INVESTIGATION OF MICROBIOTA ASSOCIATED WITH RAPID TISSUE LOSS IN CAPTIVE INDO-PACIFIC CORALS

by

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ABSTRACT

INVESTIGATION OF MICROBIOTA ASSOCIATED WITH RAPID TISSUE LOSS IN CAPTIVE INDO-PACIFIC CORALS

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Many factors are known to contribute to the unprecedented decline of coral reefs, with rapid declines resulting from coral diseases. The occurrence of coral disease in closely monitored and controlled environments offers a unique opportunity to study such events. In 2011, select corals in the Smithsonian's National Museum of Natural History Indo-Pacific coral-reef aquarium underwent rapid tissue loss in 24–48 hours. Initial results indicated the presence of Gram-negative bacteria, similar to *Rickettsia*-like organisms (RLOs) previously observed in Caribbean corals. From 2016–2018, additional samples were collected from both diseased and apparently healthy corals for histopathological and transmission electron microscopy (TEM) examination. The samples revealed interactions between RLOs and host corals, both when corals were apparently healthy and when they were diseased, i.e., losing tissue. Corresponding environmental data were analyzed to determine if environmental parameters influenced disease outbreaks in this system. It remains unclear what environmental factors contributed to disease outbreaks in this

system; however, this is the first study to attempt to analyze a combination of water quality parameters in tropical coral-reef aquariums to determine if any may be contributing factors to coral disease outbreaks. Associated organisms were present in subsamples of both apparently healthy and diseased corals, with evidence suggesting they may play a role in transmission of RLOs. Statistically significant linear relationships emerged between condition parameters in apparently healthy and diseased corals. This is the first study to histologically examine the role of RLOs in multiple Indo-Pacific coral species and explore the possible transmission of disease in a recirculating coral reef system.

1 INTRODUCTION

Corals are an integral component of marine ecosystems. Interactions among corals, microbial communities, and surrounding marine and terrestrial environments form the basis of complex marine ecosystems, under increasing pressure from direct or indirect disturbances, all linked to anthropogenic origins (Peters 2015). During the last 40 years, unprecedented mortality in reef-building corals has resulted in declines of 80% in the Caribbean and 50% in the Indo-Pacific (Pollock et al. 2011). Reef degradation and mortality can be attributed to the synergistic effects of ever-increasing human demand for shrinking resources contributing to global climate change, resulting in sedimentation, agricultural runoff, overfishing, pollution, and widespread infectious diseases and other health impairments (Knowlton 2001; Downs et al. 2005). Degraded and declining coral reefs directly or indirectly impact the organisms that interact with them, which could include 650,000 to more than nine million eukaryotes and millions of microorganisms (Knowlton 2001; Rohwer et al. 2002; Stella et al. 2011).

Corals are holobionts (Margulis 1991; Krediet et al. 2013) composed of a consortium of organisms, including corals themselves, symbiotic dinoflagellates and other algae, and microorganisms. While we are only beginning to understand how profoundly microorganisms are tied to all ecosystems, they are imperative to various

biological and physiological functions of all eukaryotic organisms (Rosenberg & Kushmaro 2011; Morrow et al. 2013; Locey & Lennon 2016). Coral-associated microbes are essential to obtaining and metabolizing nutrients, larval metamorphosis, biogeochemical cycling, and resistance to infection by pathogens (Rohwer et al. 2002; Peters 2015). The majority of studies on coral-associated microorganisms have focused on microbes colonizing the area of corals most exposed to the external environment, named the surface mucopolysaccharide layer (SML) (Krediet et al. 2013; Closek 2014).

Although little is known regarding variations in the microbial community between wild and captive corals, the microbiota does vary individually with geography, occurrences of health impairments, and environmental shifts (Morrow et al. 2013; Peters 2015). Corals in aquaria have been documented with significantly different microbiota than their wild counterparts. Kooperman et al. (2007) analyzed microorganisms associated with *Fungia granulosa* in aquaria and *in situ. Ex situ* and *in situ* corals had significantly different microbial species compositions and richness. Pratte et al. (2015) conducted a similar study on *Siderastrea siderea*. When *in situ* corals were relocated to aquaria, shifts in the microbial composition of the SML were observed, especially in the initial hours after transfer. The hologenome theory explains that shifts in microbial composition occur as a result of host coral regulation (Morrow et al. 2013). In addition to coral-mediated shifts in microbes, interspecific competition between microbial associates also influences this process (Morrow et al. 2013). It is clear the microbiota of corals is dynamic, readily shifting in response to biological or environmental factors (Ritchie & Smith 2004; Krediet et al. 2013; Bourne et al. 2016).

Health impairments to coral-reef organisms can result from either abiotic or biotic stressors acting on corals (Peters 2015). Many health impairments may begin at the cellular, or even sub-cellular level (Peters 2015) and since the 1980s, exposure to rapidly increasing physical, chemical, and biological anthropogenic stressors on coral reefs has led to more health impairments in corals, including bleaching, or the paling of coral tissue, resulting from the breakdown of the mutualism between host coral and resident algae under stress (Bleaching of Coral Reefs in the Caribbean 1987; Closek 2014; Pettay et al. 2015). Interactions among factors promoting biological or physiological damage, an organism's defense against it, and a specific or non-specific biological response (Selye 1950) have contributed to significant morbidity and mortality of corals (Selye 1950; Rützler & Santavy 1983; Weil et al. 2006; Krediet et al. 2013; Nicolet et al. 2018).

Research on coral diseases is still in its infancy, compared to the study of vertebrate diseases (Work et al. 2008). Caused by environmental stressors, compromised immunity, or pathogenic microorganisms, disease results in alteration to the structure of tissues and/or negatively affects physiological function in individuals, which ultimately affects ecosystem function and biodiversity (Rosenberg & Kushmaro 2011; Peters 2015; Nicolet et al. 2018). Diseases in corals were documented beginning in the 1970s (Peters 2015) and are continually increasing in frequency and severity (Weil et al. 2006; Closek 2014). Currently there are approximately 40 recognized coral diseases (Rützler & Santavy 1983; Weil et al. 2006; Krediet et al. 2013; Bruckner 2016a). Reports from 75 countries show that 200 species of coral are affected by disease (Bruckner 2016a).

Whereas Caribbean coral reefs have been the hotspot for coral disease, Indo-Pacific coral diseases are on the rise. Increasingly, diseases in other marine reef organisms, such as sponges, fishes, sea urchins, zoanthids, octocorals or "soft corals," sea turtles, mollusks, lobsters, sea grasses, and crustose coralline algae are also becoming more prevalent in both known and new ranges (Weil et al. 2006; Peters 2015; Bruckner 2016a). Krediet et al. (2013) questioned whether some cases of coral disease can be attributed to shifts in the microbiota of corals and if changes in coral microbial communities result from changes occurring in the surrounding environment (abiotic pathogens). Perhaps coral disease occurs secondarily to stress, as a result of opportunistic infections, rather than as a result of species-specific interactions with pathogenic microorganisms (Lesser et al. 2007; Krediet et al. 2013). While there have been strong correlations found between environmental stressors and disease in certain taxa (Friedman & Crosson 2012; Miller et al. 2019), contrary evidence exists within the available literature, where 25 viruses, 33 bacteria, 23 protists, and 21 eukaryotes (Lafferty 2017) have been identified as causative agents of disease across phyla in the oceans. However, some of these questions necessitate more analysis at a cellular level to be resolved (Work & Meteyer 2014).

Gross signs of coral disease generally fall into one of four categories, including tissue loss, tissue loss associated with microbial mats, tissue discoloration, or growth anomalies (Weil et al. 2006; Closek 2014; Peters 2015). Tissue-loss diseases are named based on the patterns, sizes and shapes of lesions, and affected species (Peters 2015) and result in high rates of mortality. The first tissue-loss disease characterized in the literature

was black-band disease. Subsequently, tissue-loss diseases, such as white-band disease (WBD), affecting both *Acropora palmata* and *Acropora cervicornis* in the Caribbean, and white plague were identified. Other tissue-loss diseases, including white patch disease, (white pox or acroporid serratiosis), and white syndrome, have also been identified (Rützler & Santavy 1983; Richardson 2004; Lesser et al. 2007; Peters 2015; Sutherland et al. 2016).

Tissue-loss diseases in the Indo-Pacific are collectively referred to as "white syndromes (WS)," and signs include patches, bands, or spots of bare skeleton where tissue has sloughed off (Bourne et al. 2016). White syndromes that have been characterized include atramentous necrosis, which affects *Montipora aequituberculata*, *Porites* ulcerative white spot disease, ulcerative white spots, and a disease appearing similar to Caribbean white plague (Bourne et al. 2016). A high proportion of Indo-Pacific corals affected by white syndromes, similar to the Caribbean, include species important to maintaining the structure of reefs, such as table *Acropora* spp. and *Montipora* spp. (Peters 2015; Bourne et al. 2016).

Emerging tissue-loss diseases in the Atlantic and Caribbean show different signs from existing "Caribbean white syndromes" (Bruckner 2016b), and could occur as a result of increased environmental stressors, increasing ranges and virulence of potential pathogens (Miller et al. 2019), or introductions of potential pathogens to new habitats (Rützler & Santavy 1983; Jones et al. 2008; Jackson et al. 2014; Pettay et al. 2015; Peters 2015; Bruckner 2016a).

Facilitated by the wildlife trade, outbreaks of novel diseases in terrestrial and marine ecosystems are increasing (Jones et al. 2008). Demand for marine ornamentals in the wildlife trade drives international importation of corals and Smith et al. (2009), showed that 33.5% of wildlife shipments to the United States over a 6-year period contained cnidarian species. Considering that corals and the seawater they are transported in may act as pathogen reservoirs, release of these corals to new environments could impact native colonies (Smith et al. 2009). Further, a high-value product sold in the aquarium industry, named "live rock," is also a concern. These pieces of rock, collected from coral reefs, are valued for their community of microorganisms, which aid aquarists in the cycling of nitrogenous wastes. Live rock often arrives at final destinations harboring both desirable nitrifying microorganisms and undesirable sessile or free-swimming organisms. Trade in live rock is largely unregulated and quarantine measures nearly non-existent, providing another pathway for the introduction of invasive microorganisms (Bolton & Graham 2006). Pettay et al. (2015) reported that invasive species and microorganisms have rapidly spread across the Caribbean region in just a few years' time, including the non-native Indo-Pacific zooxanthellae, *Symbiodinium trenchii*. Global shipping and the occurrence of disease outbreaks in close proximity to the Panama Canal further supports the idea that invasive microorganisms are being introduced to new hosts via global shipping and transportation, with potential effects on the diversity, distribution, or the ecology of communities exposed to invading species (Jackson et al. 2014; Pettay et al. 2015).

To further confound researchers, the complexity of disease dynamics has resulted in cases in which a pathogen was identified as a causative agent of coral disease, only to be refuted by subsequent research. Highly contagious white pox disease, affecting Caribbean *A. palmata,* is one of the few coral diseases for which a pathogenic causative agent has been identified—the human fecal bacterium, *Serratia marcescens* (Rosenberg & Kushmaro 2011). However, even in this case, although Koch's Postulates were fulfilled, *S. marcescens* has not been found in all cases of white pox, indicating that there may be other biotic pathogens or, perhaps, unculturable strains of *S. marcescens* playing a role in this disease (Sutherland et al. 2016). This has also occurred in the case of acroporid white syndrome. Whereas earlier research indicated apoptosis resulting in tissue loss as its cause, the results of subsequent research indicate that *Vibrio* spp. bacteria or ciliates may be associated with this disease (Peters 2015).

Ciliates are known to cause major mortalities of marine invertebrates (Cróquer et al. 2006). Sweet et al. (2014) discovered histophagous ciliates actively predating upon coral, contributing to the advancement of tissue loss in WBD and WS affected corals. Although ciliate infections generally occur secondarily to primary diseases, *Philaster lucinda* and *Varistrombidium kielum* have been found in both *ex situ* and *in situ* cases of Indo-Pacific coral that lost tissue (Sweet et al. 2014). In a study conducted by Nicolet et al. (2018) on brown-band disease (BrB) in Indo-Pacific corals, researchers also found corallivorous scuticociliates contributing to tissue loss (Sweet & Bythell 2012a).

Members of the Rickettsiales bacteria have been found associated with marine organisms including coral (Casas et al. 2004; Miller et al. 2014; Klinges et al. 2019) and

are known to have been associated with coral disease for more than 25 years (Klinges et al. 2019). Still, most research conducted on the symbiotic relationship between Rickettsiales and potential hosts has focused on terrestrial ecosystems. As of 2019, there were three known families within the order Rickettsiales: Rickettsiaceae, Anaplasmataceae, and Candidatus Midichloriaceae (Klinges et al. 2019) and all families are obligately intracellular. The presence of incomplete metabolic pathways suggest that these microbes exploit host corals, and even zooxanthellae, for their metabolic needs. Although these microorganisms take hosts' ATP for energy, they lack energy storage mechanisms, and therefore, sap energy reserves of the host, eventually causing mortality of host cells (Klinges et al. 2019). While their role in coral disease is currently under investigation, Rickettsiales have been identified in both apparently healthy corals and those affected by disease, and in other marine organisms, including sponges, hydrozoans, placozoans, sea anemones, protists, and ctenophores, worldwide (Casas et al. 2004; Miller et al. 2014; Klinges et al. 2019).

2 HISTOPATHOLOGICAL CHARACTERIZATION OF DISEASE IN INDO-PACIFIC CORALS IN AQUARIA

In 2011, a coral tissue-loss disease outbreak was documented in the National Museum of Natural History's (NMNH) 2,100-gallon (7,950-Liter) aquarium. The aquarium volume included a 700-gallon (2,650-Liter) sump, connected to an integrated water recirculation system with a 450-gallon (1,703-Liter) capacity. Histopathological examination revealed intracellular Gram-negative bacteria, some of which were similar to, and located in the same mucocytes of tentacles and cnidoglandular bands, as *Rickettsia*-like organisms (RLOs) previously found in Caribbean corals (Casas et al. 2004; Miller et al. 2014; Peters unpubl. data, 2012). Additional periodic episodes of tissue loss on corals, of unknown etiology(ies), have been documented in this system. Tissue sloughed off the skeleton in just days, resulting in mortality of affected *Acropora* and *Montipora* colonies (Figures 1 and 2).

Histopathological analysis of affected coral samples in 2017 showed the presence of intracellular Gram-negative bacteria in the cnidoglandular band epithelium of mesenteries and in the epidermis of tentacles, similar in appearance to the Rickettsiales found in Caribbean corals (Miller et al. 2014) (Figure 3). Suspect Gram-negative bacilli were found in cells of the surface body wall. In aquariums, coral losses are usually explained anecdotally as a result of poor or altered water quality, including

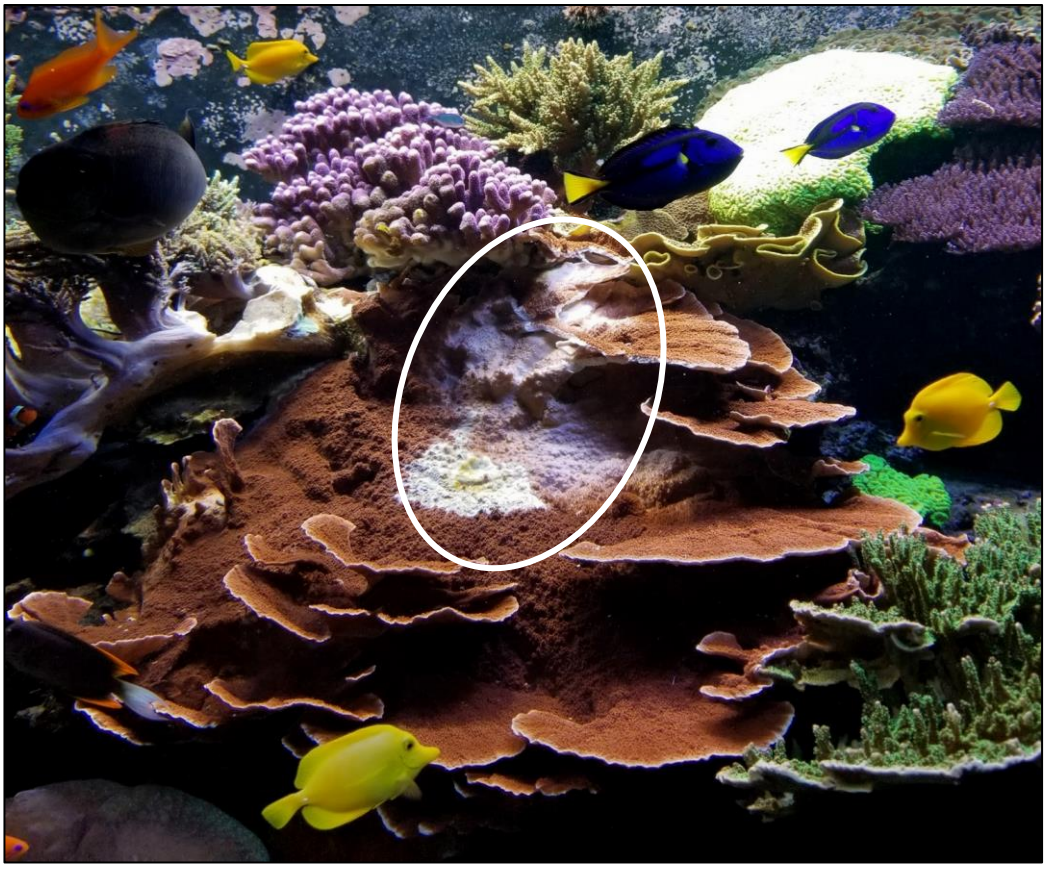


Figure 1. *Montipora capricornis* affected by tissue loss
***M. capricornis* colony during a disease outbreak in July 2017 in the aquarium at the Smithsonian's NMNH. Note the circled lesion. As tissue loss increases, the lesion grows larger, resulting in more denuded skeleton. Photo taken by Caitlin Gillis, NMNH staff.**

water flow, lighting, or lack of experience of the managing individual (Clode & Marshall 2003; Sweet et al. 2012b); however, this particular public aquarium was well-managed, with excellent water quality and relatively stable water parameters, providing an excellent model system for studying this disease. The study goal was to combine histopathology and electron microscopy with molecular analyses of potentially pathogenic

