderea*

- Currently we are following 7 tagged *S. siderea* at our site off Ft. Lauderdale (near FtL5) and they have remained healthy for the duration of the study. We are in contact with FWC to review pictures from the diseased *S. siderea* from their permanent plots.

Transmission between diseased S. siderea and healthy S. siderea and O. faveolata

- Aquaria studies examined the transmissibility of diseased *S. siderea* to healthy *S. siderea* and *O. faveolata*.
- Three of the 10 healthy *S. siderea* fragments (30%) touching a diseased *S. siderea* developed lesions after an average of 13.5 days and one of 10 non-touching *S. siderea* developed a lesion (10%) after 8 days.
- Transmission to *O. faveolata* was more successful with 6 out of 10 (60%) of the touching fragments developing a lesion after an average of 7.5 days and 2 of 10 (20%) of the non-touching fragments developing disease signs after an average of 7.5 days.

S. siderea is vulnerable to infection from diseased C. natans

- Aquaria studies examined whether *S. siderea* was vulnerable to infection by *C. natans* with acute tissue loss disease.
- All five healthy *S. siderea* fragments (100%) touching a diseased *C. natans* developed lesions after an average of 8.4 days and 80% of the non-touching fragments developed lesions after an average of 7 days.

S. siderea lesions do not respond to antibiotic treatment (kanamycin & amoxicillin)

2018

- *S. siderea* with lesions were treated with antibiotics (with an untreated control) to determine whether bacteria might be involved in the disease process.
- 6 of 11 pairs (54.5%) of diseased fragments (control and treatment) had the same and approximately equal lesion response during the experiment, e.g. antibiotic treatment did not change the course of the lesion.
    - For 3 pairs, both fragments showed signs of healing, in 2 pairs both fragments showed equal progression and in one pair neither fragment showed lesion progression.
- In 3 pairs, the treated fragment had lesion progression while the control fragment healed.
- One pair, the treated fragment had slower progression as compared to the control.
- One pair, the treated fragment progressed whereas the control showed no change.
- A treatment regime of amoxicillin and kanamycin was not effective at stopping lesion progression with *S. siderea*.

2019

- The antibiotic experiment was repeated in 2019 with a daily dose of kanamycin and amoxicillin. Seven pairs of diseased *S. siderea* were used.
- A similar result, as in 2018, was observed with no consistent pattern of lesion cessation or slowing on coral fragments treated with antibiotic.
- In 2 pairs, the disease progressed in both fragments.

- In one pair, the disease started to heal in both fragments.
- In 2 pairs, the treated fragment had lesion progression and the control showed no change.
- In one pair the treated fragment progressed and the control fragment started to heal.
- In one pair the treated fragment showed no change and the control fragment started to heal

Although *S. siderea* is not considered a primary reef builder on Florida's reefs, it is now becoming one of the more abundant species as SCTLD kills off the more susceptible species. Taking a proactive approach to understand health in this coral species would be prudent.

Task 4: To Develop Effective Probiotics to Treat and Prevent SCTLD

- A library of over 600 inhibitory isolates is being characterized that were originally isolated from apparently disease-resistant corals.
- One isolate, McH1-7, stopped or slowed tissue loss when inoculated onto disease *M. cavernosa* from Ft. Lauderdale (9/10 diseased fragments) and the Florida Keys (n=5/5 diseased fragments).
- Treatment of healthy corals with McH1-7 may protect them against disease transmission (*n*=8), but the beneficial effects of this probiotic could possibly be transferred from the treated fragment to touching diseased fragments.
- Experiments are being designed to improve the distribution delivery of probiotics to corals in the field.
- The library of inhibitory isolates is also being analyzed to identify potential strains that can be used in combinational treatments (multiple probiotics) that may be more effective than treating with a single strain.
- Targeted treatments are being developed towards *V. coralliilyticus* to improve the effectiveness of our treatments, which includes probiotics specifically found to be effective against this pathogen and potentially the development of bacteriophages specific to Florida strains of *V. coralliilyticus* (in collaboration with Dr. Gary Richards, USDA-ARS).

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