Project Title: Studies on environmental co-factors potentially influencing the disease dynamics of Florida's coral tissue loss diseases

PIs: Greta Aeby, Valerie Paul (SMS); **Co-PIs:** Jan Landsberg, Yasu Kiryu (FWRI-FWC)

Disease dynamics through time

At our two initial field sites, Looe Key and Fort Lauderdale, we found differences in lesion appearance (Ft. Lauderdale: subacute often with a bleached band vs. Keys: acute to subacute tissue loss usually lacking a bleached band) and in mortality on tagged *M. cavernosa* colonies with tissue loss disease. Tagged *M. cavernosa* colonies at Looe Key had an 84.2% mortality rate after one year but only 15% of the Fort Lauderdale colonies died after a year. There could be regional differences in environmental conditions, which could be affecting *M. cavernosa* health and immune response, or are affecting presumptive pathogen-host dynamics, allowing the presumed pathogen(s) 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 a natural progression in disease-host dynamics with presumed pathogens or other etiologic factors evolving to a less virulent state. Coral disease outbreaks are often more virulent at the start of an outbreak and then prevalence and severity decrease through time (Brandt et al. 2012, Aeby et al. 2016).

During our 2019 tagging study, we found that the mortality rate of *M. cavernosa* at Looe Key declined from 73.7 % mortality (n=17) between Nov 2018 and May 2019 to 21.4% mortality (n=14) between Nov 2019 and May 2020. There was also a shift in gross lesion morphology with all diseased colonies tagged in 2018 having tissue loss lesions whereas in 2019 the dominant lesion was that of bleached polyps (71.4%) with the remainder being subacute tissue loss. This suggests that the differences in mortality we found among diseased *M. cavernosa* colonies at Looe Key vs. Fort Lauderdale are explained, in part, by temporal processes. The 2nd site set up in the Keys (Haslun's reef) appears to be in an earlier stage of SCTLD progression with 100% of the lesions having tissue loss (n=8) and 75% mortality occurring between November 2019 and May 2020. We are continuing to monitor tagged colonies at all three sites (Table 1) to determine whether the pattern of reduced disease severity continues through time.

Task 1. Compare lesion morphology and mortality through time among diseased *M. cavernosa* and *S. siderea* colonies in the Keys and Fort Lauderdale sites

Methods

All colonies in both regions were photographed approximately every other month or as possible. Colonies in the Keys were photographed by Mote Marine Lab (11.18.19, 1.15.20, 3.23.20, 5.27.20, 7.27.20, 9.28.20, 12.5.20, 5.6.21), and colonies at Fort Lauderdale by Broward County Environmental Protection and Growth Management Department personnel (11.22.19, 4.16.20, 7.2.20, 10.12.20, 1.8.21, 3.4.21, 5.27.21).

Table 1. Sites established for monitoring disease dynamics through time.

Region	Site	Depth (m)	Latitude (N)	Longitude (E)
Ft Lauderdale	FtLT	8.2	26.14858	80.09591
Lower Keys	Tag site (Looe)	7.3	24.54599	81.404
Lower Keys	Haslun's reef	7	24.55234	81.43745

Results

Ft. Lauderdale (disease emerged in 2014-15)

At Ft. Lauderdale, disease on tagged *M. cavernosa* remains on the reef after over 3 years but continues to fluctuate at a lower prevalence through time (Fig 1). Colony survival remains high with 65% of the tagged colonies still alive after 3 years (Fig. 2). Although SCTLD remains on the reef the rate of tissue loss from disease has declined (Fig. 3). Eight healthy *M. cavernosa* were tagged at the site in June 2018 and seven out of eight colonies (87.5%) developed lesions at some point between June 2018 and October 2020 (Fig. 4). Six out of the seven developed bleached polyps (85.7%) and one out of the seven developed bleached polyps with tissue loss (14.3%). Of the seven newly infected colonies, three (42.9%) went on to develop tissue loss resulting in colony morbidity (partial colony mortality). Disease prevalence in newly infected colonies fluctuated through time (Fig. 5).

Lower Keys-Tag site (Looe Key) (disease emerged in 2018)

At the Tag site, disease prevalence declined through time and is starting to show variability similar to colonies at Ft. Lauderdale (Fig. 1). Colony mortality remains low with 78.6% of the colonies still alive in May 2021 (Fig. 2). Twenty-four healthy *M. cavernosa* colonies were tagged in Nov 2019 and 12 out of 24 colonies (50%) developed lesions at some point during the study (Fig. 4). All 12 colonies developed bleached polyps and two out of those (18.2%) developed tissue loss resulting in colony morbidity. Disease prevalence in newly infected colonies fluctuated through time (Fig. 5).

Lower Keys–Haslun's reef (disease emerged in 2019)

At Haslun's reef, prevalence remained high throughout the study (Fig. 1) and colony survival between Nov 2019 and Dec 2020 was 0% (Fig. 2). Twelve healthy *M. cavernosa* were tagged at the site in Nov 2019 and eight of those (66.7%) developed lesions at some point during the study (Fig. 4). Five of the eight newly infected colonies developed bleached polyps with tissue loss

(62.5%) and 3 of the 8 developed bleached polyps (37.5%). Six of the 8 (75%) newly infected colonies developed progressive tissue loss and suffered colony morbidity. Disease prevalence in newly infected colonies fluctuated through time (Fig. 5).

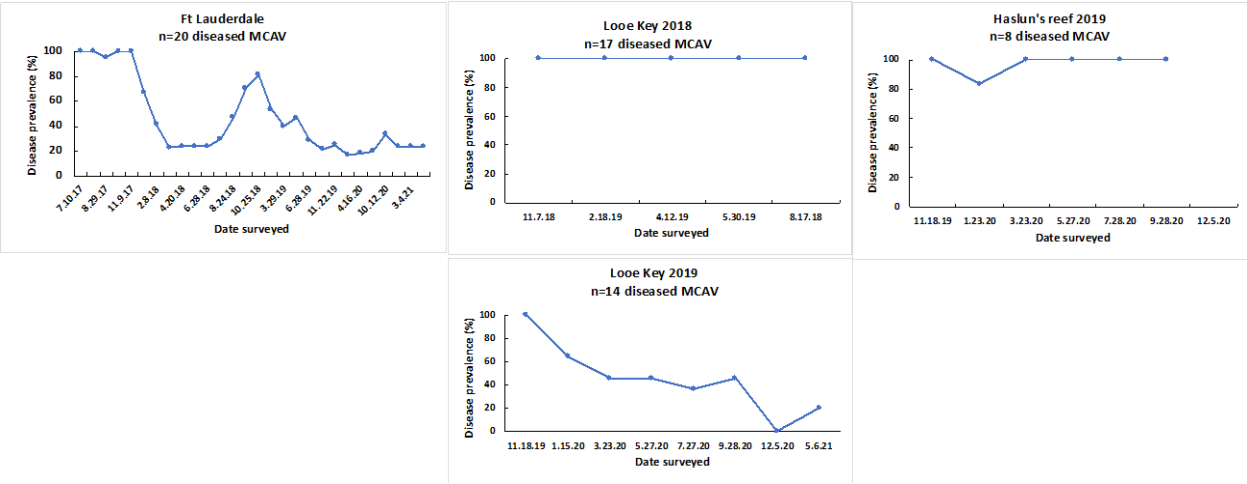

Figure 1. Changes in disease prevalence through time at three sites where SCTLD emerged at different times. Estimated times of disease emergence is 2014-15 in Ft. Lauderdale, 2018 at Tag Site (Looe Key) and 2019 at Haslun's reef.

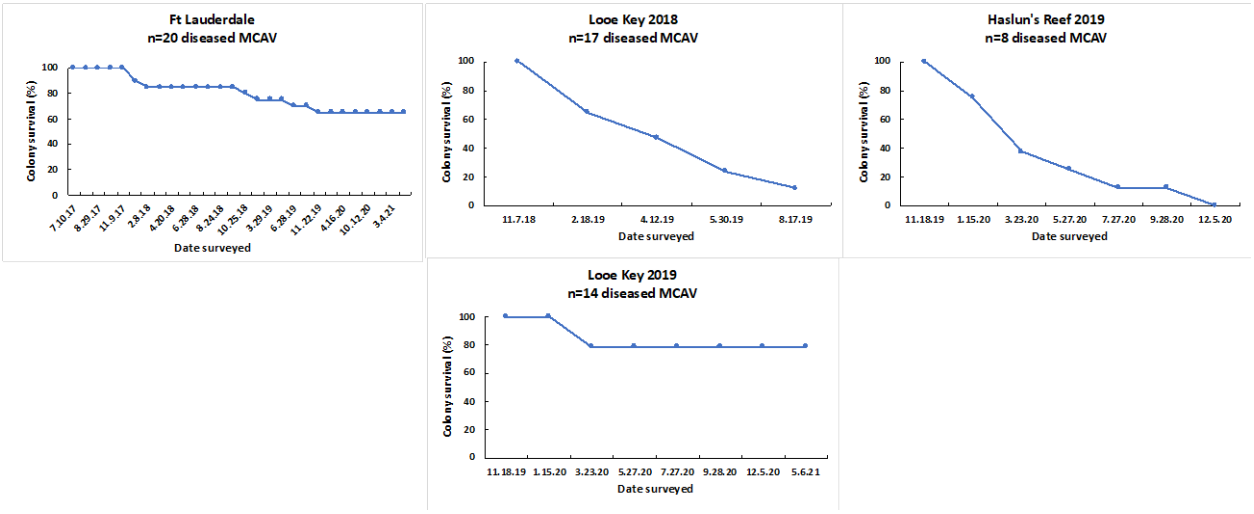

Figure 2. Differences in colony survival through time at three sites where SCTLD emerged at different times. Estimated times of disease emergence is 2014-15 in Ft. Lauderdale, 2018 at Tag Site (Looe Key) and 2019 at Haslun's reef.

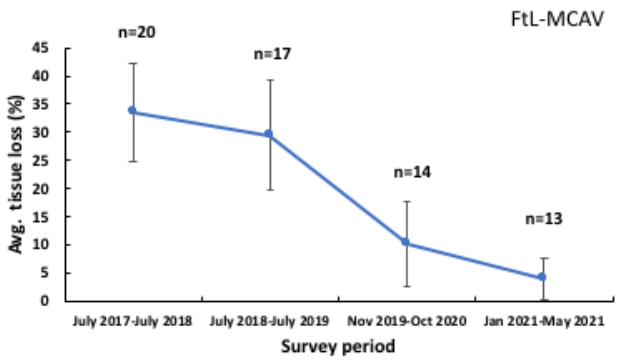

Figure 3. Changes in percent tissue loss from SCTLD through time in tagged *M. cavernosa* colonies at Fort Lauderdale. Data represent the mean and standard error.