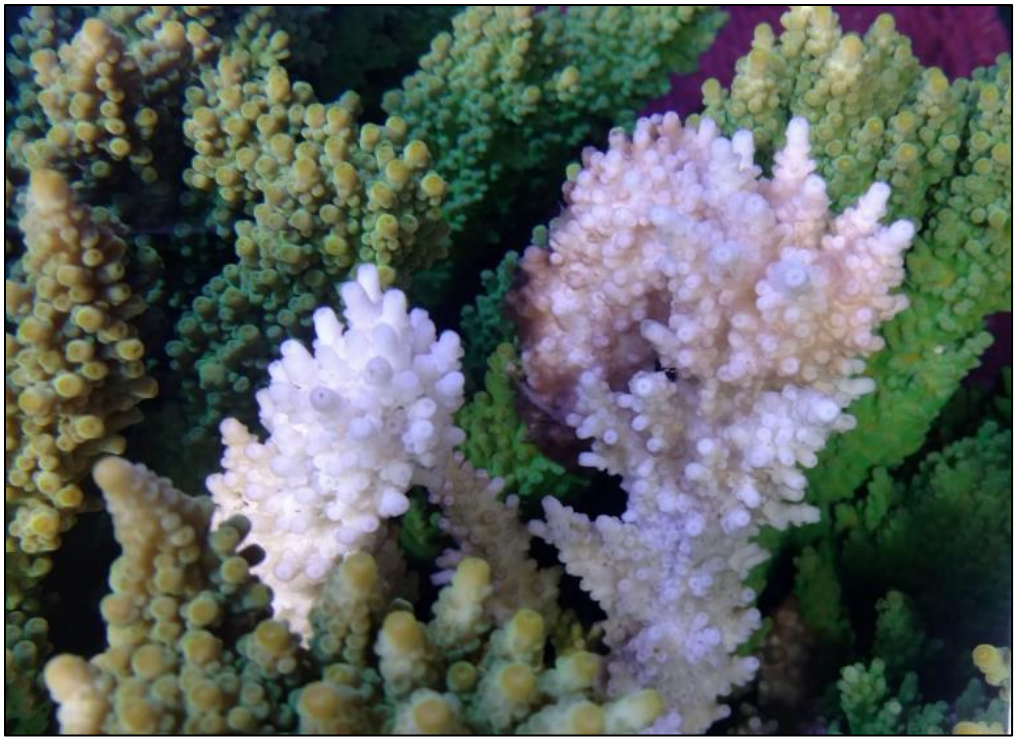


Figure 2. *Acropora* sp.'ORA® Frogskin' lesions
Tissue-loss lesions affecting a coral colony in the Smithsonian's NMNH aquarium.

microorganisms and evaluate environmental conditions in the aquarium to provide a more accurate diagnosis and avoid or better manage disease outbreaks within this closed model system. Whether in *ex situ* or *in situ*, healthy corals are essential to the sustainability of coral reef ecosystems, and shedding light on disease dynamics serves to inform management decisions in the long term to ensure better overall ecosystem health (Krediet et al. 2013).

The research reported in this thesis focused on the tissue condition and aquarium parameter analyses, specifically to investigate these questions and test these hypotheses:

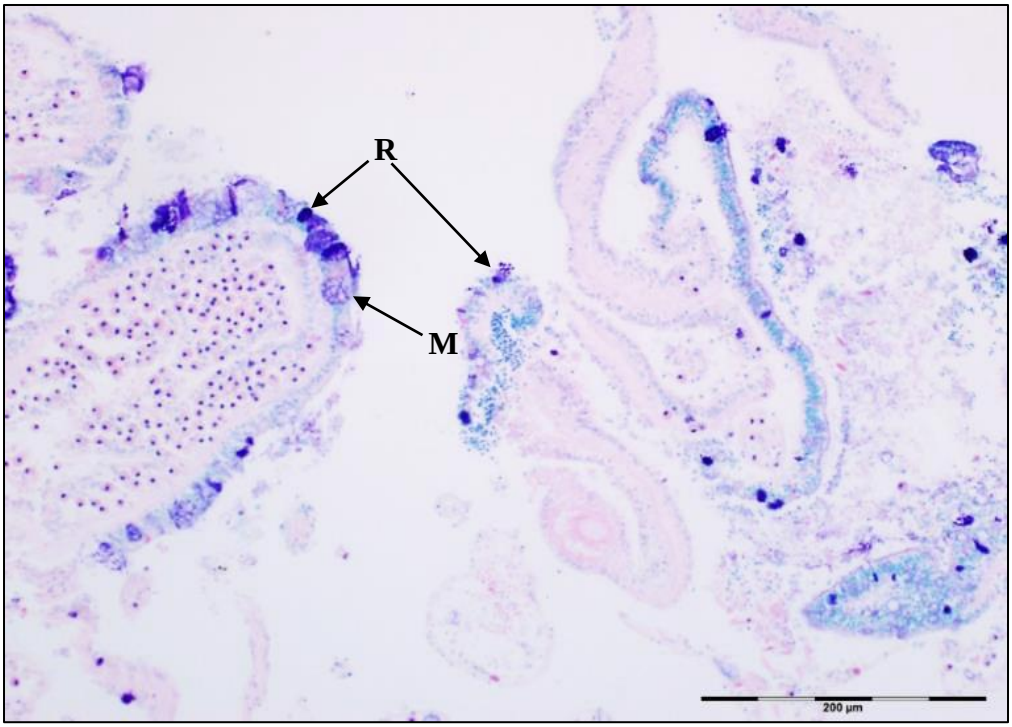


Figure 3. *Acropora* sp. infected with RLOs
Visualized with Giemsa at 20x magnification, RLOs (R) are dark purple grape-like clusters infecting both the mucocytes (M) of the epidermal epithelium, portions of the gastrodermis and cnidoglandular bands of an *Acropora* sp. collected from the Smithsonian's NMNH aquarium.

1. Can suspect RLOs or other potentially pathogenic microorganisms be detected in the different colonies of apparently healthy Indo-Pacific corals in the NMNH coral reef exhibit tank?

- H_0: Suspect RLOs are detected in apparently healthy Indo-Pacific corals in the NMNH coral reef exhibit tank.
- H_A: Suspect RLOs are not detected in apparently healthy Indo-Pacific corals in the NMNH coral reef exhibit tank.

2. Are corals in the NMNH coral reef exhibit tank presenting with tissue loss infected with RLOs or other microorganisms as determined by histopathological examinations with light microscopy?

- H_0: The composition of the microbiota is the same between diseased and apparently healthy corals.
- H_A: The composition of the microbiota differs significantly between diseased and apparently healthy corals.

3. Are any of the observed suspected pathogenic microorganisms RLOs, as determined by ultrastructure using transmission electron microscopy?

- H_0: Microorganisms identified by light microscopy do not meet the criteria to be identified as RLOs with TEM.
- H_A: Microorganisms identified by light microscopy contain ultrastructural elements that distinguish them as RLOs with TEM.

4. Are corals in the NMNH coral reef exhibit tank presenting with tissue loss doing so as a result of environmental stressors, including changes in water quality or light conditions outside the range to which they are adapted?

- H_0: Disease outbreaks in the NMNH coral reef exhibit tank do not occur following water quality or lighting perturbations.
- H_A: Disease outbreaks in the NMNH coral reef exhibit tank coincide with water quality or lighting perturbations.

3 METHODS

3.1 Coral Histopathology Surveys of Apparently Healthy and Diseased Samples

Samples from apparently healthy and diseased coral colonies were collected from affected corals during two initial tissue-loss outbreaks (Table 1) using the protocol outlined in Appendix A. Further samples were collected during disease outbreaks in November 2016, April and July 2017, and January and September 2018. All samples were fixed in Z-Fix Concentrate (Anatech, Ltd.) diluted with 4 parts of artificial seawater, except for diseased subsamples 17-001-H–J and 17-042-J, fixed in Methacarn solution, and 17-001-K–M and 17-042-K, fixed in Carnoy's solution. It is important to note that the taxonomic identification of cultured corals in the aquarium trade is difficult, unless DNA analysis is conducted. Therefore, all coral specimens were identified to genera, if possible, based on historical knowledge of the acquisition of these corals, knowledge of tank managers, and expertise of coral scientists. Those designated 'ORA®' were special cultivars obtained from Oceans, Reefs & Aquariums (Fort Pierce, Florida).

Samples were processed using standard operating procedures in the Dr. Peters histology laboratory at George Mason University (Price & Peters 2018). After fixation, samples were trimmed to fit into cassettes for processing using a Dremel®. Enrobing in agarose prior to decalcification was performed when tissue-loss margins were present.

Table 1. Tissue-loss events
Dates of tissue-loss events analyzed during this study. Species affected is noted for each individual event.

Date	Species Collected	Histology Samples
November 9, 2016	*Acropora* sp. 'ORA® Frogskin'	17-001
February 4, 2017	*Acropora* sp. 'ORA® Red Planet'	17-007
April 12, 2017	*Montipora capricornis* (Green)	17-042
July 22, 2017	*Acropora hyacinthus*(?)	17-052
	Montipora capricornis (Red)	17-053
January 29, 2018	*Acropora* sp. 'ORA® Frogskin'	18-007

Samples were decalcified, using 10% pH 7 EDTA. All samples were embedded in paraffin blocks for sectioning. 5 µm-thick sections were mounted on microscope slides and stained with both Harris's hematoxylin and alcoholic eosin Y to evaluate tissue condition and Giemsa to detect Gram-negative bacteria. According to protocols outlined by Miller et al. (2014), all histoslides were examined using light microscopy and tissue condition, lesions, and bacterial infections were recorded in a spreadsheet noting their severity or intensity. Scores ranging from 0–5 were assigned for each condition parameter, with 0 being unaffected or "within normal limits" and 5 being the worst condition or highest severity. Reproductive structures were given scores based on the stage and number of structures present. Associated organisms were rated 1, if present, and 0, if absent. NS indicates "not enough tissue to determine" condition. A full

description of scores for each category can be found in Appendix B, as adapted from Miller et al. (2014).

3.2 Statistical Analyses

All semi-quantitative data from both apparently healthy and diseased samples were organized into two boxplots. Both boxplots were compared to observe similarities and differences in the distribution and spread of condition scores in both sample sets.

The Pearson-Product Moment Correlation test was used to statistically analyze semi-quantitative data from both sample sets (Lamb et al. 2014). Correlation coefficients(r) between all categories included in the semi-quantitative rating scale for both apparently healthy and diseased samples were calculated. The resulting matrices were observed for the strength and direction of any linear relationships between condition parameters. Any $r \geq 0.70/-0.70$ was considered to be strongly linearly positively or negatively correlated, respectively. To determine the significance of correlated categories, the p-value was calculated and compared to $\alpha = 0.05$ using regression analysis.

The prevalence of all taxa of organisms found associated with all pieces of all subsamples of both apparently healthy and diseased samples was calculated and graphed to analyze differences or similarities in associated taxa between sample sets.

3.3 TEM Analysis for Microorganisms

Transmission electron microscopy (TEM) was utilized to observe microorganisms found infecting host cells and to characterize their ultrastructure. Samples were processed for TEM using methods outlined by Price and Peters (2018). The fixative solution was a

2.5% glutaraldehyde solution with a 0.05 M sodium cacodylate buffer solution. For 2017 TEM samples, decalcification occurred prior to secondary fixation. 2018 samples were placed in the primary fixative according to the method outlined above and, after 24 hours, in a secondary fixative solution of 1% osmium tetroxide with a 0.05 M sodium cacodylate buffering solution, then decalcified. Next, samples were trimmed to fit TEM BEEM® embedding capsules and dehydrated, using graded ethanols (70%, 100%, 100%), with a final dehydration in 100% acetone. The EMBed-812 kit was used to embed all samples for sectioning. Resin blocks were then prepared for sectioning. The GW Nanofabrication and Imaging Center, George Washington University, Washington, D.C., sectioned the samples into 90 nm-thick ultra-thin sections for imaging on a Leica EMUC7 ultramicrotome, using a DiATOME ultra 45^{o} diamond knife. Subsequently, post-sectioning staining used 1% uranyl acetate to increase contrast during imaging. Imaging was performed by managing senior research scientist Dr. Christine Brantner. Electron micrographs were examined for microorganisms and for any significant findings regarding cellular condition.

3.4 Environmental Parameters Related to Disease Outbreaks

Water quality data, including specific gravity, calcium, alkalinity, and magnesium levels from 2010–2018, were collected by the aquarium maintenance staff through manual titration water testing using commercially-available water testing kits 3–4 times weekly. The test kits used to obtain calcium and alkalinity values were the Hach® alkalinity and hardness (calcium) kits and, more recently, the Red Sea Pro series titration test kits. Specific gravity was measured optically by refractive index readings and

temperature was monitored electronically. Historical records were obtained and entered into digital format to be compared with tissue-loss events. Any changes in lighting and other equipment anomalies, data gaps in paper records, and available dosage records were also analyzed, with a focus on the two months prior to a disease outbreak. These environmental data were analyzed for overall trends corresponding to disease outbreaks.

4 RESULTS

For this study, ten species were sampled across five genera, with *Acropora* and *Montipora* species most affected by disease in this system. To survey apparently healthy corals, samples were collected from colonies of *Acropora, Montipora, Pocillopora, Turbinaria,* and an unknown brain coral. Results will be reported in this chapter, with further analyses and implications of these results reserved for the discussion section.

4.1 Histopathology of Apparently Healthy and Diseased Samples

4.1.1 Analysis of Apparently Healthy Samples

Although histological examination of apparently healthy samples showed portions of subsamples with cellular architecture and staining within normal limits (Tables 2 and 3), 100% of samples contained suspect RLOs in either the epidermis, or the gastrodermis and cnidoglandular bands, or in a combination of these foci. Suspect RLO infections in the epidermal epithelium and in both the gastrodermis and mesenterial filaments of samples 18-014 through 18-021 were given scores of 0.5–5 in severity, with a mean score of 2.6 for severity of epidermal RLOs and 2 for severity of RLO infections in the gastrodermis and cnidoglandular bands (Figure 4). 18-037 through 18-039 samples had slightly more severe RLO infections in the epidermis and in the gastrodermis and mesenterial filaments, with mean scores of 3.7 and 3.4, respectively.

Table 2. Semi-quantitative data of apparently healthy samples 18-014 through 18-021 Samples collected on February 23, 2018 to survey apparently healthy corals during a time where no rapid tissue loss was observed. Associated organisms were rated 0 if absent, 1 if present. Semi-quantitative scores of 0–5 were used to describe condition, with 0 being in excellent condition or no change observed and 5 being the most severe effects observed. The exception to this is the oocytes and spermaries category, where the semi-quantitative scale is used to describe maturity of oocytes or spermaries, if present.

Sample	Species	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
18-014-A	*Pocillopora* sp.	1	3	2	1	0	0	5	5	1	0	0	0	0	0	0	0
18-014-B	*Pocillopora* sp.	3	4	3	1	1	2	3	4	2	0	0	0	0	0	0	0
18-015-A	*Acropora* sp.	4	3	2	4	2	3	1	2	4	0	0	0	0	0	0	0
18-015-B	*Acropora* sp.	3.5	3	2	2	2	2.5	0.5	2.5	3	0	1.5	0	0	0	0	0
18-016-A	*M. capricornis*	2.5	3	1.5	2	2	2	1	0.5	1	0	0	0	0	0	0	0
18-016-B	*M. capricornis*	2.5	3.5	1.5	2	1	1.5	1	1	1	0	0	0	0	0	0	0
18-017-A	*M. hispida*	3	3	2	2.5	2.5	2.5	1.5	1	1.5	0	0	0	0	0	0	1
18-017-B	*M. hispida*	4	4	2	3	2	3	2	3	1	0	2	2	0	0	0	0
18-018	*Acropora* sp. Red Planet	4	5	3	3	3	3	5	1	4	0	0	0	0	0	0	1
18-019	*Turbinaria* sp.	3	3	2	2	1	1.5	3.5	1.5	1.5	0	0	0	0	0	0	1
18-020	*Brain UNKN*	3	3	3	2	2	1	4	1	3	0	0	0	0	0	0	0
18-021	*Acropora* sp. 'Frogskin	3	3	3	2	1	1	4	2	1	0	0	0	0	0	0	0
% Affected		100	100	100	100	91.7	91.7	100	100	100	0	16.7	8.3	0	0	0	17.6
Mean		3	3.4	2.3	2.2	1.6	1.9	2.6	2	2	0	0.3	0.2				
St. dev.		0.8	0.6	0.6	0.8	0.8	1	1.7	1.4	1.2	0	0.7	0.6				
Median		3	3	2	2	2	2	2.5	1.8	1.5	0	0	0				
Min		1	3	1.5	1	0	0	0.5	0.5	1	0	0	0				
Max		4	5	3	4	3	3	5	5	4	0	2	2				

Table 3. Semi-quantitative data of apparently healthy samples 18-037 through 18-039 Semi-quantitative scores of 0–5 used to describe condition, with 0 being in excellent condition or no change observed and 5 being the most severe effects observed. The exception to this is the oocytes and spermaries category, where the semi-quantitative scale is used to describe maturity of oocytes or spermaries, if present. 18-037 samples collected from *Acropora* sp. 'ORA® Frogskin', 18-038 samples collected from one colony of *Acropora* sp. 'ORA® Red Planet', and 18-039 samples collected from a separate colony of the same species. Associated organisms were rated 0 if absent, 1 if present.

Sample	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/ Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
18-037-A	2	3	1	3	3	3	5	4	2	0	0	0	0	0	0	0
18-037-B	3	3.5	3	2	1.5	2.5	5	4	1	0	0	0	0	0	0	1
18-038-A	3.3	3.3	2.3	2	2	3	1	3.3	2	0	2	1.7	1	0	0	1
18-038-B	3	3.7	2	1.3	1	1.3	1	3.3	1.3	0	2.3	1.3	0	0	0	1
18-039-A	3	3	3	2	1	1	5	3	2	0	0	0	0	0	0	0
18-039-B	4	4	3.5	2	1.5	2.5	5	2.5	1.5	0	1	0	0	0	0	0
% Affected	100	100	100	100	100	100	100	100	100	0	50	33.3	8.3	0	0	25
Mean	3.1	3.4	2.5	2.1	1.7	2.2	3.7	3.4	1.6	0	0.9	0.5				
St. Dev.	0.7	0.4	0.9	0.5	0.8	0.9	2.1	0.6	0.4	0	1.1	0.8				
Median	3	3.4	2.7	2	1.5	2.5	5	3.3	1.8	0	0.5	0				
Min	2	3	1	1.3	1	1	1	2.5	1	0	0	0				
Max	4	4	3.5	3	3	3	5	4	2	0	2.3	1.7				

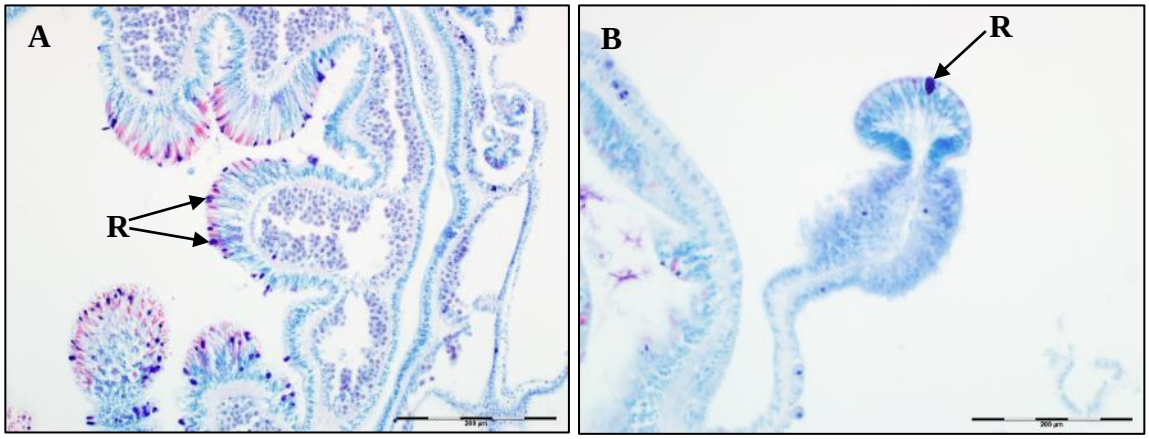


Figure 4. RLO Infections in Apparently Healthy Samples
RLOs (R) are shown infecting the A) tentacle epithelia of *Pocillopora* sp. and B) cnidoglandular bands' epithelia of *Acropora* sp. 'ORA® Frogskin'. Visualized with Giemsa, RLOs appear as dark purple grape-like clusters. Image A magnified 20x; Image B magnified 40x.

RLOs appeared as purple coccoid clusters infecting mucocytes when stained with Giemsa (Miller et al. 2014). Some subsamples exhibited portions of tissues that were in histologically poorer condition than expected, because there were no gross signs of health impairment.

The zooxanthellae of all apparently healthy samples were given scores between 1 and 4, with mean scores of 3.4 in 18-014 through 18-021 samples, and 3.4 in 18-037 through 18-039 samples. On average, the zooxanthellae were in fair condition; however, in a number of samples, they were lysed and had been released with other cell debris into the lumens of gastrovascular canals. The gastrodermis was pale-staining and, in places, discontinuous. The condition of epidermal mucocytes was rated as good to fair, with scores ranging from 1–3. 18-014 through 18-021 samples had an average score of 2.3 and 18-037 through 18-039 samples received similar ratings, with a mean score of 2.5. The

cnidoglandular bands appeared to be intact, with mild degeneration noted, and mesenterial filaments exhibited minimal to mild dissociation throughout both sample sets. Most samples had at least 50% of the costae intact, with the exception of 18-015-A and 18-018, with approximately 75% of costae exposed. 18-019 *Turbinaria* sp. samples contained a large abundance of melanin or melanin-like pigment granules throughout the surface body wall (Figure 5). Oocytes and spermaries were observed in subsamples from 18-015, 18-017, 18-038, and 18-039. Associated protozoans were observed in both apparently healthy samples sets, while crustaceans were only associated with 18-038-A.

4.1.2 Analysis of Samples Affected by Tissue Loss

17-001 Samples

The general condition of 17-001 subsamples were fair to poor, with a mean score of 3.5 (Table 4). Many samples showed necrosis occurring through a portion or, in some cases, all of the sample. Aggregations of suspect RLOs were observed in the mucocytes of the epidermal epithelium (mean score=3.1), and the gastrodermis and cnidoglandular bands epithelia (mean score=2.7) (Figure 6). RLOs were observed within mucocytes, bursting from cells when the integrity of the host cell plasmalemma was compromised. Both life stages of the RLOs were present, with putative elementary bodies appearing as small, pale-staining coccoid to pleomorphic structures and reticulate bodies as dark purple grape-like clusters (Figures 6 and 7). Samples had frothy pale-purple staining material in epidermal mucocytes, due to increased mucus production, resulting in an

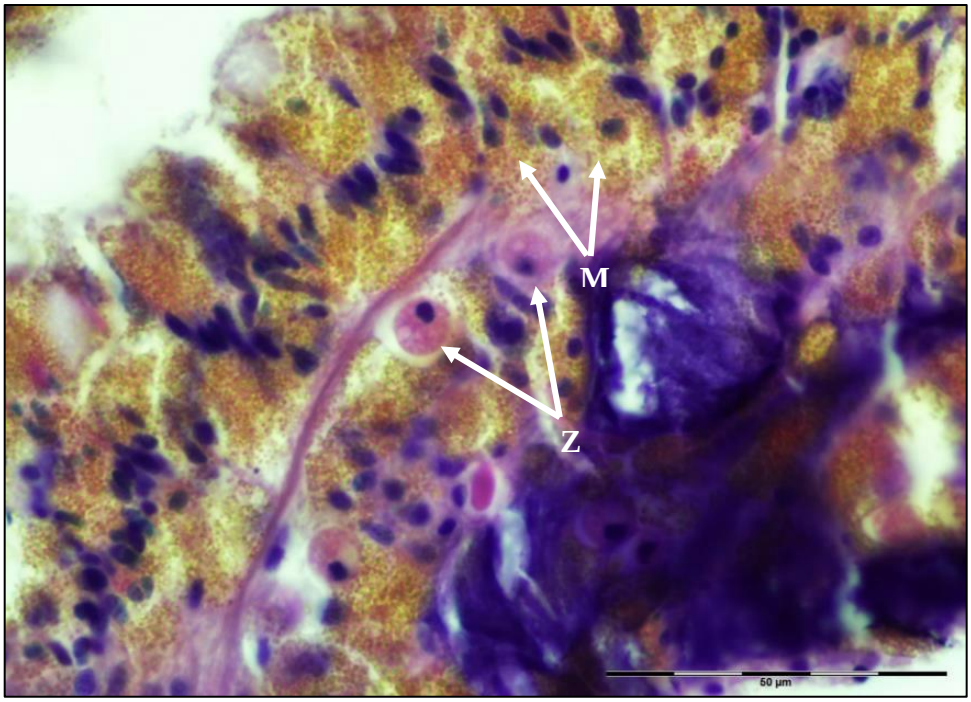


Figure 5. Melanin in *Turbinaria* sp. sample
Visualized with Harris' hematoxylin and eosin stain, the surface body wall 18-019 contains melanin (M) pigmentation, pictured here as aggregates of gold granules, 100x. Zooxanthellae (Z) is present and visible.

average score of 2.5. Zooxanthellae were in fair to poor condition (mean score=3.5). The gastrodermis was scored in the fair to poor range, due to both hypertrophy and attenuation of the gastrodermis in subsamples, hypertrophied mucocytes, heavy mucus production, and discontinuity. Mesenterial filaments' mucocytes, degeneration of cnidoglandular bands, and dissociation of mesenterial filaments, received mean scores in the fair range of 3.8, 3.1, and 3.2, respectively. Associated organisms, including protozoans and crustaceans were observed. Samples with necrosis exhibited higher densities of protozoans consuming zooxanthellae, cell debris, and RLOs. Whereas

Table 4. Semi-quantitative data of 17-001 samples affected by rapid tissue loss
Samples were collected on November 9, 2016 from *Acropora* sp. 'ORA® Frogskin', Samples A–G were fixed in Z-fix®, H–J in Methacarn, and K–M in Carnoy's Solution. Samples E–G, J, L, and U–V were all placed into a holding tank until November 12, 2016. Associated organisms were rated 0 if absent, 1 if present. Semi-quantitative scores of 0–5 used to describe condition, with 0 being excellent condition or no change and 5 being the most severe, with the exception of the oocytes and spermaries categories, where scores describe structure maturity, if present.

Sample	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
17-001-A	1.5	1.5	0.3	4.5	1.5	2.5	4.3	3.3	1.8	0	0.5	0	0	0	0	1
17-001-B	3	3	0	5	2	2	5	3	3	0	0	0	0	0	0	0
17-001-C	5	5	5	5	5	5	1.5	1	5	0	0	0	0	0	0	1
17-001-D	5	5	5	5	5	5	1	1.5	5	0	0	0	0	0	0	1
17-001-E	2.8	2.7	0.7	2.3	2.3	2	3.7	4.2	2.3	0	2.2	1	0	0	0	1
17-001-F	2.8	3.2	2.2	2.8	1.3	1.8	5	3.8	2	0	1.3	1	0	0	0	1
17-001-G	3	2.8	1	4	3.1	3	2.9	2.75	2.1	0	1.6	1	0	0	0	1
17-001-H	5	5	5	5	5	5	1	3	5	0	0	0	0	0	0	1
17-001-I	2	1.8	0.5	2.8	2	1.3	4.3	3.3	1.5	0	1.8	1.3	0	0	0	1
17-001-J	3.3	3.8	2.5	2	2.3	2.8	3.5	3.3	2.5	0	1	0	0	0	0	0
17-001-K	5	5	5	5	5	5	1	1	5	0	0	0	0	0	0	1
17-001-L	3	3.3	2.7	2.5	2	1.8	3	2.3	3.5	0	0.7	0.3	1	0	0	1
17-001-M	3.5	3.3	3.3	3.2	3.3	3.8	3.7	3.2	3.5	0	1.8	1	0	0	0	1
% Affected	100	100	92.3	100	100	100	100	100	100	0	61.5	46.2	7.7	0	0	84.6
Mean	3.5	3.5	2.5	3.8	3.1	3.2	3.1	2.7	3.3	0	0.8	0.4				
St. Dev	1.2	1.2	2	1.2	1.5	1.4	1.5	1	1.4	0	0.8	0.5				
Median	3	3.3	2.5	4	2.3	2.8	3.5	3	3	0	0.7	0				
Min	1.5	1.5	0	2	1.3	1.3	1	1	1.5	0	0	0				
Max	5	5	5	5	5	5	5	4.2	5	0	2.2	1.3				

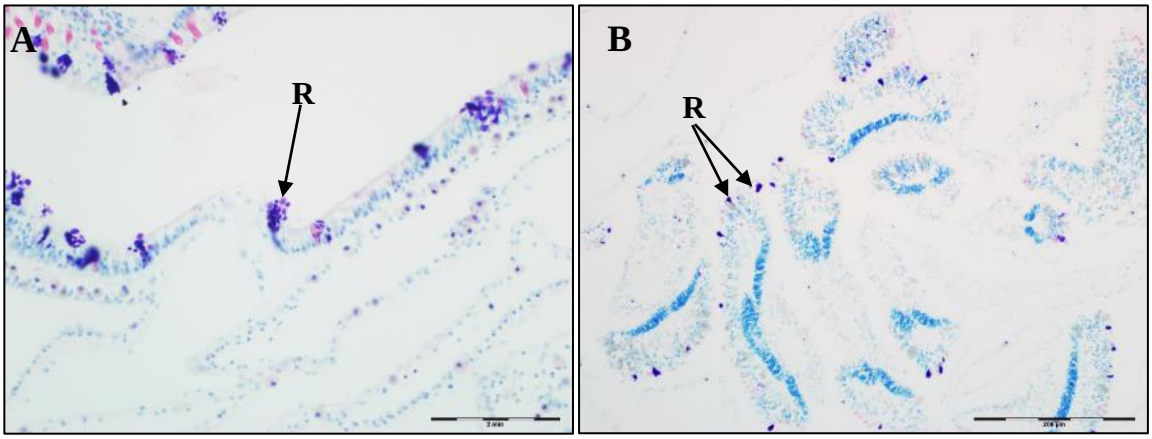


Figure 6. RLO infections in 17-001 samples
A) RLO infections (R) in mucocytes, with reticulate bodies bursting out of cells B) cnidoglandular (CG) bands of *Acropora* sp. 'ORA® Frogskin', visualized at 40x and 20x, respectively, with Giemsa.

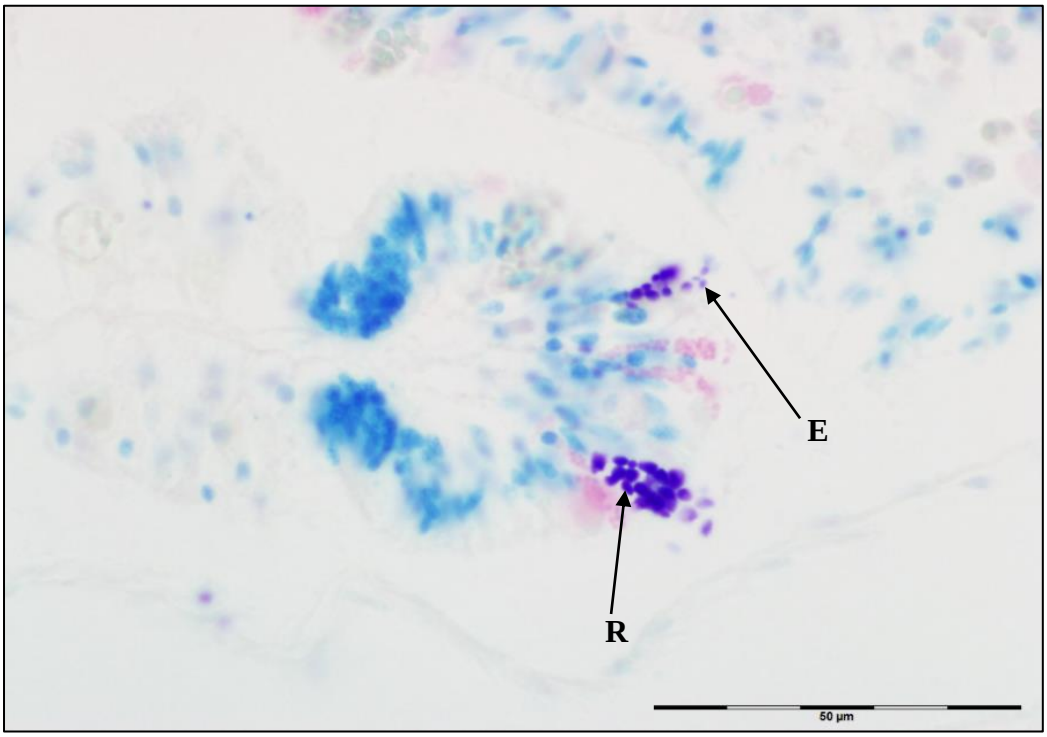


Figure 7. RLO Infections in *Acropora* sp. 'ORA® Frogskin'
RLO reticulate bodies (R) in cnidoglandular band epithelia appear as purple clusters. Elementary bodies (E) are present, appearing as pleomorphic, paler staining purple structures. Giemsa, 100x.

protozoans were prominent in 84.6% of samples, crustaceans were observed in only 7.7% of samples; 64% of samples contained oocytes and 41% contained spermaries. Although scores for condition of costal tissues varied from 1.5–5, approximately 50% of costae were exposed (mean score=3.3).

17-007 Samples

The majority of 17-007 subsamples were lost in processing and remaining subsamples were in very poor condition, resulting in a score of 4 (Table 5). Intact epidermal mucocytes were producing large quantities of dark, stringy mucus. Many were lysing or missing, and epidermis appeared attenuated, resulting in a score of 5. Moderate RLO infections were also observed in the gastrodermis and cnidoglandular band epithelia, which received a score of 3. RLOs were observed extracellularly in the lumens of gastrovascular canals in 17-007-A-2-1. RLO reticulate and elementary bodies were visible in infected mucocytes and where mucocytes had burst (Figure 8). A number of the zooxanthellae exhibited staining characteristic of lysis, which resulted in a score of 3. The gastrodermis was pale-staining and atrophied. Degeneration of mesenterial filaments and dissociation of cnidoglandular bands was marked and, therefore, resulted in a score of 4 in both categories. Approximately 50% of costae were exposed, resulting in a score of 3.

17-042 Samples

17-042 samples were in fair to poor condition with scores ranging from 2–5 (Table 6). Associated organisms, including ciliates within gastrovascular canals and externally (50%), nudibranchs (10%), crustaceans (20%), microcolonies of suspect

Table 5. Semi-quantitative data of sample 17-007-A affected by rapid tissue loss Collected on February 4, 2017 from a colony of *Acropora* sp. 'ORA® Red Planet', semi-quantitative scores of 0–5 used to describe condition, with 0 being in excellent condition or no change observed and 5 being the most severe effects observed. The exception to this are the oocytes and spermaries categories, where the semi-quantitative scale is used to describe maturity, if present. Standard deviation is not included for this single sample. Associated organisms were given a score of 1 if present, 0 if absent.