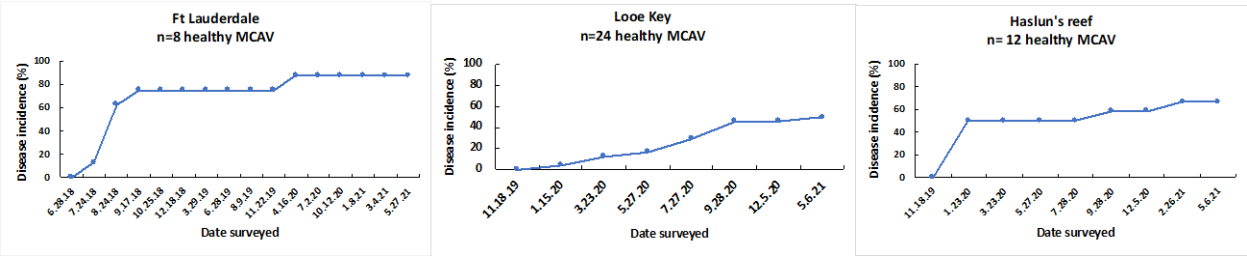

Figure 4. Cumulative disease incidence through time for healthy *M. cavernosa* tagged at three sites where SCTLD occurs.

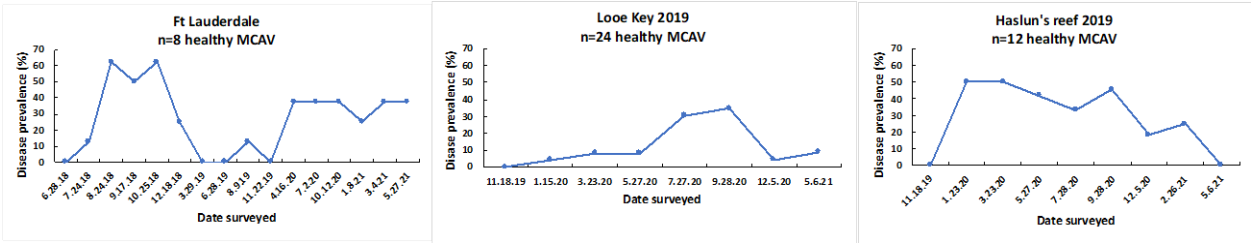

Figure 5. Fluctuations in disease prevalence on newly infected *M. cavernosa* colonies tagged at three sites where SCTLD occurs.

Disease in *S. siderea*

For the current Florida disease outbreak, the disease signs vary among affected coral species with differences in rate of tissue loss, lesion morphology and occurrence. One coral species, *S. siderea*, displays unusual lesions with multi-focal bleached spots, spots of purple discoloration or discolored spots (bleached or purple) with tissue loss. So, the question remains as to whether this species is affected by the same presumptive pathogen(s) or etiological factors

as the other coral species affected by SCTLD, but disease signs vary or if this coral has a different disease altogether. Initial histopathological surveys of *S. siderea* with one gross lesion type (multifocal tissue loss with purple pink discoloration) show that the hallmark lytic necrosis lesions common to other scleractinian corals affected by SCTLD are also present (Landsberg et al. 2020.). Through manipulative studies, we have shown that *S. siderea* with lesions is a transmissible disease, but it does not respond to antibiotics in the same manner as other coral species with SCTLD-type lesions (*Meandrina meandrites, Montastraea cavernosa, Colpophyllia natans*) that have been tested. This suggests that the etiology of the disease affecting *S. siderea* differs, in part, from the other species affected in the SCTLD outbreak, or that the antibiotics are treating secondary bacterial infections that are not present and involved in advanced tissue loss in *S. siderea*. We also noted that during our preliminary transmission studies there were differences in transmission success dependent upon the gross lesion morphology. Fragments presenting with bleaching with tissue loss transmitted more readily than fragments having other lesion types. However, our sample sizes were too small to adequately evaluate this. Some coral species respond to any irritation with a pigmentation response and such associated lesions do not usually indicate disease. For example, compromised tissues of *Porites* spp. in the Indo-Pacific express red fluorescent proteins which produce a pink lesion whether from disease or other natural processes such as algal abrasion, competition, etc. (Palmer et al. 2009). During coral reef surveys that assess the health of the reef, it is important to be able to distinguish between disease and non-disease lesions.

Results

Ft. Lauderdale – FtLT

Six tagged colonies with multi-focal bleached lesions were followed between Nov 2019 and May 2021. Disease prevalence fluctuated during that time period (Fig. 6) and no colonies died (Fig. 7). Change in percent healthy tissue on colonies ranged from -50% to +100% with an average change of +5.5% (lesions on colonies re-pigmented).

Keys – Haslun's reef

A total of 18 colonies with disease lesions (purple or dark discoloration with or without tissue loss) were followed between Nov 2019 and May 2021. Disease prevalence remained 100% throughout the study period (Fig. 6) and case fatality rate was 11.1% (Fig. 7). Average amount of healthy tissue declined by an average of 51.1% (range = +100% to -100) between Nov 2019 and May 2021.

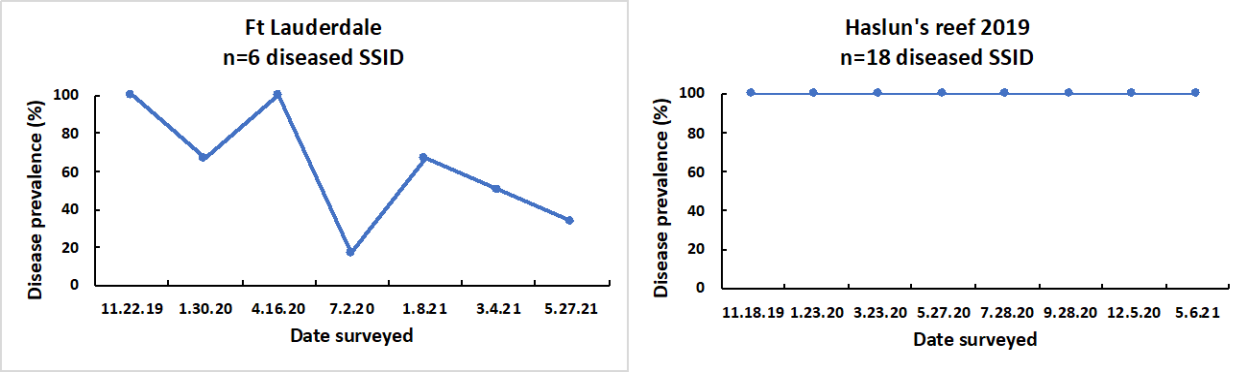

Figure 6. Regional differences in disease prevalence through time for diseased tagged colonies of *S. siderea*.

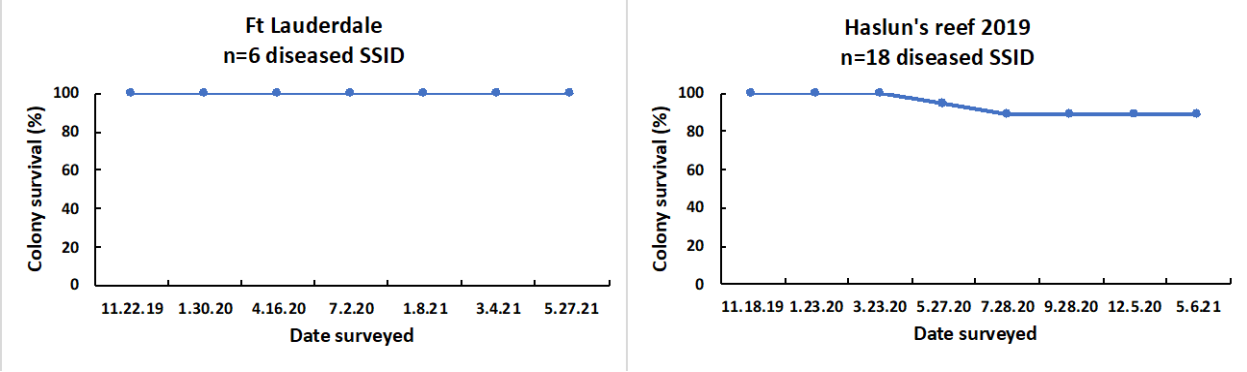

Figure 7. Regional differences in colony survival for diseased *S. siderea*.

Task 2. Test for differences in transmission success among *S. siderea* lesion types in aquaria studies using *Sidastrea siderea* and *Orbicella faveolata* as test corals.

We were able to test 7 more diseased *S. siderea* fragments with different types of lesions increasing our sample sizes from prior studies. Aquaria studies were completed using the same design as in 2018. Briefly, 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. faveolata*) 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. faveolata*) 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

Results were similar to what was found during prior studies with successful disease transmission (development of a lesion) occurring for both species, *S. siderea* and *O. faveolata* upon direct contact with a diseased *S. siderea* and through the water column. One of the 7 healthy *S. siderea* fragments (14.3%) touching a diseased *S. siderea* (lesion=chronic tissue loss) developed lesions after 13 days but none of the non-touching *S. siderea* developed lesions. Transmission to *O. faveolata* was more successful with 2 out of 7 (28.6%) of the touching fragments developing a lesion (bleached lesion) after an average of 18.5 days and 1 of 7 (14.3%) of the non-touching fragments developing disease signs after 19 days. This suggests that the lesions on *S. siderea* are transmissible (contact and through the water) and there are differences among species in susceptibility.

Differences in transmissibility among lesion types

The lesions on diseased *S. siderea* differed in morphology, and during our experiments in 2018 we noticed variability in transmission success among lesions types. We combined our results from 2018 with our 2021 experiments to get a clearer picture of these potential differences. We categorized the initial lesions on the diseased *S. siderea* as purple discoloration with bleaching, purple discoloration with tissue loss, bleaching, or bleaching with tissue loss. We then calculated the transmission success (2018 and 2021 experiments combined) among the different lesion types overall and for each test species (*S. siderea* and *O. faveolata*) and found that bleached lesions with evidence of tissue loss were the most likely to transmit under experimental conditions as compared to other lesion morphologies (Table 2).

Table 2. Differences in transmission success among lesion types in *S. siderea* and between species (*S. siderea* and *O. faveolata*). P=purple, TL=tissue loss, bl=bleaching, cTL=chronic tissue loss, SSID=*S. siderea*, OFAV=*O. faveolata*.

SSID	tot transmission=4/18=22.2%			
lesion types	2019	2021	tot x lesion	tot transmission
P+TL	1/3	0/1	1/4	25%
bl	0/1	0/2	0/3	0
bl+TL	2/4	0/1	2/5	40%
P+bl	0/3	0/2	0/5	0
cTL	0	1/1	1/1	50%
OFAV	tot transmission =9/18=50%			
lesion types	2019	2021	tot x lesion	tot transmission
P+TL	2/3	0/1	2/4	50%
bl	1/1	1/2	2/3	66.7%
bl+TL	4/4	1/1	5/5	100%
P+bl	0/3	0/2	0/5	0
cTL	0	0/1	0/1	0
tot	11 prs	7 prs	18 prs	

Siderastrea siderea histopathology

Methods

A total of 16 *S. siderea* samples were collected and preserved in 1 part Z-Fix (zinc formalin; Z-Fix concentrate; 18.5% formaldehyde; Anatech Ltd. Battle Creek, MI) mixed with 4 parts 0.2 µm–filtered natural seawater (or 35 salinity artificial seawater) for histology. These 16 samples comprised diseased specimens exhibiting diverse lesions from Florida (8 from Looe Key [LK; 24.54599°, –81.404°, collected 18 November 2019], 5 from Haslun's Reef [HR; 24.55234° – 81.43745°, collected 19 November 2019], and 3 from Ft Lauderdale, [FtLT, 26.148585°, – 80.095915°, collected 22 November 2019]. Six apparently healthy reference coral samples were also collected for histological examination from the Dry Tortugas (DT, from 3 sites: 24°35.608, –82°58.862°, 24°38.564, –82°58.161°, and 24°36.316, –82°58.148°) during 28- 29 January 2020).

Post-fixed samples were shipped to the FWC/FWRI lab at St. Petersburg, FL for processing with routine paraffin embedded histological specimens. Before processing tissue for histological slides, all fragment samples were close-up photographed using a digital camera with a macro lens (Nikon, Tokyo, Japan). Diseased fragment samples exhibiting grossly visible lesions were further examined under higher magnification with a dissecting microscope attached to an Olympus DP72 digital camera (Tokyo, Japan), and photomicrographs taken.

For ease of orientation and maintaining coral integrity, samples were enrobed with 1.5% agarose to hold tissues (under a heat vacuum oven [60°C] at 22 mm Hg (Jones and Calabresi 2007) and

associated surface biota in place after the skeleton was removed following decalcification processing with 10% ethylenediaminetetraacetic acid (EDTA, Fisher Scientific, $Na_2 \cdot 2H_2O$; MW = 372.1) solution. After all the aragonite skeletons had dissolved away, decalcified, some of selected soft tissues were again placed under a dissecting microscope and photographed prior to histological processing.

Decalcified tissues were organized for sectioning orientation at both radial (cross, parallel to the polyp mouth) and sagittal (longitudinal, perpendicular to the polyp mouth) angles. Routine paraffin embedded histologic sections were cut at 4 µm, stained with Mayer's hematoxylin and eosin (H&E) and thionin stains (Luna 1968). Tissues were also embedded with glycol methacrylate plastic resin (JB-4; Electron Microscopy Sciences, Hatfield, PA) with arbitrary angle, sectioned at 4.0 µm, and stained with Weigert's iron hematoxylin and eosin (H&E), thionin, and periodic acid–Schiff–metanil yellow (PAS-MY; Quintero-Hunter et al. 1991). Slides were examined with an Olympus BX51 light microscope equipped with an Olympus DP71 digital camera (Olympus Inc., Tokyo).

Histological parameters taken were as follows: lytic necrosis occurring at the gastrodermis, coagulative necrosis in the general tissues, changes in the symbiotic microalgae, colloquially called zooxanthellae (such as PAS reaction, hypertrophy, atrophy, necrosis, symbiosome condition), abundance of coral-acid rich protein (CARP) granules at the surface area, mucus abundance and the staining condition with Thionin (brown) and PAS (red), and presence of other miscellaneous organisms, such as endolithic sponges were noted.

Results

Siderastrea siderea colonies at the different locations sampled for histology appeared to grossly exhibit either single or multiple lesion types that were focal or multifocal. Diseased *S. siderea* colonies at the three locations in Florida were observed in the field to exhibit (1) multifocal bleached spots (bleaching discoloration, BD), (2) spots of purple discoloration (PD) (i.e., varying degrees of dark to light purple discoloration, or combinations of lesion types), (3) bleached spots with tissue loss (BD/TL), or (4) purple discoloration spots with tissue loss (PD/TL).

The gross and enlarged gross appearance of the PD and BD lesions on colonies in the field is compared to macroscopic observations of the biopsy samples post-fixed in the lab (Fig. 8, Table 3). The apparent gradation of some lesions in the field that grossly showed PD is visually compared following sample fixation and when macroscopically viewed under artificial light conditions in the lab (Fig. 8). In some cases, PD changed in degree of intensity, faded, or disappeared with time (or under fixed conditions). Grossly, reference samples collected from the Dry Tortugas (DT) appeared to be clinically healthy (i.e., none of them had PD, BD, nor TL). Generally, the epidermis surface especially at the septal ridge to the outer edge of the corallite

appeared to be white in the DT specimens. Color leached out presumably due to tissue over fixed in Z-Fix.

PD determined grossly in the field was separated macroscopically by the visual appearance of PD along the septa (septal PD) contrasted against the color of oral lesions. Grossly, oral lesions could appear grey-purple (Fig. 8A, D) or white-beige (Fig. 8B, E), but macroscopically were pink-purple (Fig. 8G) or white beige, respectively (Fig. 8H). The purple discoloration and the varying degrees of intensities reflected the development and extent of the lesion and the expansion of the lesion in individual polyps. Histologically, the oral lesions were seen to have lytic necrosis (Landsberg et al. 2020) with sloughing of necrotic tissue into the oral cavity (Fig. 9). Healthy reference samples obtained from DT exhibited no tissue necrosis including lytic necrosis at the gastrodermis nor observable bleaching of the zooxanthellae.

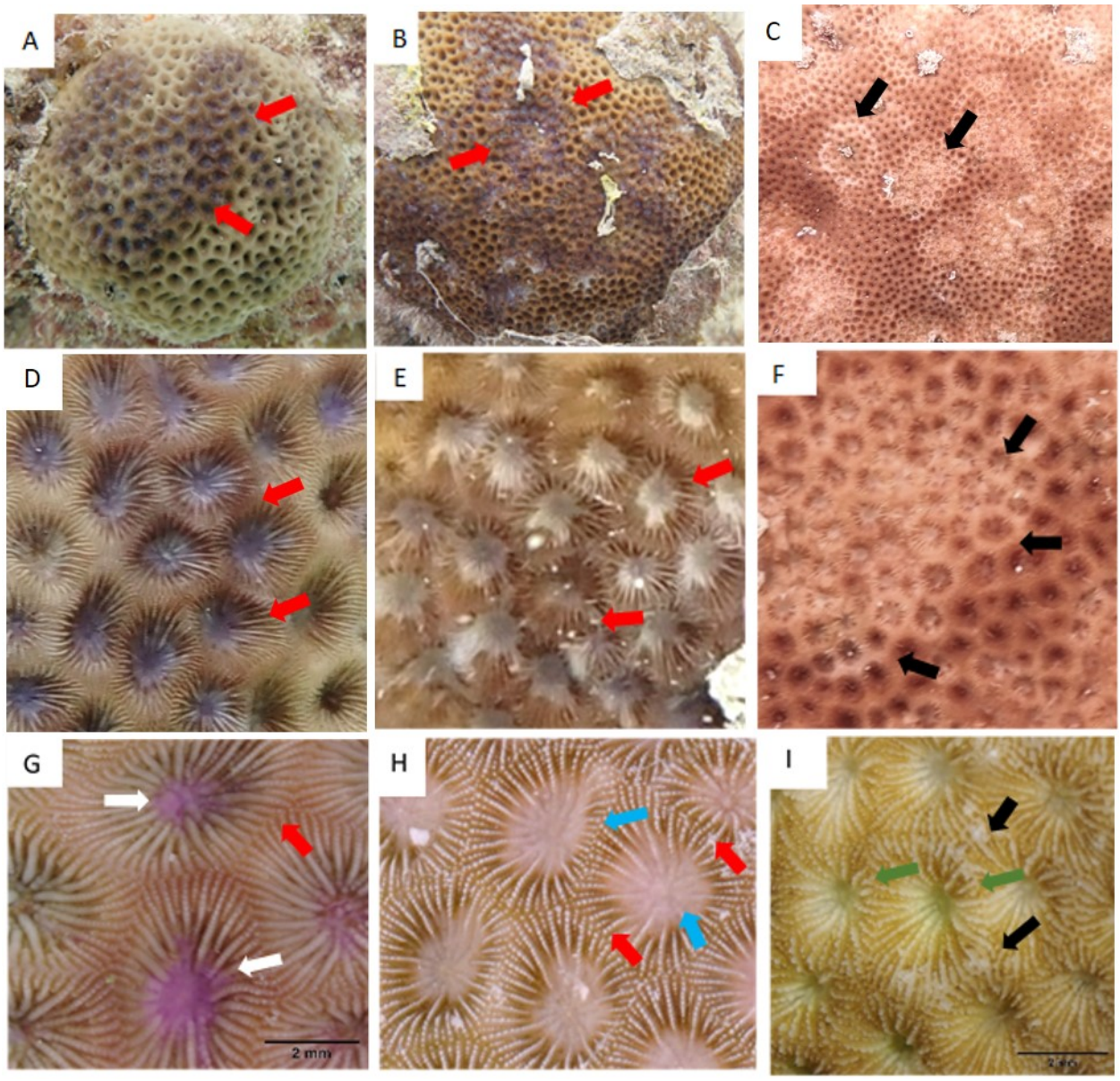

Figure 8. Gross (A-C) and enlarged gross (D-F) observations of purple discoloration (PD) (A-B, D-E) and bleaching discoloration (BD) (C, F) in colonies compared to macroscopic views (G-I) of post fixed samples showing dark coloration of septal ridges (red arrows) contrasted against grey-purple (white arrows; D, G) or white-beige oral lesions (blue arrows; E, H), or gross bleaching on septal ridges (black arrows) contrasted against white-green endolithic oral lesions (green arrows; F, I). A, D, G = specimen #7 from LK; B, E, H specimen # 1 from LK, and C, F, I, specimen #16 from FtLT.