Sample	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
17-007-A	4	3	5	2	4	4	5	3	3	0	2	2	0	0	0	0
% Affected	100	100	100	100	100	100	100	100	100	0	100	100	0	0	0	0
Mean	4	3	5	2	4	4	5	3	3	0	2	2				
Median	4	3	5	2	4	4	5	3	3	0	2	2				
Min	4	3	5	2	4	4	5	3	3	0	2	2				
Max	4	3	5	2	4	4	5	3	3	0	2	2				

bacteria in gastrovascular canals (80%), and polychaetes (30%) were observed (Figures 9–11, 13). Suspect RLOs were observed in epidermal mucocytes (mean score=0.8), and internally, in both the gastrodermis and cnidoglandular band epithelia (mean score=2.0) (Figure 12).

Although zooxanthellae were present, a large proportion exhibited signs of poor condition or were lysing, resulting in scores between 3–5, with a mean of 4.3. Internally,

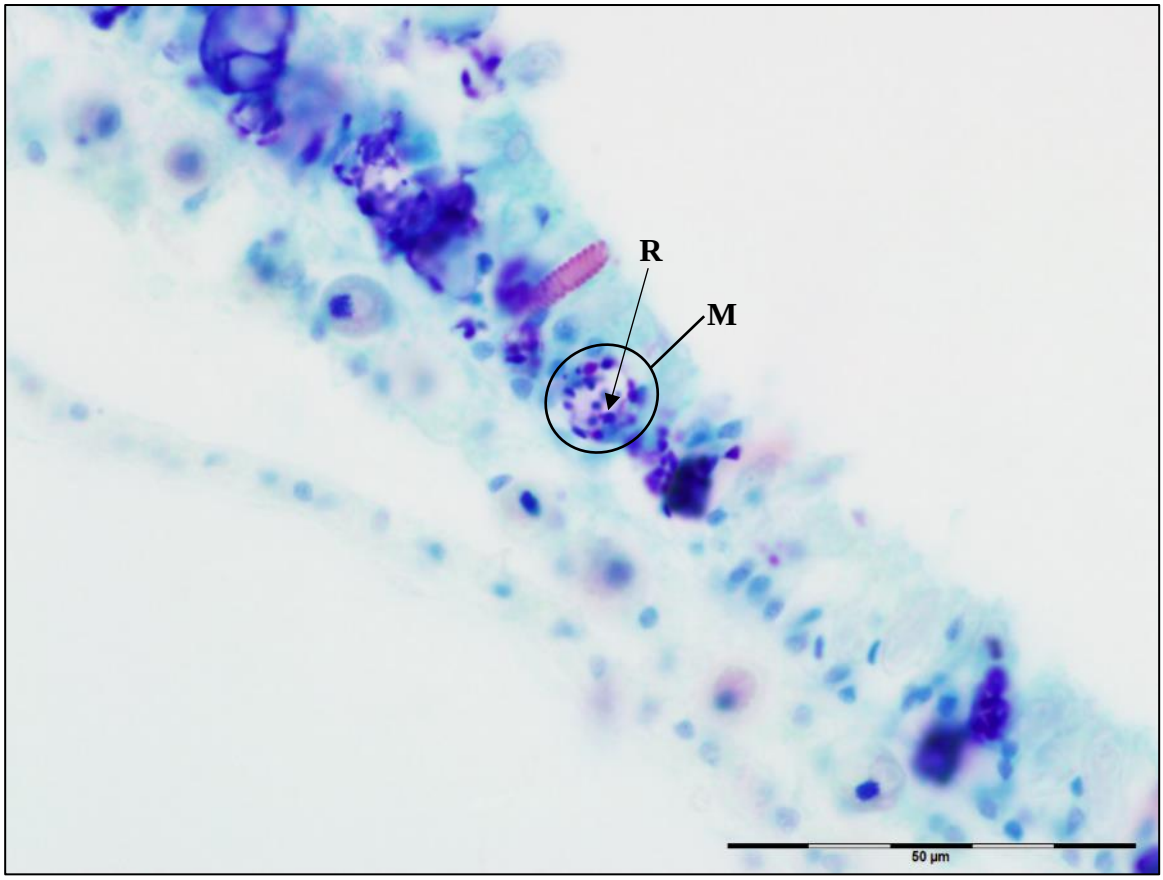


Figure 8. surface body wall of 17-007 Sample
Visualized with Giemsa, RLO (R) reticulate and elementary bodies are visible in mucocytes (M) of the epidermal epithelium of *Acropora* sp. 'ORA ® Red Planet' as dark purple coccoid or pleomorphic clusters.

degeneration of cnidoglandular bands, dissociation of mesenterial filaments, and mesenterial filament mucocytes were moderate, resulting in mean scores of 3.5, 3.7, and 3.6, respectively. Costae condition varied, but on average, approximately 75% of costae were exposed. Few early spermaries were observed in one subsample.

17-052 and 17-053 Samples

17-052 and 17-053 subsamples were in overall poor condition (Table 7). Necrosis and lysis of both the basal body wall and surface body wall left few tissues intact and

Table 6. Semi-quantitative data of samples 17-042 affected by rapid tissue loss Collected on April 23, 2017 from a colony of *Montipora capricornis,* semi-quantitative scores of 0–5 used to describe condition, with 0 being in excellent condition or no change observed and 5 being the most severe effects observed. The oocytes and spermaries category describes condition and maturity of oocytes or spermaries, if present. During this disease outbreak, *Montipora*-eating nudibranchs were observed on this colony. Samples A–I were fixed in Z-fix®, whereas sample J was fixed in Methacarn and K was fixed in Carnoy's Solution. Associated organisms were given scores of 1 if present, 0 if absent.

Sample	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
17-042-A	4	4	2	2	4	4	2	4	5	4	0	0	0	0	0	1
17-042-B	4	4	2	2	3	4	1	3	5	0	0	0	0	0	0	0
17-042-C	2	3	2	2	NS	NS	0	1	0	0	0	0	1	0	0	1
17-042-D	4.5	3.5	2.5	3.5	2	2	0.5	1	3.5	5	0	0	0	0	0	0
17-042-E	4	3	2	4	2	3	2	4	3	2	0	0	0	0	0	1
17-042-F	5	4	3	3	4	4	1	1	3	4	0	0	0	0	1	1
17-042-G	5	5	5	5	5	5	0	1	5	2	0	0	1	1	1	1
17-042-I	5	5	5	5	5	5	1	1	5	4	0	2	0	0	1	0
17-042-J	4.5	5	5	4.5	2.5	2.5	0.5	1.5	4	3.5	0	0	0	0	0	0
17-042-K	5	5	3	5	4	4	0	2	4	1	0	0	0	0	0	0
% Affected	100	100	100	100	90	90	70	100	90	80	0	10	20	10	30	50
Mean	4.3	4.2	3.2	3.6	3.5	3.7	0.8	2	3.8	2.6	0	0.2				
St. Dev	0.9	0.8	1.3	1.3	1.2	1.0	0.8	1.3	1.6	1.8	0	0.6				
Median	4.5	4.0	2.8	3.8	4.0	4.0	0.8	1.3	4.0	2.8	0	0.0				
Min	2.0	3.0	2.0	2.0	2.0	2.0	0	1.0	0	0	0	0				
Max	5.0	5.0	5.0	5.0	5.0	5.0	2.0	4.0	5.0	5.0	0	2.0				

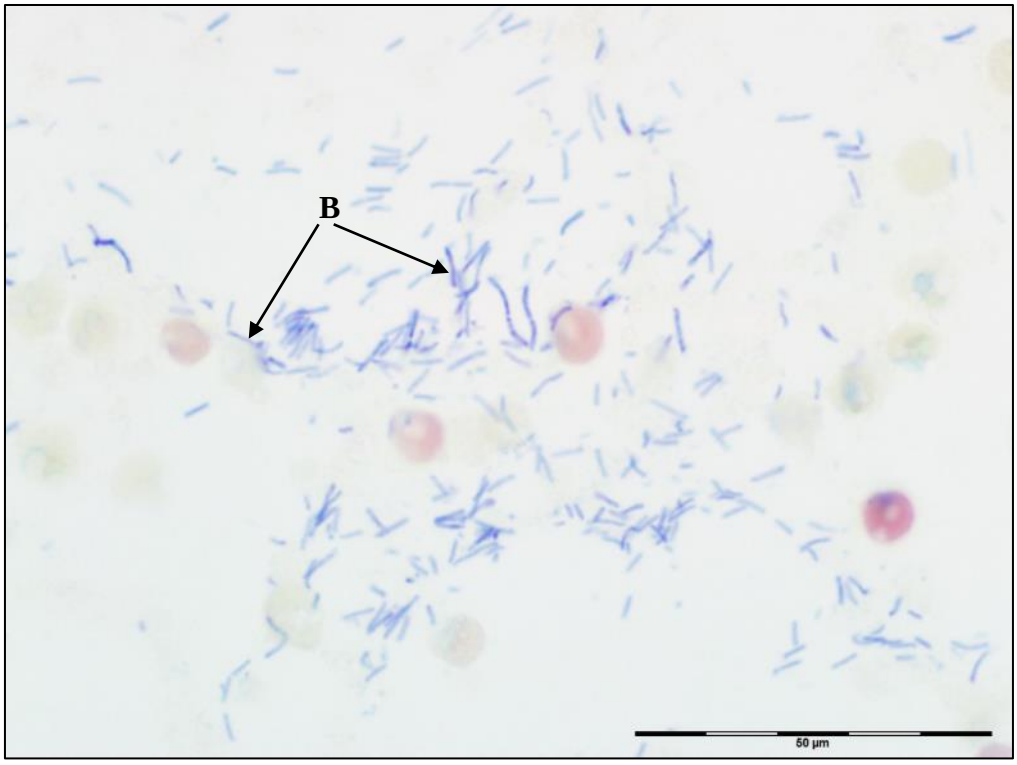


Figure 9. Suspect bacteria found in 17-042 samples
Bacteria (B) appear as blue rod-shaped structures internally in 17-042 samples of *Montipora capricornis* stained with Giemsa, magnified 100x.

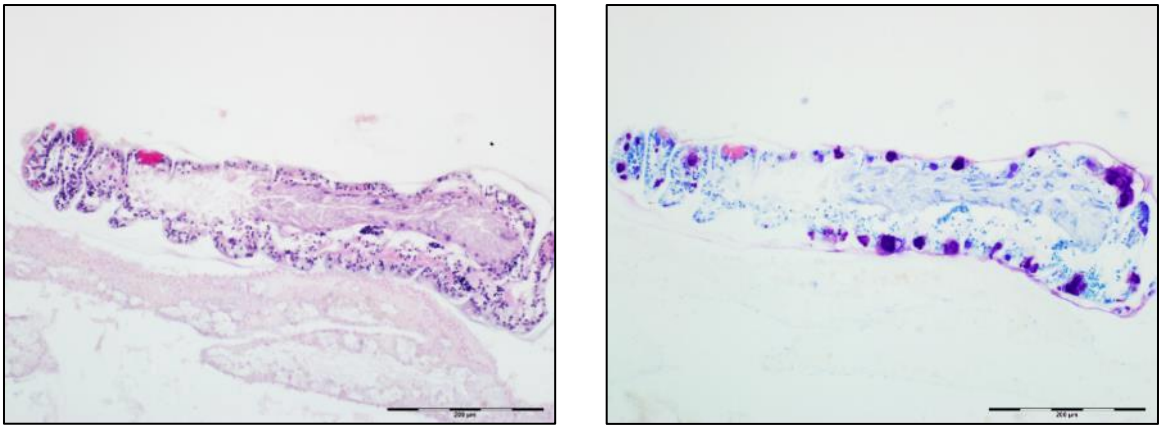


Figure 10. Polychaete worm
Longitudinal section of polychaete worm on the surface of *Montipora capricornis* stained with both Harris's hematoxylin and eosin Y (left) and Giemsa (right).

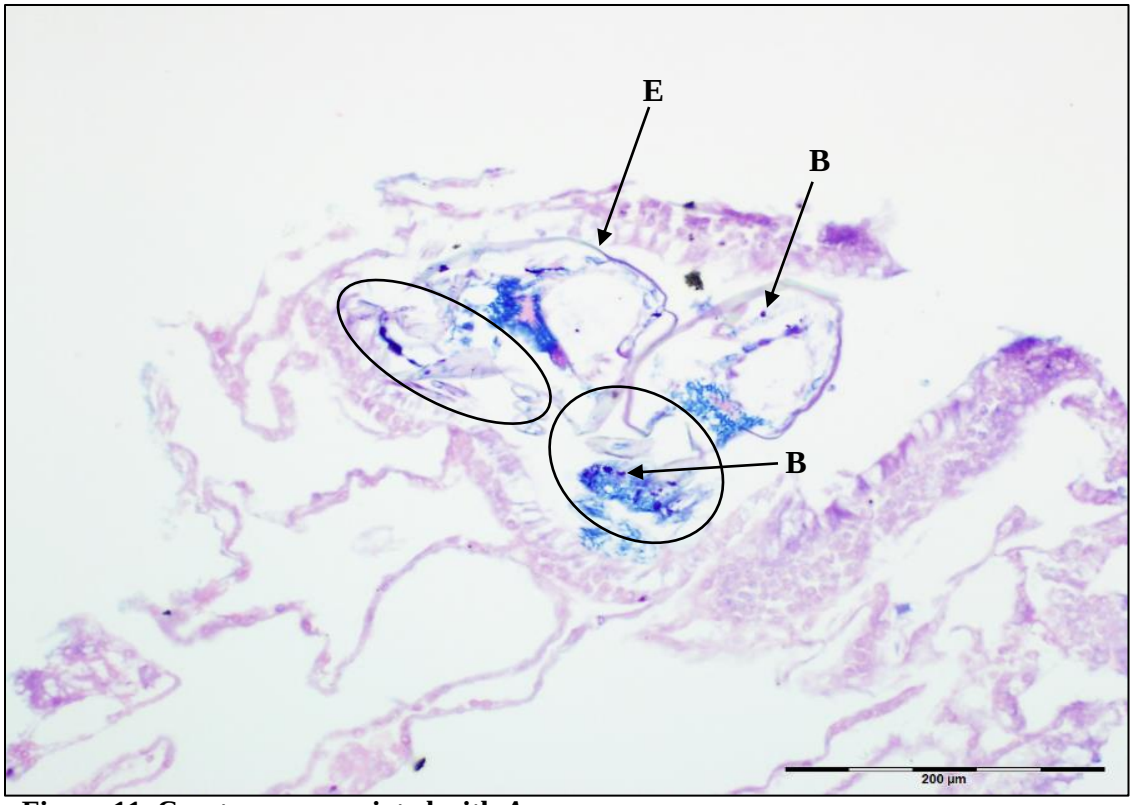


Figure 11. Crustacean associated with *Acropora* sp.
Giemsa clearly indicates the presence of Gram-negative bacteria (B) both externally, on appendages (encircled), and internally. Chitinous exoskeleton (E) is slightly refractile under light microscopy, 20x.

observed in the epidermis and the gastrodermis and cnidoglandular bands' epithelia, resulting in scores of 0–3.5 in severity, with means of 0.6 and 2.2, respectively. The degeneration of cnidoglandular bands and dissociation of mesenterial filaments was moderate to marked, receiving mean scores of 3.6 and 4.2, respectively. Costal tissue loss was significant, resulting in a mean score of 4.0. Whereas, subsamples of 17-052 contained spermaries and oocytes, subsamples from 17-053 did not. These structures in many cases, appeared deformed and pale-staining indicating that they were lysing.

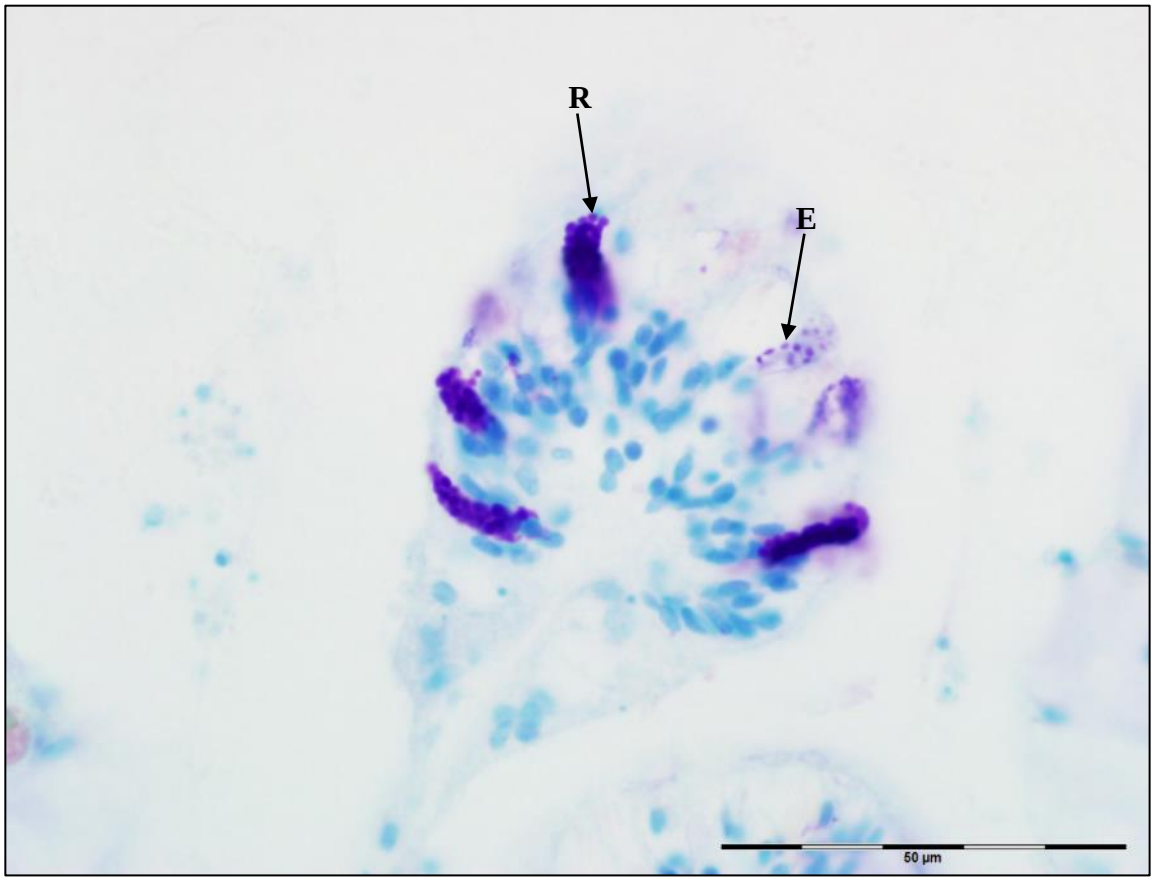


Figure 12. RLO infections in the cnidoglandular bands of 17-042 samples
RLO Infections are visualized in the cnidoglandular bands of *Montipora capricornis* with Giemsa stain. Whereas elementary bodies (E) are small, coccoid to pleomorphic structures, reticulate bodies (R) appear as dark-purple grape-like clusters.