Table 3. Comparison of gross field descriptions of lesion types with macroscopic observations of core samples post fix.

Site	N	Gross lesion			Macro/microscopic lesion post fix				
		BD	PD	TL	Septal BD	Oral lesion (PD)	Septal PD	Oral lesion (no PD)	TL
LK	8	0	5	8	2	2	6	4	8
HR	5	0	2	5	1	2	3	1	5
FtLT	3	2	2	1	2	1	2	2	3
DT	6	0	0	0	0	0	0	0	0

BD = bleaching discoloration, PD = purple discoloration, TL = tissue loss
LK = Looe Key, HR = Haslun's Reef, FtLT = Fort Lauderdale, DT = Dry Tortugas

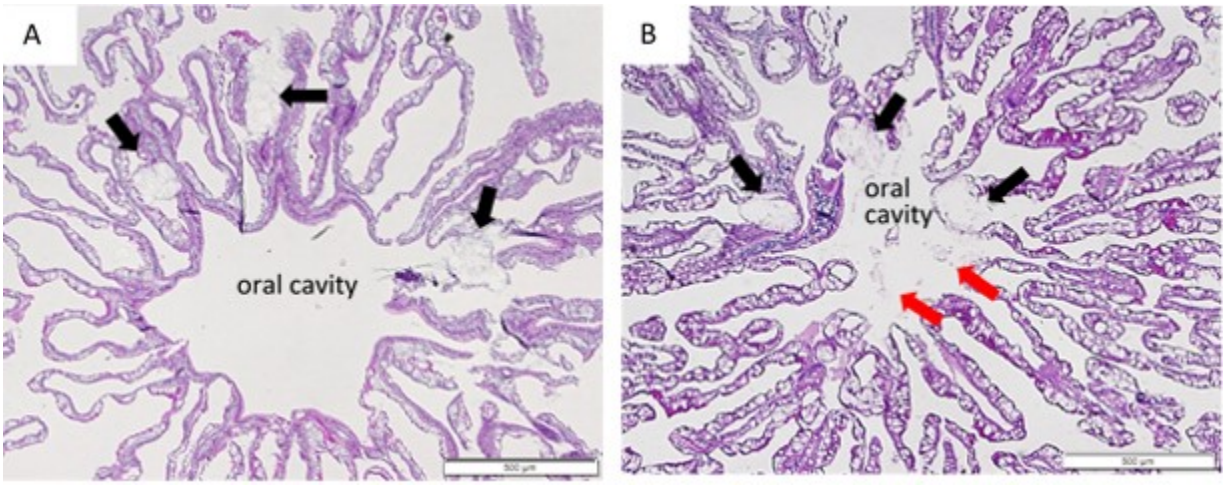

Figure 9. Radial histologic sections (H&E) across the oral cavity of specimen #8 (A) from LK compared to specimen #13 (B) from FtLT showing similar lytic necrosis lesions (black arrows) and sloughing of necrotic tissue into the oral cavity (red arrows).

Although initially observed only in two specimens at FtLT, BD was also observed macroscopically in two specimens from LK and 1 specimen from HR. There was no apparent difference between bleaching at the sites, rather BD could be a progressive stage in the development of oral lesions (Fig. 9) or bleaching along the septal ridge (Figs. 8 C, F, I; 10, 11),

both of which can lead to TL (Figs. 9-11). Bleaching (loss of zooxanthellae) was histologically confirmed at all three sites (62.5–80%; Figs. 10, 11).

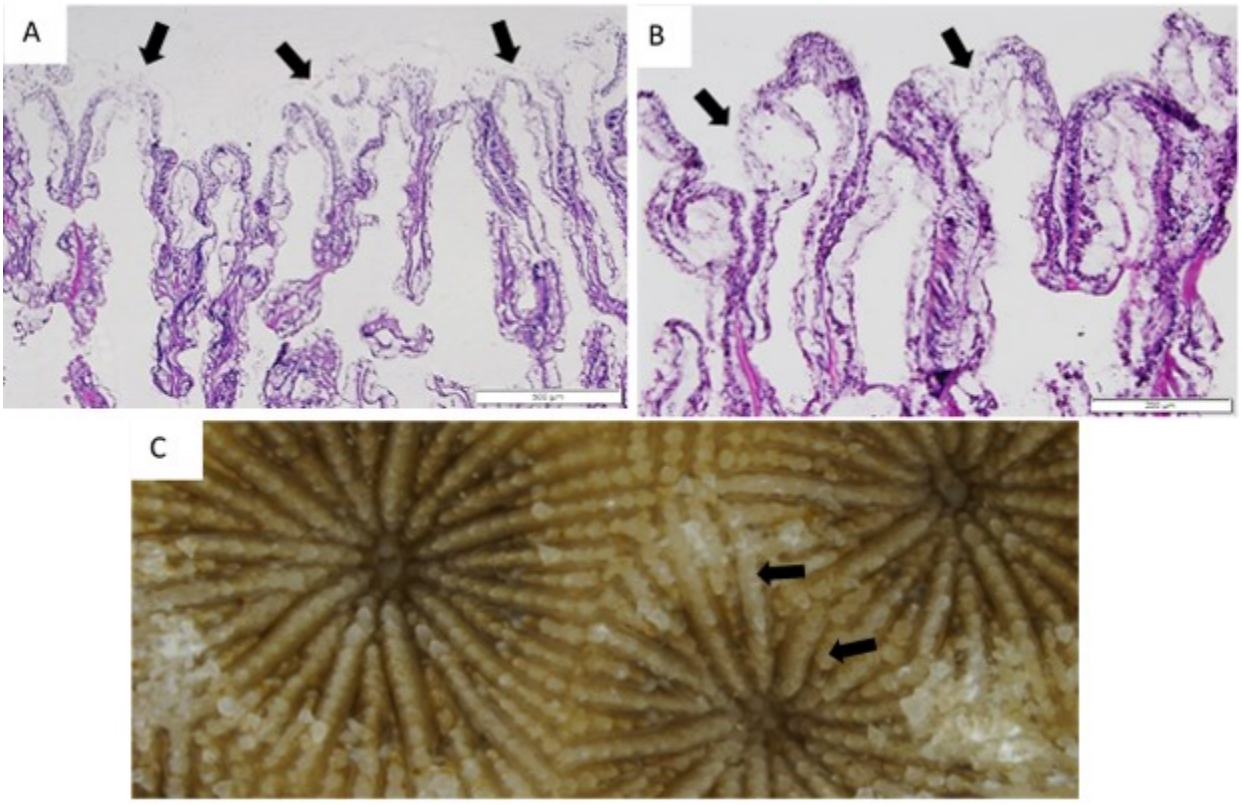

Figure 10. Sagittal histologic sections (H&E) along the septa of specimen #8 (A) from LK compared to specimen #15 (B) from FtLT showing surface bleaching and early TL lesions compared to the macroscopic appearance of the corallite septa with bleaching and TL in specimen #16 from FtLT (C, black arrows).

Additional histological observations were noted for descriptive purposes (Table 4). It is unknown from this preliminary investigation to what extent any of these were significant in the appearance or response to lesions. All diseased samples examined histologically exhibited TL, with early lesions showing lytic necrosis (LN) (n =13) characteristic of SCTLD found at the gastrodermis at both basal and surface body wall throughout three sites (Fig. 9; 62.5–100%). However coagulative necrosis (Fig. 13A; N = 10) in advanced TL was commonly found at the grossly observable lesion border, especially at LK (87.5%) with less prevalence at HR (40%) and FtLT (33.3%). This supports the progression of disease observed in the field that LK site was sub-acute compared to HR and FtLT sites that were more likely acute.

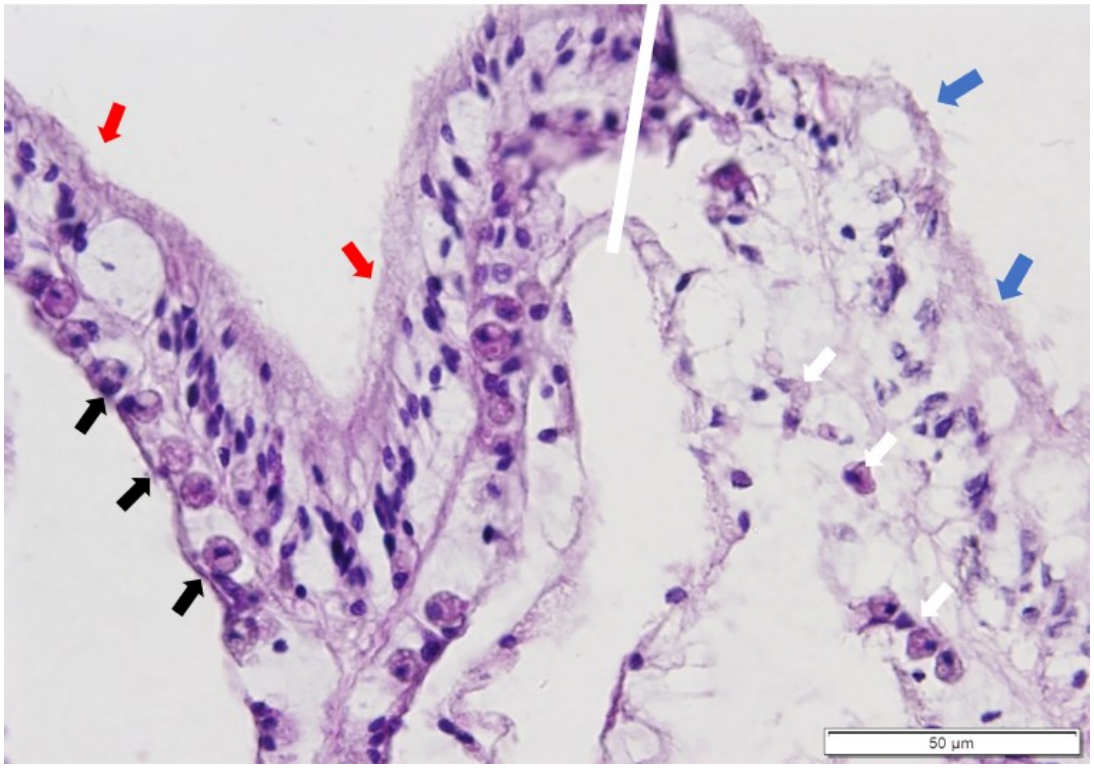

Figure 11. Sagittal histologic section (H&E) along the septa of *S. siderea* specimen #16 from FtLT showing border (white line) between surface bleaching (blue arrows) with reduced density of, loss of, or *in situ* necrosis of zooxanthellae with abnormal appearance (white arrows), and apparently healthy tissue (red arrows) with zooxanthellae (black arrows). Note abnormal appearance of remnant zooxanthellae (white arrows) and general loss of cytoplasm or increased mucus (white space) in lesioned area.

Sponges, some of which were degraded (along with spicules and sometimes associated with eosinophilic granulocytes), were detected in the skeletal tissue especially at the aboral region and found at LK (37.5%) and HR (80%). Only one sample (#11, HR) had a clionid (a boring sponge with zooxanthellae) near the septal surface and in the basal area of the skeletal tissue. Coral-acid rich protein (CARP) granules in the calicodermis were generally present in all specimens examined (characteristically present in *S. siderea*, and notably eosinophilic in H&E), and multifocally, prominently aggregated CARPs (Fig. 13B) were found in specimens from all the sites (60–66.6%). Possibly, this aggregation reaction is a host response to some of the endolithic organisms. In the samples obtained from DT, CARPs were barely present or were less dense in the calicodermal tissue located approximately 300 to 600 µm just below the surface (as viewed histologically in sagittal section). Gradually CARPs increased in density in the calicodermis in the middle of the polyp towards the aboral end and appeared to be at maximum density at the aboral side. Here, CARPs were dispersed (Fig. 12E, F).

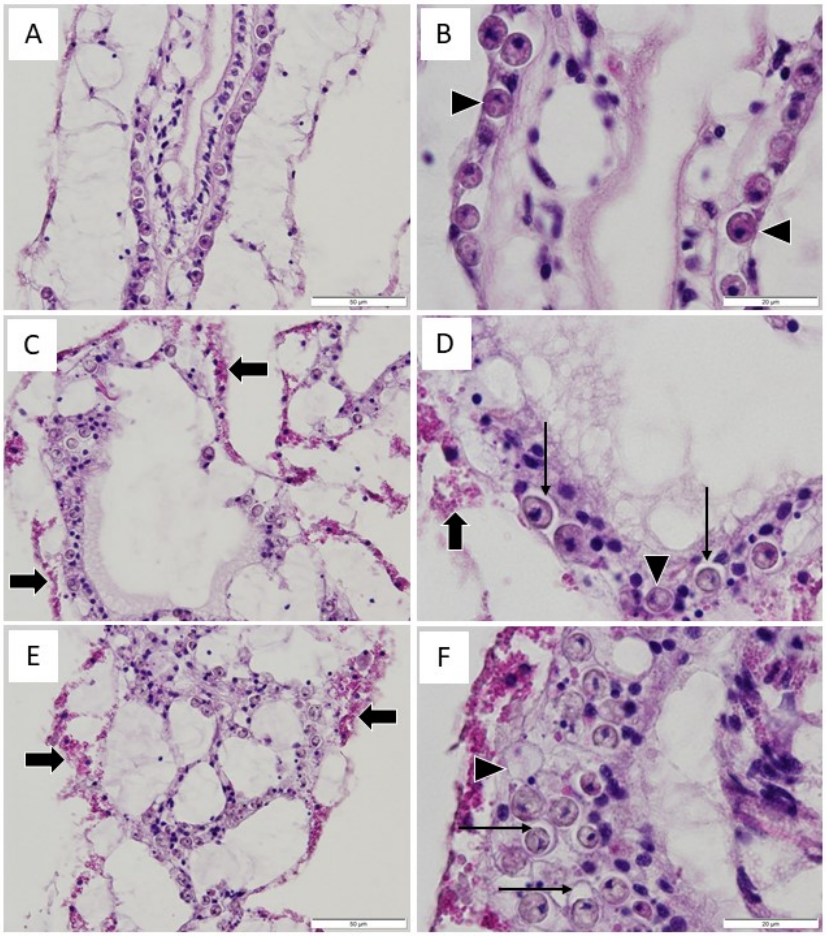

Figure 12. Histologic sections of apparently healthy *Siderastrea siderea* from the Dry Tortugas (#1 DT) showing the distributions of the coral-acid rich protein (CARP) granules resided at the surface (A [low mag] and B [high mag]), middle (C [low mag] and D [high mag]) and aboral (E [low mag] and F [high mag]) (sagittal cuts, H&E). A. Rare to less dense CARPs in the calicodermal tissue located approximately 300 to 600 µm below the surface. B. Higher magnification of A. Note also that the cytoplasm of the zooxanthellae is stained eosinophilic (arrow heads). C. CARPs (thick arrows) increase in density towards the middle of the polyp (in sagittal section). D. Higher magnification of C showing notable eosinophilic granules (thick arrow). Note also that the cytoplasm of the zooxanthellae is discolored (arrowhead), with some showing atrophy and dilated symbiosomes (thin arrows). E. Deeper tissue at the aboral side with a high density of the CARPs (thick arrows) in the calicodermis. F. Higher magnification of E. Note also that the zooxanthellae appeared to be degenerated (loss of cytoplasm [arrowhead], vacuolated symbiosomes [arrows]).

Zooxanthellae with intracytoplasmic PAS-positive materials (starch) were seen only at the LK site (Fig. 13D; 62.5%) but were not found at HR (0%) or FtLT (0%). Note that the starch collar around the pyrenoid was stained PAS-positive in zooxanthellae at all three sites (Fig. 13C, D) as well as DT. In the samples obtained from DT, the zooxanthellae were well populated at the surface body wall gastrodermis, but the zooxanthellae density decreased towards the aboral end of the polyp. Zooxanthellae at the surface gastrodermis had a brownish to reddish cytoplasm (under H&E), with a solid homogeneous appearance, or exhibited a negative PAS reaction (the

pyrenoid was PAS positive) (Fig. 14A). At the middle to aboral areas, zooxanthellae tended to exhibit a discolored cytoplasm (under H&E) or showed a lightly positive PAS reaction. At the surface, zooxanthellae generally had no starch granules. However, at the middle to aboral areas, the number of zooxanthellae with starch granules increased, while the zooxanthellae were hypertrophied at the aboral end. Possibly the difference in starch storage reflects the nutritional health status of the zooxanthellae. Histologically, for the diseased specimens examined from LK, HR, and FtLT sites, mucus appeared to be less abundant (and stained brown to thick purple with thionin, not shown) on the oral side compared to the aboral side. In the samples obtained from DT, large amounts of mucus exhibited PAS positive for all samples (Fig. 14B, C). For these apparently heathy specimens, mucus did not stain brown with Thionin at the oral side and throughout the tissue area. Presumptively, the brown color staining with thionin may be indicative of degraded mucus along with debris in the dead tissues.

Table 4. Comparison of histologic features at four locations. Examples of LN are shown in Fig. 9, BD in Figs. 8, 11 and the other features in Fig. 13. CARPs = coral acid-rich proteins, zx = zooxanthellae.

Site	Lytic necrosis (LN) N (%)	Coagulative necrosis (CN) N (%)	CARPs aggregates heavy at surface N (%)	Zx with PAS + cytoplasm (starch granules) N (%)	BD N (%)
LK	5/8 (62.5)	7/8 (87.5)	8/8 (100)	5/8 (62.5)	5/8 (62.5)
HR	5/5 (100)	2/5 (40)	4/5 (80)	0/5 (0)	4/5 (80)
FtLT	3/3 (100)	1/3 (33.3)	2/3 (66.6)	0/3 (0)	2/3 (66.6)
DT	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)

LK = Looe Key, HR = Haslun's Reef, FtLT = Fort Lauderdale, DT = Dry Tortugas

In conclusion, PD and BD was associated with tissue loss in *S. siderea*.

Field observations and histopathological results generally matched well. However, it was not possible to determine the cause of the PD. PD was noted to be lost over time during histological processing and was retained in formalin fixative and EDTA solution but was dissolved in alcohol and solvent. This may possibly suggest that PD is associated with hydrophobic chemicals. Grossly, purple pigments appeared to adhere to the oral disc area and they were apparently located deeper in the polyp more towards the aboral tissue.