18-007 Samples

18-007 subsamples were in generally fair condition, with a mean score of 2.9 (Table 8). Zooxanthellae were in good condition, with a mean score of 2.1. Epidermal mucocytes were numerous and releasing abundant frothy mucus (mean score=1.9). Internally, subsamples exhibited minimal to mild degeneration of cnidoglandular bands and dissociation of mesenterial filaments, with some hypertrophy, resulting in scores of

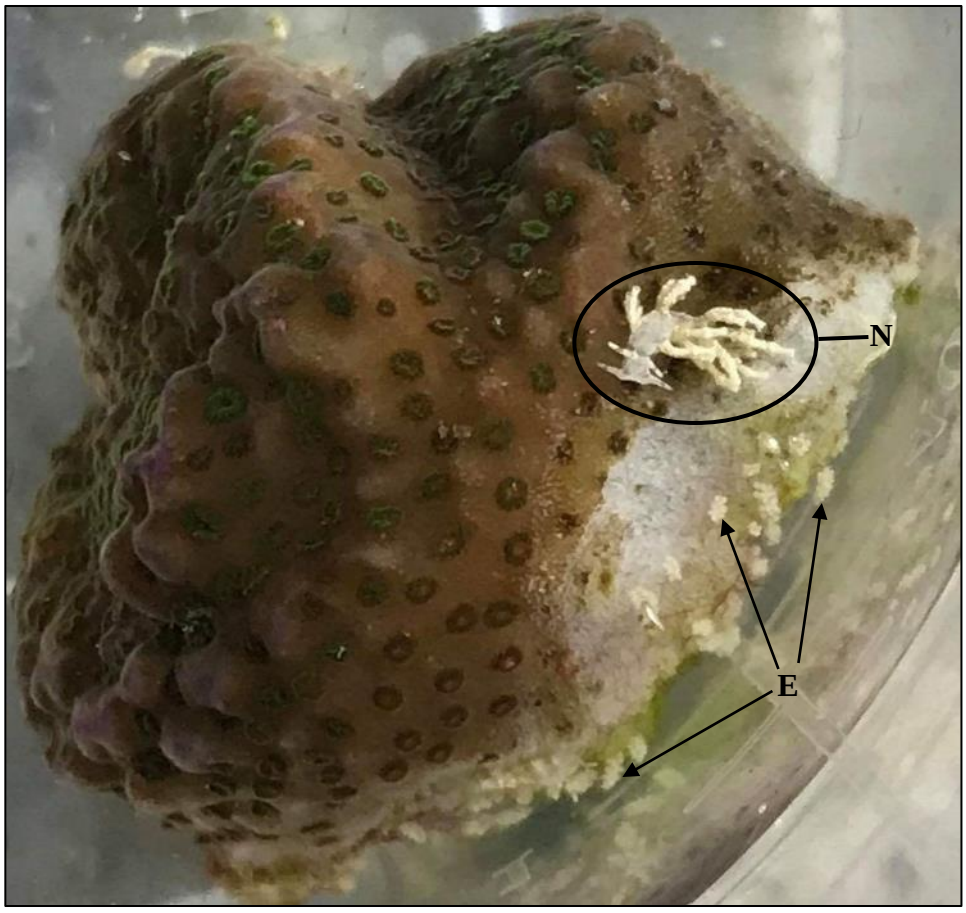


Figure 13. Nudibranch adult and egg masses present on *Montipora* colony "Montipora-eating nudibranch" (N), approximately 9 mm can be observed here, along with numerous egg masses (E), approximately 1 mm laid on denuded skeleton.

1.4 and 2, respectively. Whereas the suspect RLO infections in the epidermal epithelium were marked (mean=4.1), RLOs were observed in minimal numbers in the gastrodermis and cnidoglandular bands' epithelia (mean=1.5). Approximately 25% of costae were exposed, resulting in scores of 1–2. Early to mid-stage oocytes and spermaries were observed. Ciliates were found aggregating in large numbers in all 18-007 subsamples

Table 7. Semi-quantitative data of samples 17-052 and 17-053 affected by rapid tissue loss Collected July 22, 2017, semi-quantitative scores of 0–5 describe condition, with 0 being in excellent condition or no change observed and 5 being the most severe effects observed. The exception to this is the oocytes and spermaries categories, where the semi-quantitative scale is used to describe maturity of oocytes or spermaries, if present. NS indicates not enough tissue to determine. Samples 17-052 were collected from a colony of *Acropora hyacinthus*, and 17-053 samples were collected from the similarly affected *Montipora capricornis*. Associated organisms were rated 1 if present, 0 if absent.

Sample	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
17-052-A	5	5	5	5	5	5	NS	NS	5	0	3	3	0	0	0	1
17-052-B	5	5	5	5	5	5	1	1	5	0	2	0	0	0	0	1
17-052-C	5	5	5	5	5	5	1	3.5	5	0	3	3	0	0	0	1
17-053-A	4	3	4.5	2	2.5	4	0	2.5	3	0	0	0	0	0	0	0
17-053-B	1	1	2	4	1	3	1	2	1	0	0	0	0	0	0	1
17-053-C	4	4	5	5	3	3	0	2	5	0	0	0	0	0	0	0
% Affected	100	100	100	100	100	100	50	83.3	100	0	50	33.3	0	0	0	66.7
Mean	4	3.8	4.4	4.3	3.6	4.2	0.6	2.2	4	0	1.3	1				
St. Dev	1.6	1.6	1.2	1.2	1.7	1	0.6	0.9	1.7	0	1.5	1.6				
Median	4.5	4.5	5	5	4	4.5	1	2	5	0	1	0				
Min	1	1	2	2	1	3	0	1	1	0	0	0				
Max	5	5	5	5	5	5	1	3.5	5	0	3	3				

Table 8. Semi-quantitative data from samples 18-007 affected by rapid tissue loss collected on January 29, 2018 from a colony of *Acropora* sp. 'ORA® Frogskin', semi-quantitative scores of 0–5 used to describe condition, with 0 being in excellent condition or no change observed and 5 being the most severe effects observed. The exception to this is the oocytes and spermaries category, where the semi-quantitative scale is used to describe maturity of oocytes or spermaries, if present. Samples were also collected for TEM imaging and analysis at this time.

Sample	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries	Associated Crustaceans	Associated Nudibranchs	Associated Polychaete Worms	Associated Protozoans
18-007-A	2.8	2.2	1.6	2.6	1.2	2	4.2	1.6	2.2	0	0.8	0.4	0	0	0	1
18-007-B	3	2	2	2	2	2	3	1	1	0	2	2	0	0	0	1
18-007-C	3	2	2	2	1	2	5	2	3	0	2	0	0	0	0	1
% Affected	100	100	100	100	100	100	100	100	100	0	100	66.7	0	0	0	100
Mean	2.9	2.1	1.9	2.2	1.4	2	4.1	1.5	2.1	0	1.6	0.8				
St. Dev.	0.1	0.1	0.2	0.4	0.5	0	1.0	0.5	1.0	0	0.7	1.1				
Median	3	2	2	2	1.2	2	4.2	1.6	2.2	0	2	0.4				
Min	2.8	2	1.6	2	1	2	3	1	1	0	0.8	0				
Max	3	2.2	2	2.6	2	2	5	2	3	0	2	2				

(Figure 14), and some had consumed RLOs, which were still exhibiting normal staining characteristics (Figure 15).

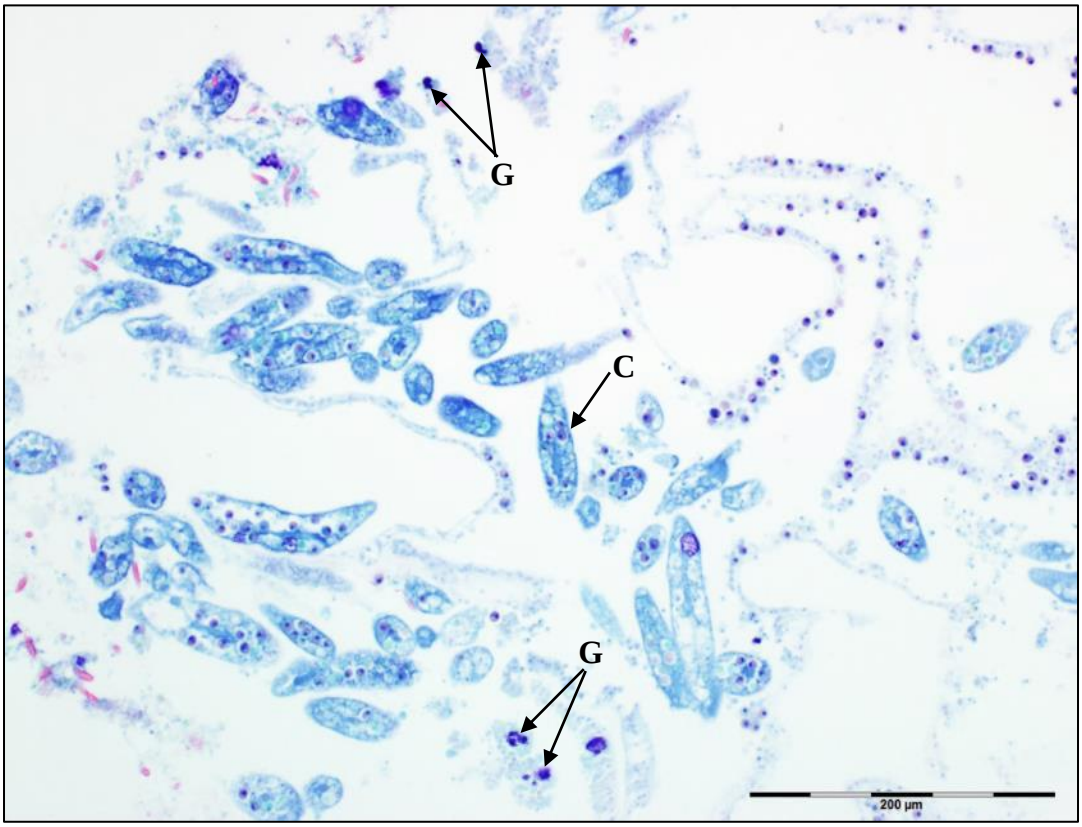


Figure 14. Ciliate aggregations in 18-007 samples, 20x
Visualized with Giemsa stain, here ciliates (C) are blue cigar-shaped structures in gastrovascular canals consuming coral tissues, zooxanthellae, and cell debris. Gastrodermal RLO (G) infections are still visible in remnant tissue.

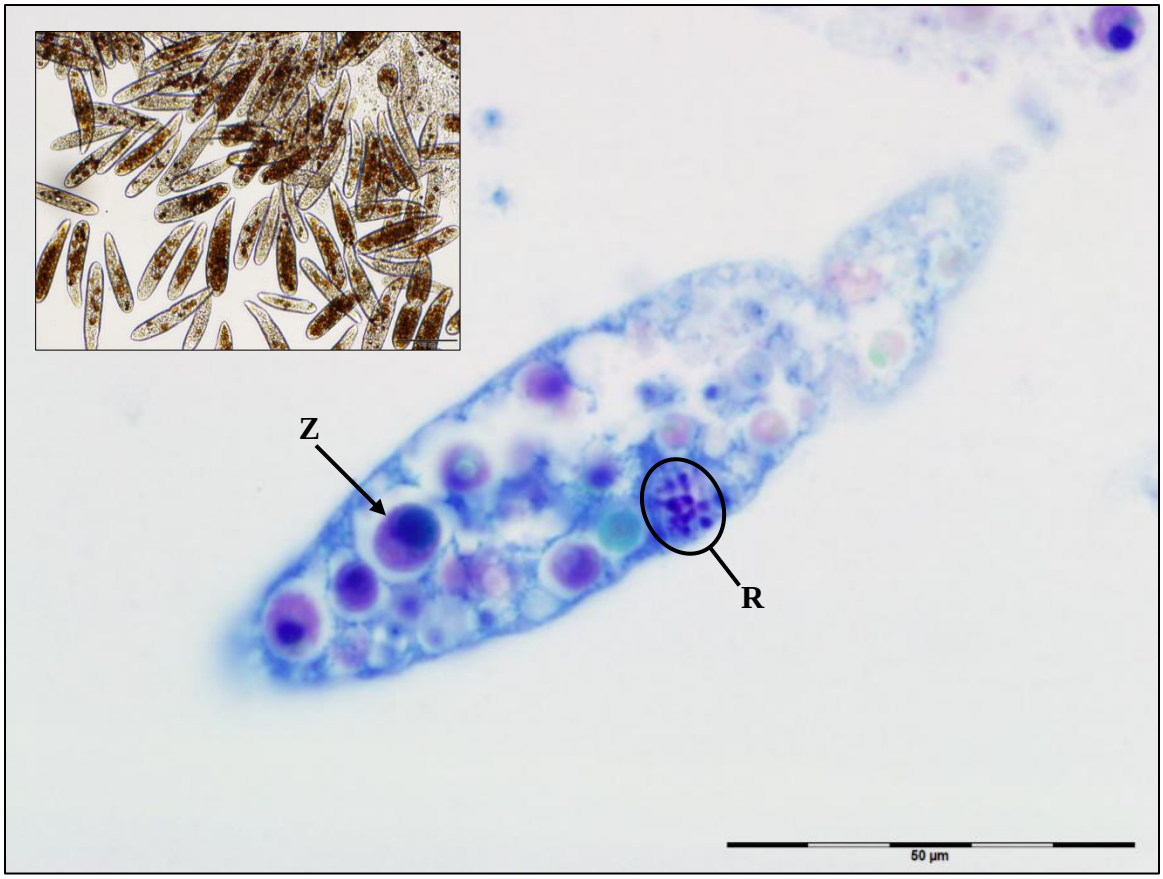
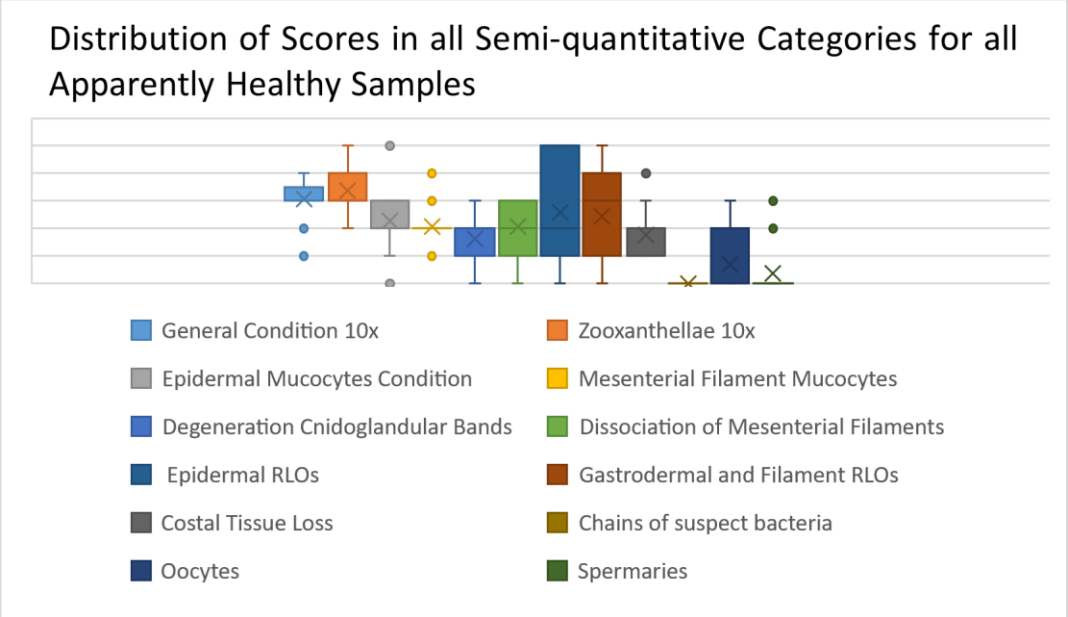


Figure 15. *Philaster lucinda* ciliate in 18-007
Longitudinal section of ciliate magnified 100x, visualized with Giemsa. Actively consuming zooxanthellae (Z), cell debris, and RLOs (R), the purple-staining, grapelike cluster within the organism. The insert is a light micrograph of these organisms, by Dr. Brent Whitaker, prior to fixation and processing.