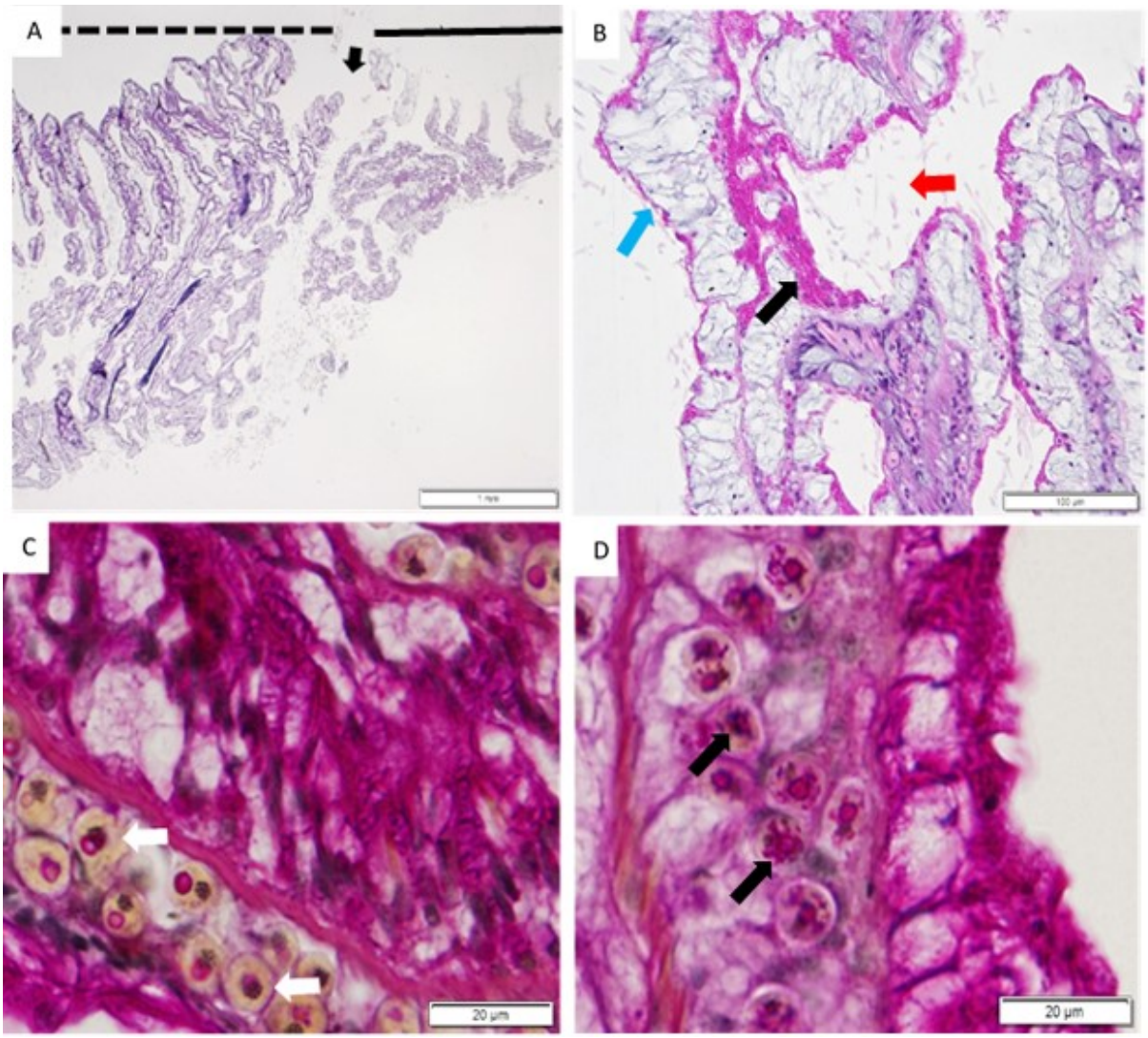

Figure 13. Histologic sections of *Siderastrea siderea* (A-B, H&E; C-D, PAS) showing various histopathological features. A. Sagittal section showing TL border (black arrow) with area of coagulative necrosis (solid line) and apparently healthy tissue (dotted line) (#2, LK). B. Mesenterial filament at close to the surface area showing prominent coral-acid rich protein (CARP) granules (black arrow) in the calicodermal layer, possibly reacting to nearby endoliths (red arrow) in the skeleton. Compare with apparent (subjective) lower density of CARPs (blue arrow) on the other side of the mesentery with less endoliths (#2, LK). C. Gastrodermal layer close to the surface area showing zooxanthellae with a clear cytoplasm (brown, yellow color, white arrows) (#15, FtLT). D. Gastrodermal layer close to the surface area showing zooxanthellae filled with PAS-positive (pink, red) starch granules (black arrows) (#1, LK).

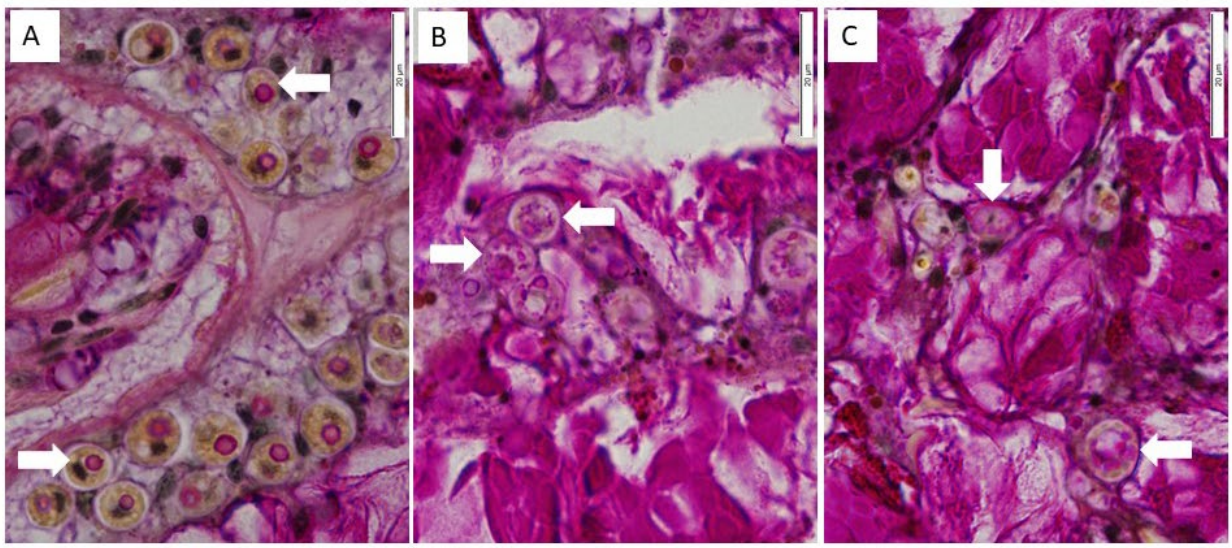

Figure 14. Histologic sections of apparently healthy *S. siderea* from Dry Tortugas (#3 DT) showing PAS reaction of zooxanthellae in the surface gastrodermis (A), middle (B) and aboral (C) tissue areas (PAS). A. Gastrodermal layer at close to the surface area showing zooxanthellae with clear cytoplasm (i.e. PAS-negative; brown yellow color, white arrows). B. Gastrodermal layer at the middle area showing zooxanthellae filled with PAS-positive (pink-red, white arrows) starch granules. Note abundant mucus, stained PAS-positive as well. C. Gastrodermal layer at the aboral area showing zooxanthellae filled with PAS-positive (pink-red, white arrows) starch granules. Note abundant mucus stained with PAS-positive as well.

The role of excess nitrogen in disease processes in Florida's coral

A major stressor for corals on Florida's Coral Reef is increased anthropogenic nitrogen loading (Zhao et al. 2013, Lapointe et al. 2019). The coral-zooxanthellae symbiosis is especially sensitive to disruption from excess nitrogen which stimulates cell division in zooxanthellae shifting the balance between nitrogen and phosphate. This in turn results in a destabilization of zooxanthellae integrity (Wiedenmann et al. 2013), which can be visualized at the cellular level (Rosset et al. 2017). Disruption of the coral-zooxanthellae relationship can leave corals less resistant to environmental challenges (lower bleaching threshold) (Wiedenmann et al. 2013) and disrupt coral microbiomes leaving corals more susceptible to disease (Zaneveld et al. 2016, Wang et al. 2018). Nutrient enrichment has also resulted in increased severity in some coral diseases (Bruno et al. 2003). However, this association between excess nitrogen, zooxanthellae health and coral health has only been investigated in a handful of coral species. To determine whether excess nitrogen is playing a role in coral health and disease processes in Florida's corals we **1)** compared zooxanthellae health in corals from Dry Tortugas vs. Florida sites, **2)** used aquaria studies to directly test the effect of nitrogen enrichment on zooxanthellae health in *S. siderea*, and **3)** used aquaria studies to directly test the effect of nitrogen enrichment on disease progression.

Task 3. Effect of nitrogen enrichment on zooxanthellae health

Ten healthy *S. siderea* fragments were collected in January 2020 from the Dry Tortugas where water quality is optimal compared to nearshore Florida waters. Fragments were transported to the Smithsonian Marine Station (SMS) in Ft. Pierce, FL and held in water tables until the start of the experiment (March 2021). Just prior to the experiment, a band saw was used to cut two small pieces off each fragment which were preserved in 10% Z-fix and Trump's fixative for histological analyses as outlined above. The remainder of each fragment was then cut in half for use in the experiment. One half of each fragment was exposed to nitrate enriched (10µM $NaNO_3$) seawater and the other half placed in plain seawater as a control. Each fragment was housed in individual 5 L aquaria which were placed in larger temperature-controlled water tables with circulating freshwater adjusted with a cooling and heating system to maintain water temperatures at 26–27°C. Aquaria were filled with seawater filtered through a 0.22 µm pore filter (FSW) and a bubbler placed to create water motion. All tanks received a complete water change triweekly, at which time, experimental tanks were dosed so that they had a concentration of 10µM NaNO3. Water samples were collected from a subset of the tanks (3 control and 3 experimental) and submitted to the Broward County Environmental Monitoring Lab for confirmation of nitrate levels. Pulse Amplitude Modulated Fluorometry (Model: Walz Diving-PAM; LED emission maximum 650 nm) was used to measure the *in situ* photochemical efficiency of all *S. siderea* fragments two times per week. Internal settings on the diving-PAM were as follows: measuring intensity = 6, saturation intensity = 8; saturation width = 0.8 s; actinic width = 0.15; actinic intensity = 4. Fluorescence measurements were conducted in duplicate for each fragment, after 60 min of dark acclimation. Upon completion of the experiment, small fragments of experimental and control pieces of *S. siderea* were preserved in 20% Z-fix and Trump's fixative for follow-up histological examination. The experiment was run March 10 to May 10, 2021. Percent change in F_V/F_m values through time of experimental fragments, in comparison with the corresponding control fragments, was calculated and used as an indicator of response to nitrate enrichment.

Results

The maximum quantum yield of PSII (F_V/F_m) is a proxy for photochemical efficiency (Jones et al. 1999; Abrego et al. 2008) and we found that both groups of corals (experimental and control) showed increased average F_V/F_m until around week 3 of the experiment. This is likely in response to enhanced light levels in the outdoor water tables where they were housed for the experiment. After 3 weeks, the avg. F_V/F_m leveled off for both groups but experimental corals exposed to nitrates maintained a lower photochemical efficiency as compared to their controls (Fig. 15).

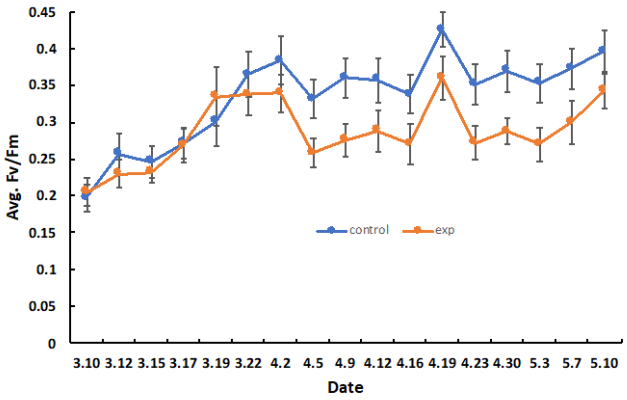

Figure 15. Photochemical efficiency of zooxanthellae in healthy *S. siderea* through time when exposed to chronic 10µm nitrate.

Task 4. Use aquaria studies to directly test the effect of nitrogen enrichment on disease progression in *M. cavernosa*, *S. siderea* and *C. natans*.

For each species tested, lesioned fragments of diseased colonies were collected in the field and transported to SMS where fragments were cut in half with a rock saw. One half of each fragment was exposed to nitrate enriched (10µM $NaNO_3$) filtered seawater and the other half placed in filtered seawater as a control. Fragments were housed in individual 5L aquaria and 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 and partial water changes conducted daily. For the experimental fragments, water was replaced with FSW pre-mixed with the corresponding concentration of $NaNO_3$. *Montastraea cavernosa* and *C. natans* showed progressive tissue loss during the experiment and so rate of tissue loss was measured using ImageJ. *Siderastrea siderea* lesions did not progress to tissue loss and so were rated as lesion progressed, stayed the same or appeared to heal (re-pigmentation or loss of purple discoloration). Six diseased fragments of *C. natans* were collected from the Middle Keys on January 26, 2021 and subsequently processed and set up in experiments on Jan 29, 2021 at SMS. The experiment ran until Feb 17, 2021. Seven diseased *S. siderea* fragments were collected in the Middle or Lower Keys between April 30 and May 18, 2021. Fragments were set up into experiments within days after each collection date. Eight fragments of diseased *M. cavernosa* were collected from the Middle Keys on May 11 or 18th and set up in the experiment within days after each collection date. Pulse Amplitude Modulated Fluorometry (Model: Walz Diving- PAM; LED emission maximum 650 nm) was used to measure the *in situ* photochemical efficiency of all *S. siderea* and *M. cavernosa* fragments two times per week as described below in Obj. 4b. PAM was not conducted on *C. natans* due to the rapid rate of tissue loss.

C. natans

There was no effect of short-term exposure to 10µM nitrate on the rate of tissue loss on *C. natans* with SCTLD (Fig 16). By day 7, ten of the twelve fragment (5 control, 5 experimental) had complete tissue loss limiting the duration of exposure to excess nitrates.

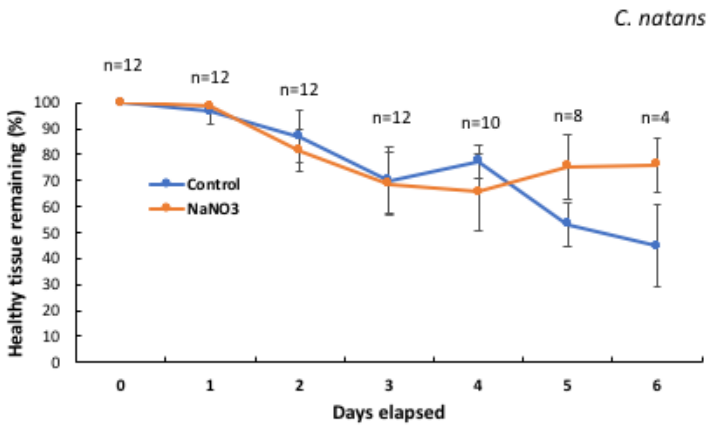

Figure 16. Change in amount of healthy tissue remaining on *C. natans* with SCTLD with and without nitrogen enrichment (n=6/treatment). Five of six pairs had complete tissue loss by day 7.

S. siderea

Lesion progression on *S. siderea* fragments was scored at the end of the experiment as lesion progressing (+), stasis (=) or healing (re-pigmentation in bleached lesions or pigment regression) (-). Nitrate enrichment had no consistent effect on lesion progression regardless of lesion type with lesions showing all three types of response (Table 5). There was also no effect on nitrates on the photochemical efficiency of zooxanthellae in healthy or lesion tissue on diseased *S. siderea* (Fig. 17). However, the effective quantum yield (F_v/F_m) of zooxanthellae in the nitrate treatment was lower than their paired controls with more variability in response among the lesion tissue (Fig. 17).

Table 5. Response of lesions on *S. siderea* to nitrate enrichment. '+' indicates an increase, '-' indicates a decrease, p=purple discoloration, bl=bleaching, tl=tissue loss.

frag i.d.	nitrate	control
1	p- bl+	p- bl+
2	p= tl+	p= tl+
3	p= tl=	p= tl+
4	p= bl+	p= bl+
5	p= bl+	p= bl+
6	p-	p= bl+
7	p+ bl+	p= bl+

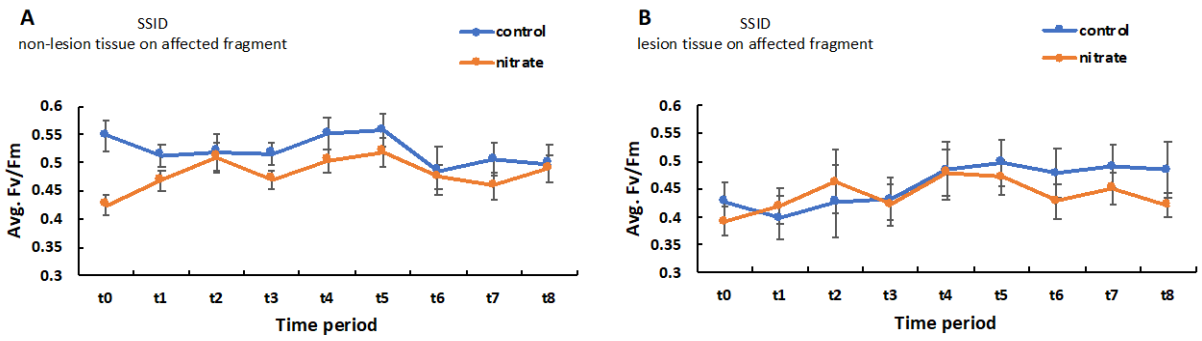

Figure 17. Effect of excess nitrates (10µM) on the health of zooxanthellae in diseased *S. siderea* as measured in non-lesion (A) and lesion tissue (B) of affected fragments, (n=7 fragment pairs).

M. cavernosa

M. cavernosa with SCTLD exposed to excess nitrates had a higher rate of tissue loss compared to controls but there was variability in response among fragments (Fig. 18). It must be noted that tissue loss was calculated after 2-3 weeks following the start of nitrate enrichment and rate of tissue loss will be calculated again after a total of 6 weeks nitrate exposure. There was also no significant effect of excess nitrate on the photochemical efficiency of zooxanthellae in *M. cavernosa* with SCTLD in either lesion or non-lesion tissue (Fig. 19) but there was a trend of nitrate exposed corals having a lower avg. Fv/Fm.

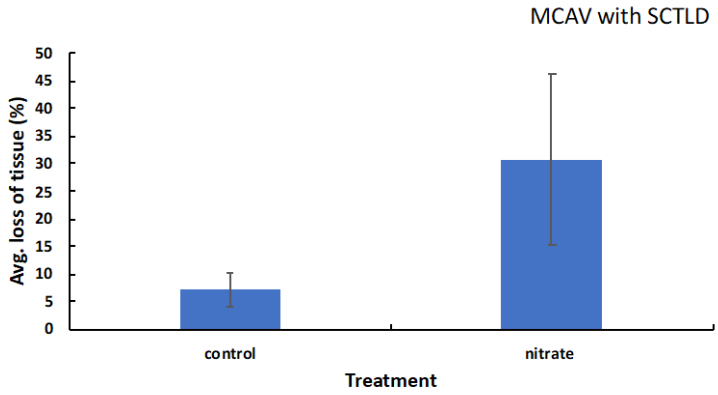

Figure 18. Differences in tissue loss in *M. cavernosa* fragments with SCTLD with and without nitrate enrichment.

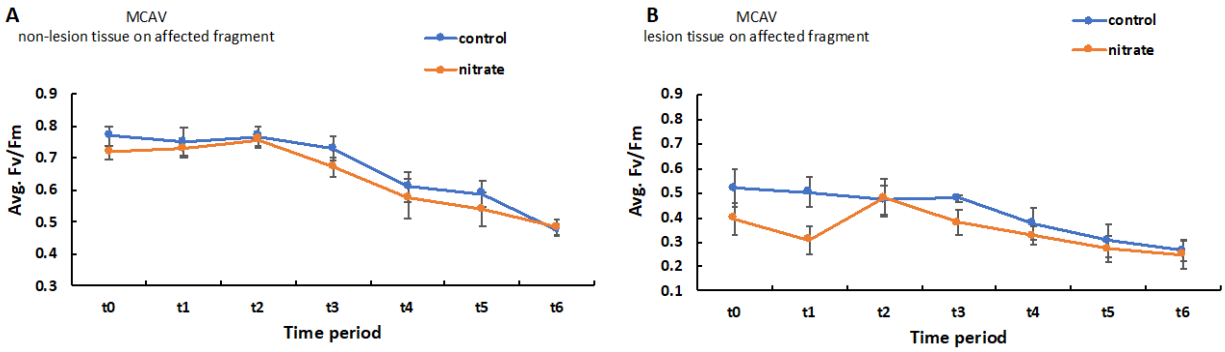

Figure 19. Effect of excess nitrates (10µM) on the health of zooxanthellae in diseased *M. cavernosa* as measured in non-lesion (A) and lesion tissue (B) of affected fragments, (n=7 fragment pairs).

Summary

Regional comparisons in lesion morphology, disease prevalence and colony mortality for *M. cavernosa*

Ft. Lauderdale (disease emerged in 2014-15)

- SCTLD remains on tagged colonies on the reef after 3 years (July 2017–May 2021).
- Disease prevalence on tagged colonies remains at lower endemic levels with fluctuations through time.
- Colony survival remains high with 65% of the colonies alive after more than 3 years (July 2017–May 2021) without any disease treatment.
- Tissue loss on infected colonies has declined with tagged colonies in 2021 having an average of 3.9% (SE±3.8%) compared to an average of 33.6% (SE±8.7%) in the first year of the study (July 2017-July 2018)
- SCTLD is continuing to spread to formerly uninfected *M. cavernosa* on the reef albeit with a different lesion morphology (bleached polyps or edges vs. tissue loss lesions) and less colony morbidity (partial colony loss).
- 7 out of 8 healthy tagged *M. cavernosa* developed lesions between June 2018 and May 2021 (disease incidence=87.5%).
- 6 of the 7 (85.7%), newly infected colonies, developed bleached polyps or edges and 1 out of the 7 (14.3%) developed bleached polyps with tissue loss.
- 3 of the 7 (42.9%) newly infected colonies developed progressive tissue loss during the study period

Lower Keys- Tag site (Looe Key) (disease emerged in 2018)

- The temporal pattern of SCTLD on tagged *M. cavernosa* is similar to the pattern which emerged from tagged *M. cavernosa* on the Ft Lauderdale reef.
- SCTLD remains on tagged colonies on the reef after over 2 years.