4.2 Statistical Analysis

Apparently healthy samples showed a narrower distribution for most condition parameters, except for epidermal RLO infection severity (Figure 16A). Overall, samples collected during a disease outbreak had a wider distribution of scores. In addition, mean scores were generally slightly higher in diseased samples (Figure 16B). Generally, the

A)



B)

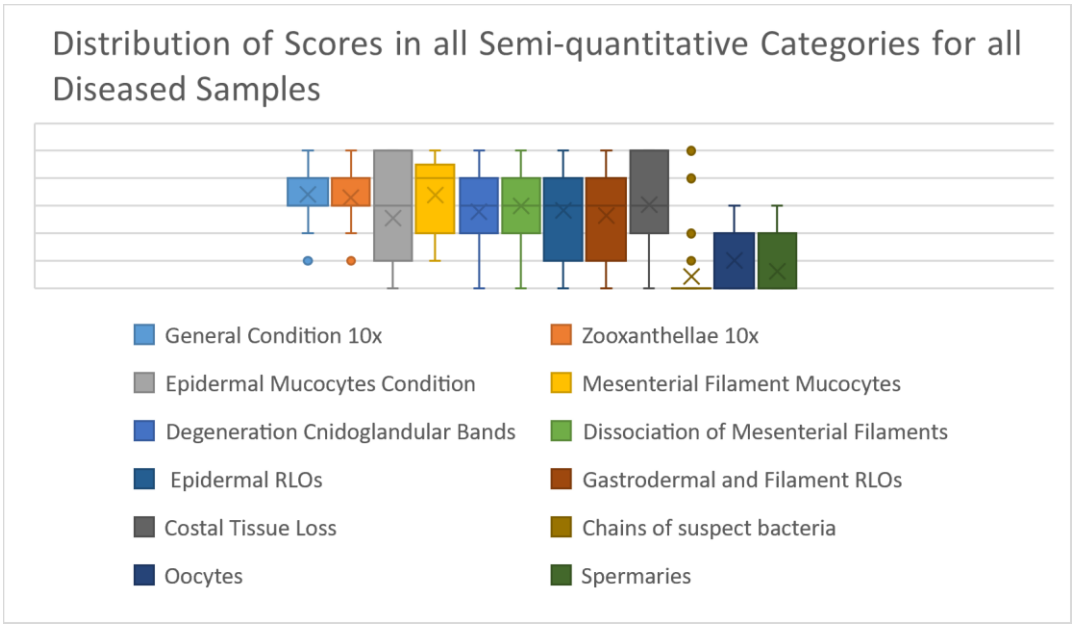


Figure 16. Visual comparison of semi-quantitative data distribution
A) Shows the distribution of data produced from all apparently healthy samples, and B) shows the distribution of all scores generated for all diseased samples.

scores for both diseased and apparently healthy samples exhibited non-normal, mostly positively skewed distributions of data, with more outliers present in apparently healthy samples (Figure 16A).

The Pearson Product-Moment Correlation (PPMC) was calculated, and a correlation matrix constructed. When apparently healthy samples were analyzed, three strong positive relationships were found between semi-quantitative categories (Table 9). The numbers of strong linear relationships increased when analyzing all diseased samples, with 16 strong relationships present between all semi-quantitative categories (Table 10). All strong relationships were statistically significant, with $p \leq 0.001$.

The most prevalent taxa of associated organisms across all subsamples from both diseased and apparently healthy samples were protozoans, such as ciliates, found in 55% and 24.1% of all histoslides analyzed, respectively (Figure 17). Crustaceans were found externally in 3.6% of diseased subsamples and 3.4 % of apparently healthy subsamples, showing little difference in prevalence between the two sample types. Nudibranchs were the least prevalent, found in only 1.1% of all diseased subsamples, all in the 17-042 samples, with none observed in apparently healthy subsamples. Suspect bacteria, as seen in Figure 9, and polychaete worms (Figure 10) were also found only in 17-042 subsamples.

4.3 Analysis of TEM Images

Six samples were collected and processed for TEM (Table 11). Samples which were secondarily fixed in osmium subsequent to decalcification (M, MM1, & MM2) exhibited artifacts, including compression of cells, causing cells to tear away from the

Table 9. PPMC correlations for apparently healthy samples
The correlation coefficients, *r*(16), were calculated for all semi-quantitative categories used for analysis of apparently healthy samples to determine if any strong linear relationships between categories exist. Any *r* values ≥ 0.70 indicate a strong linear relationship and have been highlighted in light green. Values of 1.00 indicate the same categories within the matrix, and are highlighted in dark green. Regression analysis was completed for any categories exhibiting strong relationships for significance and are displayed within the matrix.

	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries
General Condition 10x	1.00											
Zooxanthellae 10x	0.49	1.00										
Epidermal Mucocytes Condition	0.43	0.40	1.00									
Mesenterial Filament Mucocytes	0.51	0.06	-0.22	1.00								
Degeneration Cnidoglandular Bands	0.43	0.22	-0.18	0.71 *p* =.001	1.00							
Dissociation of Mesenterial Filaments	0.63	0.38	-0.12	0.67	0.79 *p*<.001	1.00						
Epidermal RLOs	-0.21	0.19	0.50	-0.10	-0.10	-0.25	1.00					
Gastrodermal/ Filament RLOs	-0.35	0.00	0.04	-0.35	-0.37	-0.10	0.32	1.00				
Costal Tissue Loss	0.47	0.20	0.17	0.52	0.52	0.37	-0.02	-0.24	1.00			
Chains of suspect bacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00		
Oocytes	0.36	0.22	-0.07	-0.09	0.03	0.27	-0.46	0.25	-0.10	0.00	1.00	
Spermaries	0.28	0.24	-0.14	0.04	0.05	0.27	-0.39	0.25	-0.21	0.00	0.85 *p* <.001	1.00

Table 10. PPMC correlations for diseased samples
The correlation coefficients, *r*(31), were calculated for all semi-quantitative categories used for analysis of apparently healthy samples to determine if any strong linear relationships between categories exist. Any *r* values ≥ 0.70 indicate a strong linear relationship and have been highlighted in light green. Values of 1.00 indicate the same categories within the matrix, and are highlighted in dark green. Regression analysis was completed for any categories exhibiting strong relationships for significance and are displayed within the matrix.

	General Condition 10x	Zooxanthellae 10x	Epidermal Mucocytes Condition	Mesenterial Filament Mucocytes	Degeneration Cnidoglandular Bands	Dissociation of Mesenterial Filaments	Epidermal RLOs	Gastrodermal/ Filament RLOs	Costal Tissue Loss	Chains of suspect bacteria	Oocytes	Spermaries
General Condition 10x	1.00											
Zooxanthellae 10x	0.91 $p<.001$	1.00										
Epidermal Mucocytes Condition	0.78 $p<.001$	0.77 $p<.001$	1.00									
Mesenterial Filament Mucocytes	0.49	0.57	0.46	1.00								
Degeneration Cnidoglandular Bands	0.79 $p<.001$	0.84 $p<.001$	0.74 $p<.001$	0.52	1.00							
Dissociation of Mesenterial Filaments	0.74 $p<.001$	0.76 $p<.001$	0.76 $p<.001$	0.51	0.92 $p=.001$	1.00						
Epidermal RLOs	-0.55	-0.60	-0.58	-0.42	-0.55	-0.60	1.00					
Gastrodermal/ Filament RLOs	-0.32	-0.29	-0.42	-0.27	-0.30	-0.28	0.50	1.00				
Costal Tissue Loss	0.85 $p<.001$	0.85 $p<.001$	0.71 $p<.001$	0.55	0.71 $p<.001$	0.70 $p<.001$	-0.45	-0.13	1.00			
Chains of suspect bacteria	0.37	0.27	0.10	0.06	0.11	0.06	-0.34	-0.24	0.23	1.00		
Oocytes	-0.11	-0.16	-0.05	-0.18	-0.01	-0.10	0.45	0.30	-0.13	-0.41	1.00	
Spermaries	0.08	0.04	0.16	0.01	0.22	0.11	0.19	0.18	0.01	-0.13	0.75 $p<.001$	1.00

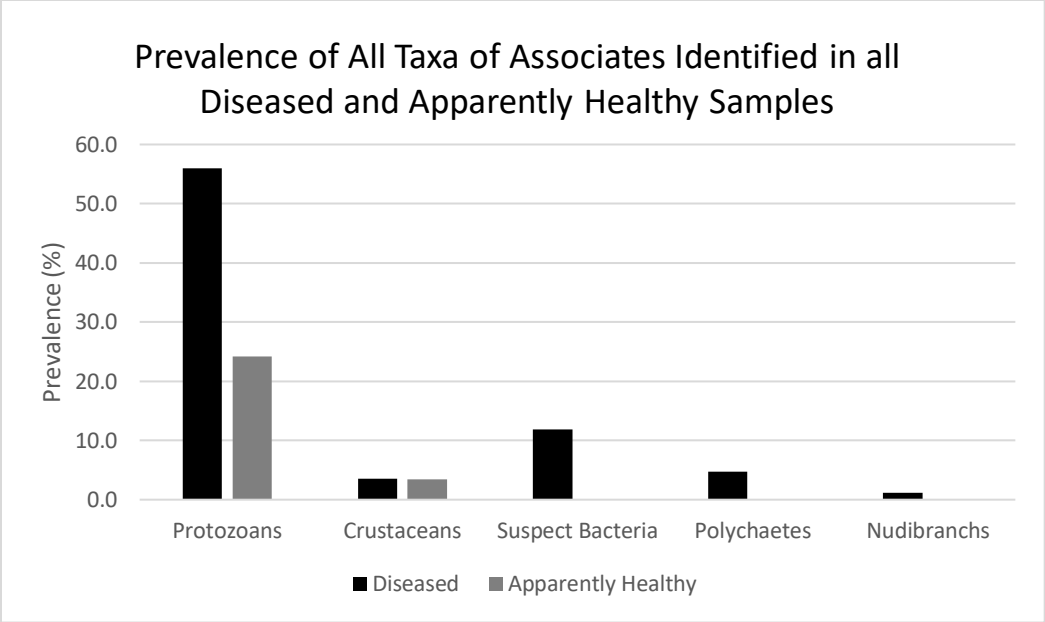


Figure 17. Prevalence of associated organisms
Compares the prevalence of the varying taxa of associated organisms found in section between all apparently healthy and all diseased samples

extracellular matrix, holes in the resin, and precipitation of either uranyl acetate or osmium tetroxide. Osmophilic particles and granule aggregates were present, which could include artifacts of fixation and staining.

TEM images were read using Harrison & Westfall (1991) as a detailed resource. Both bacilli and coccobacilli were found either singularly or in small aggregations. Images were obtained of these bacteria from RP2 collected from an apparently healthy acroporid (histology samples 18-039) and diseased sample MM1, collected during a disease outbreak affecting a colony of *Montipora* (histology samples 17-053). The observed bacteria were electron dense and approximately 1 µm long. Cell walls were

Table 11. Samples collected for TEM
All samples collected for TEM and the corresponding histology sample numbers, and whether they were collected from an apparently healthy colony (AH) or a colony affected by disease (D)

Sample Number	Species	Date Collected	AH or D?	Histology sample number
M	*Montipora capricornis*	July 22, 2017	D	17-053
MM1	*Montipora capricornis*	July 22, 2017	D	17-053
MM2	*Montipora capricornis*	July 22, 2017	D	17-053
FS	*Acropora* sp. 'ORA® Frogskin'	September 30, 2018	AH	18-037
RP1	*Acropora* sp. 'ORA® Red Planet'	September 30, 2018	AH	18-038
RP2	*Acropora* sp. 'ORA® Red Planet'	September 30, 2018	AH	18-039

clearly visible, with differential densities (Figure 18). The coccobacilli observed had wavy cell walls, with a more distinct electron-lucent periplasm than the bacilli observed. These bacteria were larger in size, measuring between 1.5–2 µm.

4.4 Analysis of Environmental Impact on Rapid Tissue Loss Disease Outbreaks

These data were analyzed for trends in combination plots of all the available specific gravity, temperature, calcium, and alkalinity data. Specific gravity and temperature from 2010–2018 were plotted together (Figure 19A), with calcium and alkalinity data from 2010–2018 plotted together on a separate graph (Figure 19B). Resulting trends indicate that specific gravity values have been 1.024–1.028, with low standard deviation and coefficient of variance (CV) of 0.0004 and 0.04%, respectively.

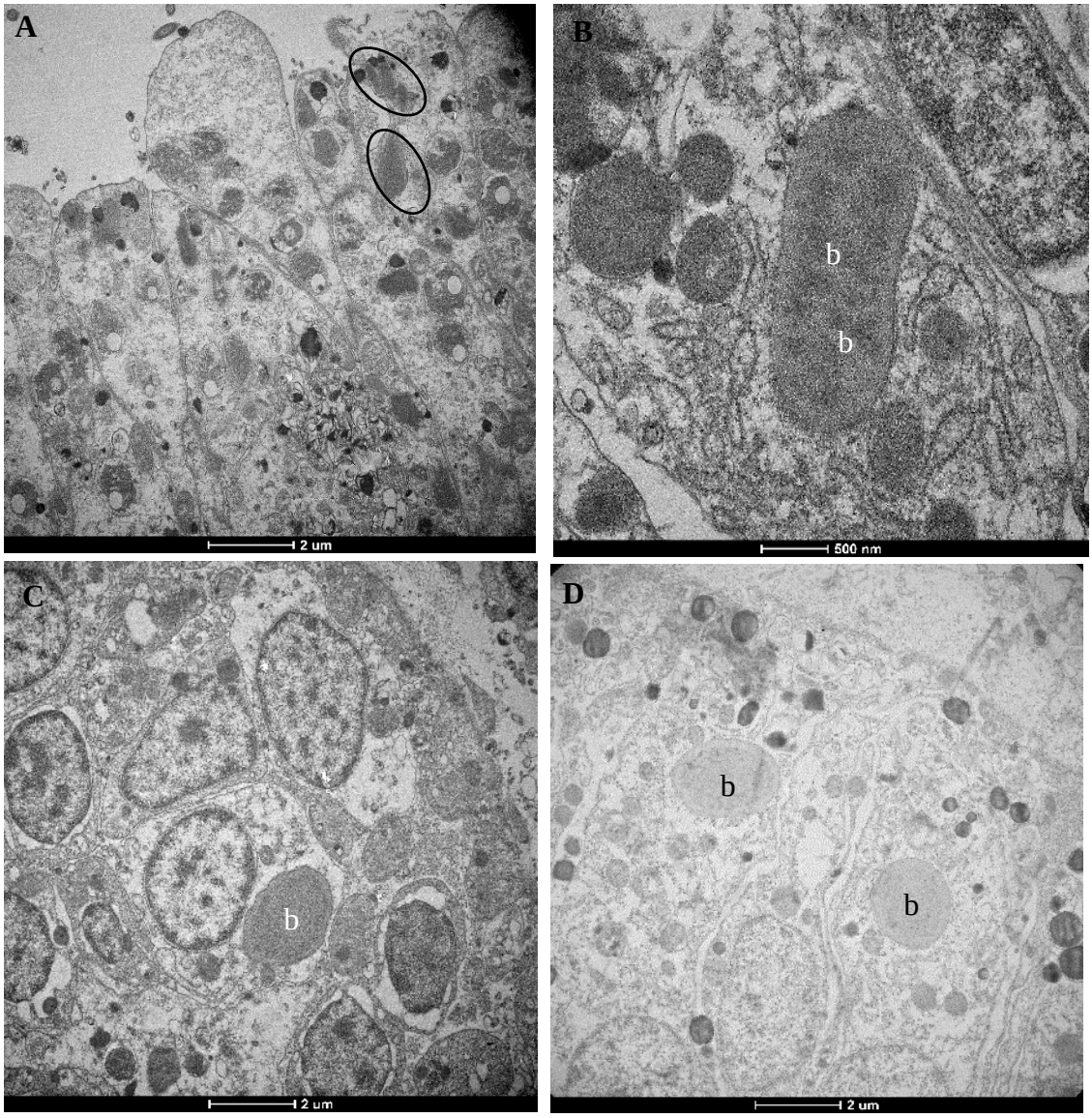


Figure 18. TEM images of bacteria
A) Shows a low-magnification image of a small bacilli aggregation (circled) present in *Montipora capricornis* B) Higher magnification image of bacilli (b) in *Montipora capricornis.* C) Coccobacilli (b) in *Montipora capricornis* with a wavy cell wall. D) Coccobacilli (b) with wavy cell wall in apparently healthy *Acropora* sp. 'ORA® Red Planet'.

The temperature fluctuated (73–79°F); however, both the standard deviation and coefficient of variance of these values were also low at 0.53 and 0.69%, respectively. Calcium and alkalinity values were much more widely distributed than the previous parameters. Whereas alkalinity (2.3–6.4 meq/l) had a standard deviation of 0.40 but a much higher CV of 10.1%, the distribution of calcium values (336–536 ppm) yielded a standard deviation of 26.79 and a CV of 6.4%.

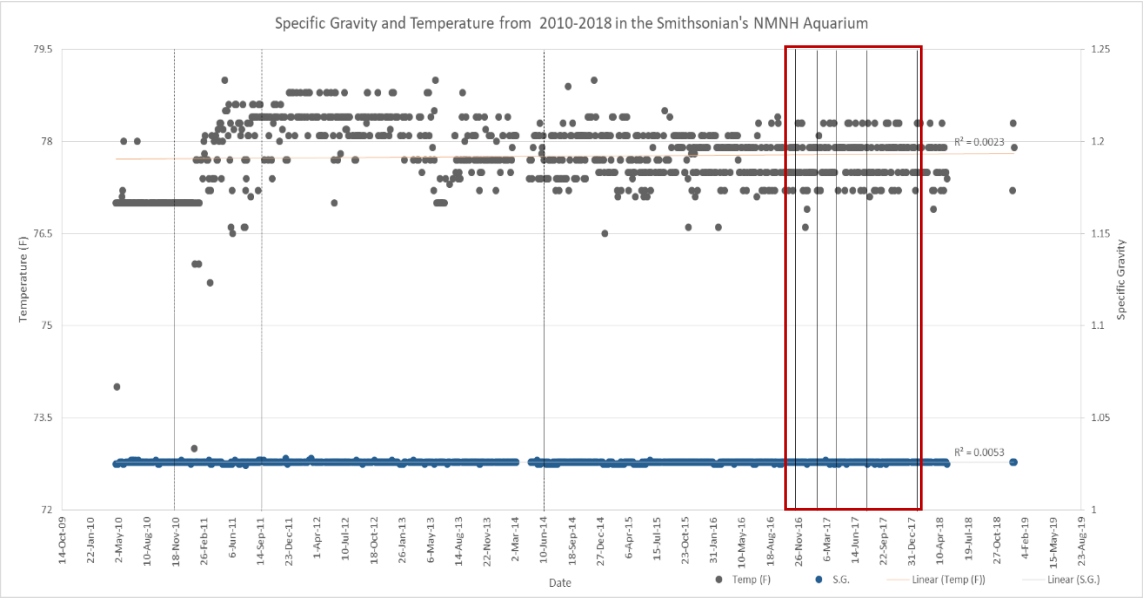


Figure 19A. Specific gravity and temperature from 2010–2018 in the Smithsonian's NMNH aquarium

Specific gravity and temperature values recorded in historical water quality records. Historical disease outbreaks are noted by black dashed lines, and those from which samples were collected for this study are indicated by the dates enclosed in the red box.