- Disease prevalence on tagged colonies declined through time this year in contrast to the high prevalence that occurred the prior year.
- Colony survival in tagged infected *M. cavernosa* was higher this year (78.6%) compared to the prior year (11.8%).
- SCTLD is continuing to spread to formally uninfected *M. cavernosa* on the reef albeit with a different lesion morphology (bleached polyps or edges vs. tissue loss lesions) and less colony morbidity.
- 50% of the healthy *M. cavernosa* (12 out of 24) developed lesions during the study (Nov 2019-May 2021) and all lesions were bleached polyps or edges.
- 2 of the 11(18.2%) colonies that developed lesions progressed to tissue loss but colony morbidity was confounded by BBD co-infections during summer months.

Lower Keys – Haslun's reef (disease emerged in 2019)

- The temporal pattern of SCTLD on tagged *M. cavernosa* is similar to the pattern that emerged from tagged *M. cavernosa* on Looe Key reef on year one.
- Prevalence remained high throughout the study.
- Tagged infected colony survival between Nov 2019 and Dec 2020 was 0%.
- Disease spread amongst healthy colonies with 8 out of 12 (66.7%) developing lesions.
- Lesions on newly infected colonies were dominated by bleached polyps with tissue loss (71.4%).
- 85.7% of the newly infected colonies had lesions that resulted in progressive tissue loss.

S. siderea **lesions through time**

Lesion morphology, temporal pattern of occurrence and colony morbidity differed between diseased *S. siderea i*n Ft. Lauderdale and Lower Keys (Haslun's reef).

Ft. Lauderdale – FtLT

- *S. siderea* colonies presented with multi-focal bleached lesions.
- Disease prevalence fluctuated through time.
- Between Nov 2019 and May 2021 there was 100% survival.
- Through time colonies alternated between signs of re-pigmentation and healing and lesion progression.
- Two colonies progressed onto partial tissue loss.

Keys – Haslun's reef

- *S. siderea* colonies presented with purple or dark discoloration with or without tissue loss.
- Disease prevalence remained 100% throughout the study (Nov 2019-May 2021).
- Avg. tissue loss was 64.2% (4.9%/month).
- Case fatality rate during the study was 11.1%.

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

- Aquaria studies examined the transmissibility of different lesion types in *S. siderea* to healthy *S. siderea* and *O. faveolata*.
- One *S. siderea* out of seven developed lesions (14.3%) after contact with *S. siderea* with lesions showing chronic tissue loss after 13 days but none of the non-touching *S. siderea* developed lesions.
- Transmission to *O. faveolata* was more successful with 2 out of 7 (28.6%) of the touching fragments developing a lesion (bleached lesion) after an average of 18.5 days and 1 of 7 (14.3%) of the non-touching fragments developing disease signs after 19 days.
- Combining our 2021 and 2018 results we find that the most transmissible *S. siderea* lesions, under experimental conditions, were bleached lesions with tissue loss. Five out of 5 fragments (100%) had successful transmission to *O. faveolata* and 2 out of 5 (40%) transmitted to *S. siderea*. Purple lesions with some bleaching (n=5 pairs) showed no successful transmission in either species.

***S. siderea* histopathology**

- 13 diseased *S. siderea* from the Keys and 3 diseased *S. siderea* from Ft. Lauderdale were collected and processed by FWRI-FWC for histological examination.
- Oral lesions typically seen first with SCTLD in *S. siderea.*
- Oral lesions radiate out on the septa to the outer edge of the corallite.
- Oral lesions are grossly grey-purple (pink-purple macroscopically) or white-beige (in field/macroscopically).
- Histologically oral lesions manifest as lytic necrosis (LN) of basal body wall septa with sloughing of necrotic tissue into the oral cavity.
- All samples (n = 16) had tissue loss (confirmed macroscopically/histologically with LN [n =13] and/or coagulative necrosis [n =10).
- Purple discoloration (PD) determined in the field was separated macroscopically by septal PD (contrast with color of oral lesion) and oral lesions (with or without PD).
- Bleaching can be associated with early oral lesions and on the septa.
- Oral lesions (PD/no PD) and BD can lead to tissue loss (SCTLD).
- BD observed grossly at FtLT (n = 2), and detected histologically in LK (N = 2) and HR (n =1).
- PD observed grossly on septal ridges, contrasted against grey-purple or white-beige oral lesions.
- PD cannot be characterized histologically and is lost over time during processing.
- Reference apparently healthy coral specimens from the Dry Tortugas did not exhibit any lesions, and had minimal CARPs (coral acid-rich proteins) compared to lesioned specimens

- Initial histological observations of zooxanthellae revealed apparent differences in PAS-positive intracytoplasmic staining for *S. siderea* with bleached lesions from Ft Lauderdale with bleached lesions vs. *S. siderea* with dark/purple lesions from Lower Keys.
- The PAS-positive staining possibly reflects the difference in starch storage and therefore the nutritional health status of the zooxanthellae.
- The staining feature of zooxanthellae maybe a useful tool to differentiate the health state of corals.

Nitrate enrichment and zooxanthellae health

- 10 healthy *S. siderea* fragments were collected in the Dry Tortugas and set up in nitrate enrichment experiments.
- Chronic nitrate enrichment reduced the photochemical efficiency of zooxanthellae in *S. siderea* after approximately 3 weeks of exposure.

Nitrate enrichment and lesion progression

- There was no effect of nitrate enrichment on the rate of tissue loss in diseased *C. natans* (n=6 pairs). However, experiment duration was limited by rapid tissue loss in fragments with 10 out of 12 fragments dead by day 7 of the experiment.
- Diseased *S. siderea* with different lesions types showed no differences in lesion progression in nitrate enriched vs. control fragments. Zooxanthellae health was lower in fragments exposed to nitrate but the response was not statistically significant.
- *M. cavernosa* with SCTLD exposed to nitrates had higher rates of tissue loss compared to controls but there was variability in response. Zooxanthellae health was lower in fragments exposed to nitrate but the response was not statistically significant.
- Nitrate enrichment affected the photochemical efficiency of zooxanthellae in healthy *S. siderea* more than diseased *S. siderea* or diseased *M. cavernosa*. This could be due to the response of healthy vs. diseased colonies and/or that the healthy *S. siderea* were collected in the Dry Tortugas which has minimal nitrate pollution and the diseased fragments were collected from offshore Florida which has known nitrogen pollution.

References Cited

Abrego D, Ulstrup K, Willis B, van Oppen M (2008) Species-specific interactions between algal endosymbionts and coral hosts define their bleaching response to heat and light stress. Proc R Soc B 275:2273–2282

Aeby GS, Callahan S, Cox EF, Runyon C, Smith A, Stanton FG, Ushijima B, Work TM (2016) Emerging coral diseases in Kāneʻohe Bay, Oʻahu, Hawaiʻi: two major disease outbreaks of acute *Montipora* white syndrome. Dis Aquat Org 119(3):189-198

Brandt M, Ruttenberg B, Waara R, Miller J, Witcher B, Estep A, Patterson M (2012) Dynamics of an acute coral disease outbreak associated with the macroalgae *Dictyota* spp. in Dry Tortugas National Park, Florida, USA. Bull Mar Sci 88(4):1035-1050

Bruno J, Petes L, Harvell CD, Hettinger A (2003) Nutrient enrichment can increase the severity of coral diseases. Ecol Letters 6:1056-1061

Jones MV, Calabresi PA (2007) Agar-gelatin for embedding tissues prior to paraffin processing. Biotechniques 42:569-570

Jones R, Kildea T, Hoegh-Guldberg O (1999) PAM chlorophyll fluorometry: a new *in situ* technique for stress assessment in scleractinian corals, used to examine the effects of cyanide from cyanide fishing. Mar Pollut Bull 38:864–874

Landsberg JH, Kiryu Y, Peters EC, Wilson PW, Perry N, Waters Y, Maxwell KE, Huebner LK, Work TM (2020) Stony coral tissue loss disease in Florida is associated with disruption of host–zooxanthellae physiology. Front. Mar. Sci. 7: 576013. doi: 10.3389/fmars.2020.576013

Lapointe BE, Brewton RA, Herren LW, Porter JW, Hu C (2019) Nitrogen enrichment, altered stoichiometry, and coral reef decline at Looe Key, Florida Keys, USA: a 3-decade study. Mar Biol 166:108

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

Palmer C, Roth M, Gates R (2009) Red fluorescent protein responsible for pigmentation in trematode-infected *Porites compressa* tissues. Biol. Bull. 216:68-74

Quintero-Hunter I, Grier H, Muscato M (1991) Enhancement of histological detail using metanil yellow as a counter-stain in periodic acid Schiff's hematoxylin staining of glycol methacrylate tissue sections. Biotech Histochem 66: 169−172. doi: 10.3109/10520299109109964

Rosset S, Wiedenmann J, Reed AJ, D'Angelo C (2017). Phosphate deficiency promotes coral bleaching and is reflected by the ultrastructure of symbiotic dinoflagellates. Mar Pollut Bull 118, 180-187

Wang L, Shantz AA, Payet JP, Sharpton TJ, Foster A, Burkepile DE, Vega-Thurber R (2018) Corals and their microbiomes are differentially affected by exposure to elevated nutrients and a natural thermal anomaly. Front Mar Sci 5:101

Wiedenmann J, D'Angelo C, Smith EG, Hunt AN, Legiret FE, Postle AD, Achterberg EP (2013) Nutrient enrichment can increase the susceptibility of reef corals to bleaching. Nat Clim Change 3(2):160–164

Zaneveld JR, Burkepile DE, Shantz AA, Pritchard CE, McMinds R, Payet JP, Welsh R, Correa AM, Lemoine NP, Rosales S, Fuchs C (2016) Overfishing and nutrient pollution interact with temperature to disrupt coral reefs down to microbial scales. Nat Commun 7:11833

Zhao J, Hu C, Lapointe B, Melo N, Johns EM, Smith RH (2013) Satellite-observed black water events off Southwest Florida: implications for coral reef health in the Florida Keys National Marine Sanctuary. Remote Sens 5(1):415–431