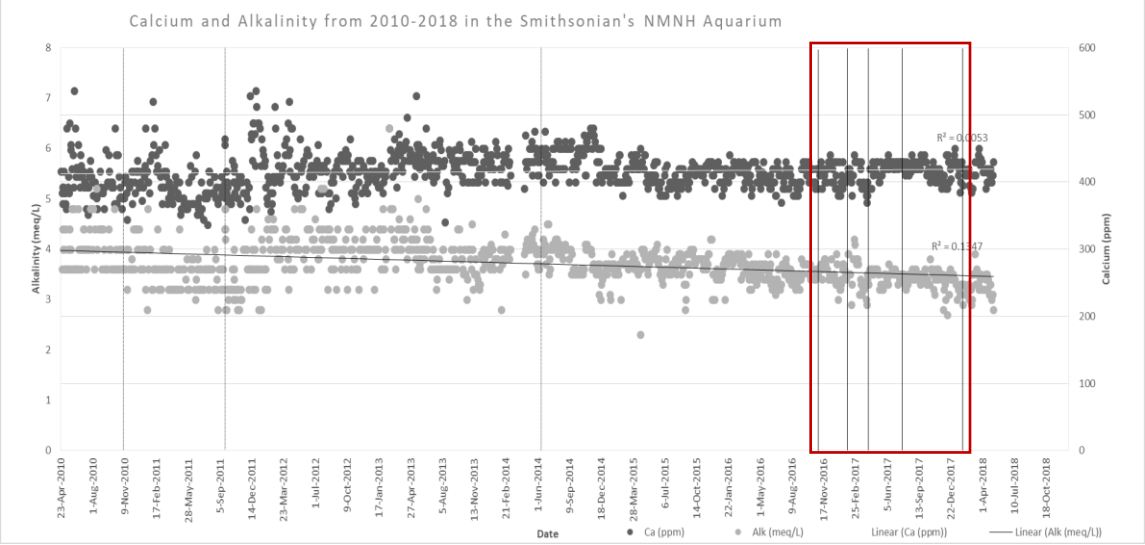


Figure 19B. Calcium and alkalinity from 2010–2018 in the Smithsonian's NMNH aquarium

Calcium and alkalinity values recorded in historical water quality records. Historical disease outbreaks are noted by black dashed lines, and those from which samples were collected for this study are indicated by the dates enclosed in the red box.

5 DISCUSSION

Multiple genera of Indo-Pacific corals from the NMNH aquarium contained RLOs when showing clinical signs of disease, but also when apparently healthy. TEM was attempted to visualize the morphology(ies) of RLOs, but they were not found; however, other bacteria were observed. Analysis of environmental data revealed that while certain water parameters were stable over the lifetime of the aquarium, others fluctuated.

5.1 Analysis of Histopathology Samples

5.1.1 Analysis of Apparently Healthy Samples

Suspect RLOs were observed in epidermal mucocytes, in mucocytes of the gastrodermis and cnidoglandular bands or in a combination of loci in 100% of apparently healthy samples 18-014 through 18-021 and 100% of 18-037 through 18-039 subsamples. Indeed, interactions between pathogenic microbes and hosts can occur on a continuum, even without gross signs of disease (Lesser et al. 2007; Work et al. 2012; Miller et al. 2014; Peters 2015; Gignoux-Wolfsohn et al. 2020). Miller et al. (2014) also found that apparently healthy samples had mucocytes infected with RLOs. Three possible roles of RLOs in corals were suggested, with the first being that RLOs are commensal or mutualistic to corals and transition to pathogenic in nature. Alternatively, results suggested that corals were in an early stage of infection, and not yet showing gross signs

of disease. The last possibility was that RLOs were altering mucus secretions of host mucocytes, thus increasing susceptibility of corals to disease. The findings of Klinges et al. (2019) supported the first postulation made by Miller et al. (2014). Finding RLOs unaccompanied by gross signs of disease in this study further supports the idea that RLOs may be mutualistic or commensal with corals before a relationship shift occurs and RLOs become pathogenic.

While potential pathogens may be primary or opportunistic, these states are not always mutually exclusive (Lesser et al. 2007). Pathogenic infections, whether resulting from primary or opportunistic pathogens, are managed by corals' innate immune defenses through mechanisms that parallel those observed in other invertebrate and vertebrate taxa (Mullen et al. 2004; Palmer et al. 2011; Kelly et al. 2016). The presence of stressor(s) could exhaust corals metabolically, inhibiting the upregulation of genes responsible for expression of immune defenses and preventing the initiation of cellular healing mechanisms (Van de Water et al. 2015; Klinges et al. 2019).

Despite harboring intracellular RLOs, the presence of melanin or melanin-like pigment in 18-019 subsamples of *Turbinaria* sp. could indicate an immune response to the presence of pathogens (Mullen et al. 2004; Palmer et al. 2011; Rosenberg & Kushmaro 2011). Innate immunity enables corals to recognize microbe-associated molecular patterns (MAMP), which trigger immune defenses, such as the prophenoloxidase-activating system, responsible for melanin deposition (Cerenius et al. 2008; Van de Water et al. 2015; Kelly et al. 2016). Melanin deposition is a well-documented defense against potential pathogens across taxa. By acting as a barricade,

melanin immobilizes foreign microbes for phagocytosis by amoebocytes and simultaneously releases toxic quinone compounds (Van de Water et al. 2015; Palmer et al. 2011; Kelly et al. 2016). Palmer et al. (2011) was the first to document melanin deposition during wound-healing processes in a scleratinian coral using *Porites cylindrica*. This study is the first to document melanin deposition in another scleratinian, *Turbinaria* sp. Dalton & Godwin (2006), Dalton &Smith (2006) and Godwin et al. (2012) all demonstrated that *Turbinaria* species are susceptible to white syndromes, likely caused by pathogens. This relationship between immune response in scleratinian corals, disease, and melanin deposition in response to potential pathogens requires more study.

Scores for RLO infections in the epidermis, gastrodermis, and mesenteries were similar in both apparently healthy and diseased samples, also reported by Casas et al. (2004), Miller et al. (2014), Klinges et al. (2019) and Gignoux-Wolfsohn et al. (2020). Apparently healthy samples exhibited slightly lower average scores for RLO infection severity in all locations. Zaneveld et al. (2017) suggested the "Anna Karenina Principle" of animal health to describe transitions from stable states to dysbiosis resulting from microbiome shifts. Dysbiosis increases susceptibility of apparently healthy corals to disease and while few studies have examined this phenomenon in aquaria, Kooperman et al. (2007) and Sweet et al. (2013) noted distinctions between microbial communities of corals from *in situ* and *ex situ* sources, including the loss of bacteria known to produce antibiotic compounds, such as *Actinobacteria,* and increases in potentially pathogenic microbial taxa in corals in aquaria, when compared to corals *in situ* —even in apparently

healthy samples (Kooperman et al. 2007; Sweet et al. 2013). Because RLO infections were slightly less severe in apparently healthy corals, perhaps these microbial shifts promoting dysbiosis occur along a continuum and are subject to both individual response and external factors.

5.1.2 Analysis of Samples Affected by Tissue Loss

This is the first study to histologically examine RLOs as potential pathogens causing tissue loss in multiple Indo-Pacific coral species. Casas et al. (2004) observed RLOs in Pacific acroporids from an aquarium supplier in San Diego; however, most similar studies have focused on Caribbean corals (Miller et al. 2014; Klinges et al. 2019; Gignoux-Wolfsohn et al. 2020) In this *ex situ* setting, *Acropora* colonies were the most affected genera, exhibiting tissue loss in four disease outbreaks, followed by *Montipora* colonies. Acroporids are particularly susceptible to disease, due to the dedication of resources to skeletal growth and low energetic investment in immune defense mechanisms (Miller et al. 2019). *Montipora* has been affected by tissue loss on multiple reefs *in situ*, with multiple etiologies indicated. The Pacific species, *Montipora capitata*, showing signs of white syndrome, now *Montipora* white syndrome (MWS), demonstrated limited immune responses while showing gross signs of disease (Work et al. 2012). The findings of this *ex situ* study support both Work et al. (2012) and Miller et al. (2019) demonstrating increased susceptibility of both *Montipora* and *Acropora* species to tissue loss *in situ*.

Diseased samples exhibited necrosis and lysing of cells and zooxanthellae. Tissue-loss margins were clear in some subsamples, where acute lysing was occurring.

There was also more costal tissue loss observed than in apparently healthy subsamples. Internally, structures on the mesenteries fared only slightly worse than those in apparently healthy samples, despite the fact that apparently healthy samples exhibited similar scores for gastrodermal and filament RLO infections. Diseased subsamples were observed to have more oocytes and spermaries than apparently healthy samples; however, in many cases, these structures appeared to be nonfunctional.

Because RLOs were detected in both apparently healthy and diseased samples, they cannot be definitively identified as the cause of disease in this aquarium. However, in cases of pathogen transmission resulting in disease, reservoir hosts are necessary for pathogens to remain in a population (Lesser et al. 2007; Ben-Horin et al. 2013). Furthermore, there may be a critical threshold of infection severity (Ben-Horin et al. 2013; Bossart et al. 2014), beneath which corals can cope with low-level, chronic infections. In cases of chronic RLO infections, even without gross signs of disease, the upregulation of genes associated with maintaining a stable state (Peters 2015; Van de Water et al. 2015) to avoid massive bacterial infection—would be an immense energy drain on cnidarian systems, slowing or even halting basic functions, such as growth and reproduction, contributing to increased susceptibility of coral disease in this system (Work et al. 2012; Bossart et al. 2014; Peters 2015; Kelly et al. 2016; Gignoux-Wolfsohn et al. 2020).

How are RLOs infecting coral colonies without contact between one another? Studies of Rickettsiales infections in mollusks by Le Gall et al. (1991), Friedman et al. (2002), and Ben-Horin et al. (2013) all suggest that RLOs have the ability to survive for

short periods in seawater. Water-borne transmission of Rickettsia was demonstrated in the 1991 and 2002 studies. Observations of extracellular RLOs on histoslides from this study (Figures 6 and 7), further support a mechanism for water-borne transmission. Because RLOs are obligate intracellular parasites, specific pathogen-free coral cell cultures are needed to determine the infectivity and virulence of these released cells. If cells remain viable extracellularly, they must have mechanisms to target host cells. A study conducted by Banin et al. (2002) demonstrated that *Vibrio shiloi* exhibited chemotaxis towards the mucus of its host, *Oculina patagonica*, using it to adhere to and penetrate the epidermis, causing bleaching in this species until 2004 (Rashef et al. 2006). Because RLOs infect mucocytes, perhaps they are similarly targeting components of mucus to find hosts. Once a host is identified, a study conducted by Klinges et al. (2019) suggests that a novel Rickettsiales, *Candidatus* Aquarickettsia rohweri may use genetic signatures to hijack a host coral's amoebocytes to gain entry into mucocytes, where the life cycle repeats.

Vectors could also be involved in the transmission of RLOs between coral colonies. Copepods, polychaete worms, nudibranchs, and ciliates were all found associated with subsamples in this study. Corals provide habitat for a diversity of associated organisms; however, associates can be a source of chronic stress to corals, inhibiting growth and sexual reproduction, resulting in increased mortality (Zaneveld et al. 2016; Rice et al. 2019). Copepods are crustacean reservoirs for potential pathogens, including *Vibrio* spp. found on their exoskeletons and in their guts and may transmit suspect bacteria through corallivory (Shelyakin et al. 2008; Cheng Dai 2010; Certner et

al. 2017). Slides stained with Giemsa revealed copepods in 17-001, 17-042, and 18-038 subsamples, with Gram-negative bacteria on appendages and in their guts, which could harbor potential pathogens (Figure 11).

17-042 samples were unique in that they were the only samples observed with suspect bacterial aggregations, polychaete worms, and nudibranchs. While the nature of associations between some polychaete worm species and corals is unclear, (Stella et al. 2011; Certner et al. 2017; Rice et al. 2019), nudibranchs found associated with these samples are predatory and are anecdotally known as *Montipora*-eating nudibranchs in the aquarium hobby. Although currently undescribed taxonomically (A. Fritts-Penniman, personal email communication, August 15, 2019), their small size and crypsis allow them to elude discovery until signs of damage to coral tissues are discovered (Enochs & Glynn 2016). Historical aquarium records note that they had been periodically seen for years. Nudibranchs can effectively transmit pathogenic microorganisms (Dalton & Godwin 2006); however, multiple genera were affected by RLOs in this study and not just *Montipora*, indicating that these nudibranchs likely do not play a significant role in transmission of RLOs in this system.

Large aggregations of ciliates were observed on the surface of 18-007 samples collected in January 2018. Protozoans were also observed in lesser numbers in diseased samples collected from *Acropora* sp. 'ORA® Frogskin' and *A. hyacinthus* during multiple tissue-loss events in 2017 and few were observed in samples of apparently healthy *Pocillopora* sp., *M. hispida*, and *Turbinaria* sp. While protozoans are generally opportunistic, certain species of ciliates have been associated with coral disease (Nicolet

et al. 2018). In 18-007 samples, ciliates were tentatively identified as *Philaster lucinda* (Dr. B.R. Whitaker, unpubl. data 2018), suspected vectors of brown-band disease in Indo-Pacific corals (Sweet & Bythell 2012a; Nicolet et al. 2018), and they contained zooxanthellae, cell debris, and Gram-negative bacteria appearing morphologically similar to RLOs (Figure 15). It is unknown if the bacteria survive digestion in the ciliate to be transmitted to other coral polyps and is another question warranting further investigation.

5.2 Statistical Analysis

Apparently healthy samples exhibited a narrower distribution of scores across most condition parameters examined during semi-quantitative analysis, compared to diseased samples. The exceptions were the scores for RLO infection severity in the epidermis, RLO infection severity in the gastrodermis and cnidoglandular bands, and both spermaries and oocytes, which exhibited a similar spread of scores in both sample sets. Diseased samples showed a wider distribution of scores, with slight increases in both mean and median scores (Figures 16A and 16B). The optimum envelope of health model (Peters 2015) describes the effect of stable and unstable states, affected by "exposures," on the health of organisms. Corals attempt to maintain stability even under the influence of exposures; however, multiple or long-term exposure to biotic or abiotic stressor(s) may limit resistance to destabilizing forces, and mechanisms employed to remain in a stable state vary with individual fitness. This model could explain the wider distributions of scores across most parameters seen in samples showing gross signs of disease, especially under the influence of additional stressor(s) in this system (Brown &

Bythell 2005; Lesser et al. 2007; Palmer et al. 2011; Bossart et al. 2014; Peters 2015; Zaneveld et al. 2016; Klinges et al. 2019).

PPMC showed that apparently healthy samples exhibited few strong positive significant relationships; however, diseased samples exhibited many strong positive significant relationships between condition parameters. When corals show gross signs of disease, they are in an unstable state along the continuum of health (Peters 2015). Corals rely on their innate immune system as a first line of defense to respond in unstable states in an effort to return to a stable state. Damage that occurs to host cells when RLO infections reach a critical threshold directly affects the efficacy of this system (Lesser et al. 2007; Peters 2015). The collapse of functional cells contributes to multi-system failure, as corals struggle to simultaneously compensate for and repair damaged cells (Van de Water et al. 2015). The cascade of effects observed in this study, beginning with damage to epithelial mucocytes and wound-healing, followed by reduced efficacy of the epithelium as a barrier (Palmer et al. 2011; Peters 2015) and resulting in damage to internal structures, explains the higher number of strong correlations observed between condition parameters in diseased samples.

As mucocytes are lost due to disease, mucus secretions are altered. While normal mucus production is affected by both abiotic factors and physiological tolerance of individuals (Kramarsky-Winter 2004), maintaining mucus production also depends on the ability to obtain the energy needed to replace mucocytes killed by the RLOs (Brown & Bythell 2005; Peters 2015; Kelly et al. 2016). On the SML, mutualists provide hosts with protection from potential pathogens through interspecific competition, production of

antimicrobial compounds and antifouling substances, and the production of nitrogen and phosphorous for their hosts' use. In turn, corals support their microbial symbionts with mucus, which is important for microbial metabolism (Ducklow & Mitchell 1979, Brown & Bythell 2005; Ritchie & Smith 2004; Kooperman et al. 2007; Sweet et al. 2013). Alterations to mucus production affects not only the normal microbiota on the SML of corals, but also deprives corals of this substance, which is vital to feeding, sediment removal, and defense against many stressors, which all increase susceptibility to morbidity and mortality (Brown & Bythell 2005).

The prevalence of associated organisms was calculated to compare the taxa of associated organisms found in both apparently healthy and diseased subsamples (Figure 17). Currently, there are few scientific studies focusing on dynamics between potential vectors, coral hosts, and disease transmission (Stella et al. 2011; Certner et al. 2017; Nicolet et al. 2018). Higher prevalence of all taxa was observed in diseased samples, indicating the possibility that associated organisms play a role in pathogen transmission in the NMNH aquarium. Work et al. (2012) also discussed finding associated organisms in samples from a disease outbreak of MWS. Further evidence supporting this idea is the observation of Giemsa-stained suspect RLOs within ciliates. Studies of disease vectors are critical to the mitigation of infectious disease, and may provide crucial information about the life cycle of potential pathogens in aquaria (Nicolet et al. 2018).

5.3 Analysis of TEM Images

Samples processed for TEM were in poor condition, and while histology samples were also in poor condition (Table 7), this was likely due in part to processing and

embedding. Bacilli were found in one apparently healthy and one diseased TEM sample, either singularly or in small numbers. Their morphological differences from bacteria observed with light microscopy indicated they were not suspect RLOs. Coccobacilli were also observed, appearing with a wavy cell wall. Beveridge (1999) suggested this cell wall configuration as characteristic of some Gram-negative bacteria subsequent to fixation for TEM and a later study by Amezaga-Madrid et al. (2003) discussed wavy cell walls appearing in Gram-negative *Pseudomonas aeruginosa,* as a result of irradiation. Yet a third study by Beltramini et al. (2009) suggests that genetic deletions for important signaling molecules resulted in morphological changes in bacterial cells walls, including a wavy, interrupted, membrane. These studies with contrasting results confirm that there are unresolved questions surrounding bacterial cell wall ultrastructure. Although suspect bacilli were observed in 17-042 samples, none were observed in histology samples corresponding with TEM samples. Given the poor condition of histology samples, the inundation with ciliates, and the fact that very few bacteria were observed in TEM samples, this is not unexpected. In future studies involving TEM, primary and secondary fixation promptly upon collection and prior to decalcification may diminish artifacts. Ensuring enough replicates to obtain intact tissues sections is also essential. TEM has been used successfully to investigate potential pathogens in cases of Caribbean coral diseases, such as BBD (Miller et al. 2011), the role of viruses in white plague disease (Soffer et al. 2014), and pathogen reservoirs of coral disease (Negandhi et al. 2010). Work & Aeby (2014) observed intracellular microbial aggregations in Indo-Pacific corals with both light microscopy and TEM, however, the staining and aggregation

characteristics are different than suspect RLO aggregations observed in this study. Combining TEM with molecular techniques, similar to Correa et al. (2016) would be highly beneficial to parsing out the identities and roles of RLOs found in this system.

5.4 Analysis of Environmental Impact on Rapid Tissue Loss Disease Outbreaks

Individual colony stress responses, virulence of potential pathogens, individual host microbiota, and even opportunistic infections are all affected by environmental conditions (Lesser et al. 2007; Peters 2015; Bourne et al. 2016). In addition, it has been demonstrated that Rickettsiales increased in abundance when environmental parameters shifted (Klinges et al. 2019).

Historical water quality data was incomplete, but still revealed important trends corresponding to disease outbreaks. The standard deviations and CV values were low for temperature and specific gravity, but were higher for both alkalinity and calcium, with calcium showing the highest variation. Temperature is provided by an electronic data monitor, making these measurements straightforward. While specific gravity is measured manually, the low variation in values across eight years of data indicated that the equipment was kept well-calibrated. The nature of manual titration tests makes higher variation among calcium and alkalinity values an expected phenomenon. However, while there was significantly higher standard deviation and CV values of calcium measurements, the variation has decreased contemporarily, as the aquarium has been made increasingly automated.

Shifts in both alkalinity and manipulations of the calcium reactor coincided with tissue-loss events. Particularly, in the 6 to 8-week period before a tissue-loss outbreak,

alkalinity swings occurred, first dropping below the target range, followed by the dosage of alkalinity buffer solution to bring alkalinity back into an accepted range. In these cases, due to the maintenance schedule, alkalinity may be overcorrected, so that this value has dropped into the accepted range by the next scheduled maintenance visit. Alkalinity, a measure of the concentration of mostly bicarbonate and carbonate, is essential to the calcification process of corals (Brockmann & Janse 2008). A drop in alkalinity, followed by an overcorrection through chemical additives could be a stressor to corals. In this same time frame, 6–8 weeks prior to a disease outbreak, maintenance or adjustments to the calcium reactor system occurred. While calcium reactors are generally advantageous to aquariums, they can leach nutrients, and anything more than very fine adjustments can be a stressor on the system (Brockmann & Janse 2008). Water quality logs showed frequent maintenance of this equipment, although the nature of the adjustments was not always clear. A delay of weeks to months between infections in hosts that reach a critical threshold and the appearance of gross signs of disease can occur in host-pathogen interactions and has been linked to periodic occurrences of environmental stressor(s) or "events" (Ben-Horin et al. 2013; Sweet et al. 2013). It remains unclear if these subtle perturbations contributed to disease outbreaks in this system, but this is the first study to attempt to analyze the combination of water quality parameters specific to tropical coral-reef aquariums to determine how they may be related to coral disease outbreaks, and therefore, is worth noting here.

The resilience of corals residing in this aquarium is an important consideration. Not all instances of low alkalinity and calcium reactor manipulation coincided with

disease events and while water parameters were generally within target ranges, destabilizations occurred when fluctuations in water parameters and significant events of change occurred. Events noted in the historical records included calcium reactor maintenance, replacement of lighting and system upgrades, changes to flow due to pump upgrades, and the installation of automated dosing systems for calcium, alkalinity buffer, and micronutrients. The microbiota of corals can shift under the influence of stressors (Ritchie & Smith 2004), but corals are resilient enough to recover a healthy microbiota once a stressor is eliminated (Sweet et al. 2013). However, disease outbreaks, with the exception of the tissue-loss event on January 29, 2018, where large aggregations of *P. lucinda* were discovered, took place cyclically 2 to 3 months apart. Work et al. (2012) stated that healing processes in corals require weeks. Sweet et al. (2013) cited a period of approximately four months for corals to show signs of recovery from an unknown disease in a different public aquarium. This chronology and supporting evidence from previous studies indicates that stressor(s) may have been continually occurring or not fully eliminated between disease outbreaks, negatively impacting the resilience of these corals.

Environmental perturbations affected other aquarium inhabitants, as well. A metal halide fixture providing lighting to the left side of the tank was not functional for a period in from early April 2017 until the lighting system was upgraded to LED lighting over the late summer through winter of 2017. In addition, in June 2017, two T-5 bulbs were reported burnt out for several days. Two coral disease outbreaks occurred during this period—in April and July 2017—affecting both *Acropora* and *Montipora* species (Table 7). Although photosynthetically active radiation (PAR) readings were not recorded

during this time, the change in lighting was so significant that a lack of crustose coralline algae growth under this area was noted and there was mortality of a large marine clam (*Tridacna gigas*) in August 2017, as well. While the clam had a prior laceration to its mantle, clams harbor algal symbionts, similar to corals. And while it is not clear what the cause of mortality was, studies show that decreased lighting reduces photosynthesis in the clam dinoflagellates reducing its nutrition, and this prolonged stress could have contributed to its mortality (Elfwing et al. 2003). It should be noted that after the lighting was replaced with an upgraded system, all corals were noted to be healthier and growing faster than before.

In early 2018, corals had grown to the extent that interspecific competition with neighboring colonies was evident. Space with access to adequate lighting and energy sources is a limiting resource for sessile invertebrates (Chadwick &Morrow 2011), so interspecific competition occurs in the form of direct aggression toward neighboring colonies or indirect defensive behavior (Connell et al. 2004). Although corals are strategically placed in aquariums by humans, these interactions still occur when neighboring colonies are near or in contact with one another. As shown in a study by Lang (1971), *Scolymia lacera* and *S. cubensis* that were kept in aquaria without light and feeding for 155 days still displayed aggression towards one another. Even under physiological stresses, corals prioritize colony expansion (Lang 1971; Connell et al. 2004). The aquarium was renovated in November 2018, at which time a considerable amount of corals was trimmed and removed. Since this time, no disease outbreaks have occurred. Not only did the reduction of biomass change the community dynamics by

lessening interspecific competition, but it reduced the demand for calcium carbonate, as well. Despite studies showing that competition may be energetically low-cost to more dominant species (Connell et al. 2004), subordinate corals experience reduced growth, reproduction, and survival as demonstrated in studies by Lapid & Chadwick (2006) and Romano (1990). Reducing interspecific competition and calcification needs may have finally eliminated enough sources of stress to allow corals to recover and return to a more stable state of health, even while harboring RLOs.

6 RESEARCH CONCLUSIONS AND FUTURE DIRECTIONS

The results of this study showed the presence of RLOs in both apparently healthy and diseased corals. While TEM did not reveal RLOs during this study, other bacteria were observed. Finally, while no statistically significant environmental factors were definitively linked to disease in this study, similar fluctuations in water parameters and anecdotal equipment adjustments existed 2 to 3 months prior to each disease outbreak sampled for this study. Could a complex link exist between subtle shifts in water chemistry that increases susceptibility to disease or, alternatively, encourage increased virulence or infectivity of RLOs present in reservoir hosts? Environmental factors are well-monitored in public aquariums, and if the data collected are reliable, they can answer fundamental questions surrounding the impact of environmental factors on disease dynamics. While corals in the NMNH aquarium have remained stable, without signs of disease, since its renovation in 2018, there are still many unanswered questions, with continued opportunity to study microorganisms and their interactions with corals across the continuum of health in this model system.

Merging classically utilized diagnostic techniques with more contemporary methods can provide a more complete picture of disease dynamics within an organism or a community (Burge et al. 2016). Immunohistochemistry, *in situ* hybridization, or high-

throughput sequencing would provide more information about the identity(ies) of the suspect RLOs observed in samples from this study. Even though the ultrastructure of RLOs was not observed, future studies would benefit from continued efforts to view these organisms with TEM. Analyzing possible transmission modes by examining both the ability of RLOs to remain viable and virulent in seawater and the role of coral-associated organisms with laser capture microdissection of bacteria found in the guts or on appendages of associated organisms could demonstrate how RLOs spread from colony to colony. While "*Montipora*-eating nudibranchs," a common pest of Indo-Pacific montiporids in aquaria, have not yet been taxonomically identified, it would be beneficial to understand the stressor(s) on corals from predation.

Preparation for and response to wildlife disease is strengthened by prior knowledge and understanding of disease events (Mörner et al. 2002).While this study demonstrated that disease surveillance of this system is effective at identifying and mitigating the effects of disease outbreaks in a highly monitored system, reliable water quality logging systems could improve monitoring efforts in this aquarium moving forward.

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APPENDICES

Appendix A

NMNH Coral Sampling Procedure

1. The collector wears nitrile gloves when collecting samples and changes gloves between collections to prevent cross contamination.

2. Samples of coral tissue with skeleton may be removed using heavy duty shears or another cutting device that has been soaked in a 10% bleach solution for 20–30 minutes and allowed to air dry OR is individually packaged and sterile
    a. Conversely, samples may be taken, in this manner, from larger pieces that have already been trimmed

3. Once the sample has been collected from the tank, it should be placed on the clean cutting board (which had also been soaked in 10% bleach solution and allowed to air dry).

4. Using the cutting tool (new, clean single edge razor blade, heavy duty shears, or bone-cutters that have been cleaned in 10% bleach solution and allowed to air dry or sterile), obtain 1–2 samples per colony for histopathology survey.

5. Label the tubes containing the samples with the species and collection date using standard masking tape and permanent marker

6. Replace the samples to remain upright in a Styrofoam tube rack if possible, to keep sample submerged in the fixative.

Appendix B

Semi-Quantitative Scale, Adapted from Miller et al. (2014)

Parameter Viewed at 100x	**Numerical Score Intensity or Severity Score**				
0 (No Change)	**1**	**2**	**3**	**4**	**5**
Low Magnification	**Very Good**	**Good**	**Fair**	**Poor**	**Very Poor**
General Condition 0 = Excellent, thick epithelia and mesoglea, mucocytes not hypertrophied, highly cellular	epithelia and mesoglea not as thick, epidermal mucocytes slightly hypertrophied	Hypertrophy of epidermal mucocytes, intact epithelia and mesoglea, mesentery and filament architecture still normal	Hypertrophy of epidermal mucocytes, minimal to mild attenuation of epithelia and mesoglea	Loss of mucocytes, moderate attenuation of epithelia and mesoglea, cellular architecture degenerating	Severe attenuation of epithelia and mesoglea, necrosis and dissociation of mesenterial filaments, necrosis and lysing of cells
Zooxanthellae 0 = Gastrodermal cells packed with well-stained algal symbionts in surface body wall, tentacles	thick layer of well-stained zooxanthellae in gastrodermis of surface body wall, tentacles	Thick layer of well-stained zooxanthella, but not as abundant	Zooxanthellae fewer in gastrodermis (atrophied), some still stain appropriately	Markedly fewer zooxanthellae in surface body wall gastrodermis and tentacle gastrodermis, some have lost acidophilic staining as proteins no longer produced or lysed	No zooxanthellae present in gastrodermal cells of colony (bleached)
High Magnification	**Minimal**	**Mild**	**Moderate**	**Marked**	**Severe**
Epidermal Mucocytes 0 = uniform distribution and not taller than ciliated supporting cells, pale mucus	Slightly hypertrophied, numerous, slightly hypertrophied, numerous, pale-staining frothy mucus	Many cells hypertrophied, abundant release of pale-staining mucus	Uneven mucocytes, some hypertrophied but some reduced in size and secretion, darker staining mucus	Some epidermal foci lack mucocytes entirely, attenuation of epidermis evident, darker staining and stringy mucus	Loss of many mucocytes, epidermis is attenuated to at least half of normal thickness or more, if mucus present, it stains dark
Cnidoglandular Band	Less than half the area of	About half the area is	About half the area is	About three quarters of the	Loss of mucocytes,

Parameter Viewed at 100x	Numerical Score Intensity or Severity Score				
0 (No Change)	**1**	**2**	**3**	**4**	**5**
Epithelium Mucocytes 0 = Oral portion lacks mucocytes, increasing in number aborally, may be abundant with pale mucus	cnidoglandular band is mucocytes, size of mucocytes variable	mucocytes, some hypertrophied	mucocytes, all hypertrophied	area is mucocytes, mucus production reduced, some vacuolation present	vacuolation and necrosis of cells present
Degeneration of Cnidoglandular Bands 0 = Ciliated columnar cells, nematocytes, acidophilic granular gland cells, and mucocytes abundant tall, thin columnar cells contiguous	Mild reduction in cell height	Cell height more reduced, mild loss of mucocytes or secretions	Attenuation (atrophy), loss of cells	Moderate attenuation of epithelium, some granular gland cells stain dark pink and are rounded, loss of cells by detachment and sloughing	Severe atrophy of epithelium, detachment from mesoglea and loss of cells, necrosis or apoptosis of remaining cells, loss of cilia
Dissociation of Cells on Mesenterial Filaments 0 = All cells intact and within normal limits, contiguous, thin columnar morphology, cilia visible along apical surface	Minimal loss of cilia, but will not be present where mucocytes are predominant	Minimal to mild loss of cells, loss of ciliated cells	Attenuation of cells, vacuolation, reduced cilia, but filament still intact	loss of granular gland cells, cell loss evident, terminal web (junctions) between cells lost, starting to spread apart along cnidoglandular band	Marked to severe separation of cells, most necrotic, vacuolated, lysing and loss of mucocytes, nematocysts, granular gland cells and ciliate columnar cells
Costal Tissue Loss 0 = Tissue covering costae intact, epidermis similar in thickness to epidermis of	Attenuation of epidermis, but still intact over costae	Up to one-quarter of costae exposed due to loss of epithelia and mesoglea	Up to one-half of costae exposed	About three quarters of costae exposed	Most costae exposed or gaps in surface body wall, tissues atrophied

Parameter Viewed at 100x	**Numerical Score Intensity or Severity Score**				
0 (No Change)	**1**	**2**	**3**	**4**	**5**
surface body wall with gastrodermis as it covers the costae, although this may vary with location					
Epidermal RLOs 0 = Not present	One infected cell on oral disks or tentacles of polyps	Several infected cells on oral disks or tentacles of polyps, numerous mucocytes present	About half of mucocytes infected on oral disks or tentacles of polyps, loss of some mucocytes	More than half of mucocytes infected on oral disks or tentacles of polyps, loss of mucocytes	Nearly all remaining mucocytes infected (may have lost many as infected cells die and lyse)
Gastrodermal/ Filament RLOs 0 = Not present	One infected cell in gastrodermis and/or on cnidoglandular bands	Several infected cells in gastrodermis and/or on cnidoglandular bands present in tissue section	Infected cells present on about half of sections through cnidoglandular bands, few infected mucocytes in gastrodermis lining gastrovascular canals	A few infected cells present on almost all sections through cnidoglandular bands, more infected cells in gastrodermis lining gastrovascular canals	Nearly all remaining mucocytes infected, but many lost as infected cells die and lyse, as mucocytes of gastrodermis or mesenteries infected
Gonad Staging	**1**	**2**	**3**	**4**	**5**
Oocytes 0 = None present	Single germ cell surrounded by mesoglea in mesentery	Early oocyte, nucleus with distinct nucleolus but little development of lipid and protein in cytoplasm	Mid-development, uniform distribution of lipid droplets and protein granules, nucleus and cytoplasm enlarge	Mature, development of cortical granules and vitelline membrane, beginning to separate from mesoglea	Spawned, hole present where ovum released
Spermaries 0=none present	Germ cells aggregate in mesoglea, forming one or a few clusters	Early spermaries, multiplication of germ cells	More spermaries present, spermatids fill lumen	Mature spermatozoa fill lumen	Spawned, remnants of spermatozoa endocytosed by absorptive gastrodermal cells on mesentery

Parameter Viewed at 100x	**Numerical Score Intensity or Severity Score**				
0 (No Change)	**1**	**2**	**3**	**4**	**5**
Chains of Suspect Bacteria 0=Not present in tissue	One bacterial focus found in tissue on slide	Two to five bacterial foci found in tissue on slide	Density increases, 6 to 10 bacterial foci found in tissue on slide	Bacteria observed in marked abundance in multiple foci	Density increases, bacteria seen throughout tissue on slide
Associated Organisms (Crustaceans, Nudibranchs, Protozoans, Polychaete Worms) 0=Not present	1=Present				

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BIOGRAPHY

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