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Ultrastructural and Histological Analysis of Dark Spot Syndrome in Siderastrea siderea and Agaricia agaricites

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Ultrastructural and histological analysis of Dark Spot Syndrome in *Siderastrea siderea* and *Agaricia agaricites*

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Abstract. Dark Spot Syndrome (DSS) typically manifests in scleractinian corals as lesions of varying color, size, shape and location that can result in skeletal changes and tissue death. A causative agent for DSS has not yet been identified. The objective of this study was histological and ultrastructural comparison of the cellular and skeletal characteristics of DSS-affected and healthy *Siderastrea siderea* and *Agaricia agaricites*. The greater resolution possible with transmission electron microscopy (TEM) revealed microbial activity and tissue changes not resolvable utilizing histology. DSS-affected tissue had less integrity, with increasing cellular degradation and vacuolization. A high concentration of electron dense inclusions, which appear to be zymogen granules, was concentrated in the calicodermis and adjacent gastrodermal layer. Numerous endolithic fungal cells were observed directly adjacent to the calicodermis in DSS-affected *S. siderea*. Numerous unidentified endolithic cells were observed directly adjacent to the calicodermis in DSS-affected *A. agaricites*. These observations suggest that the coral may be using a digestive enzyme as a defensive mechanism against endolithic cellular invasion.

Key words: Dark Spot Syndrome, *Siderastrea siderea*, *Agaricia agaricites*, ultrastructure, TEM.

Introduction

A significant increase in the incidence and prevalence of coral diseases and the number of species affected has been observed worldwide and likely plays a major role in the decline of reef-building species (Rosenberg & Loya 2004). Most coral diseases are not well characterized, and effects of these diseases on corals at the cellular level have yet to be fully elucidated. Thus, the use of multiple approaches, including molecular, ultrastructural and histological techniques is needed in the investigation of coral disease and pathology.

Siderastrea siderea is an important scleractinian reef-building coral, commonly found throughout south Florida and the Caribbean. Of the several species of Caribbean corals affected by Dark Spot Syndrome (DSS), *S. siderea* is most frequently affected (Gil-Agudelo et al. 2004, Weil 2004, Borger 2005, Gochfeld et al. 2006). DSS typically manifests as black, brown or purple lesions of varying size and shape that can occur across the coral colony surface and at colony edges. DSS does not always cause tissue death; the lesions may disappear without any evident damage, or they can resolve and reappear on a different area of the colony (Gochfeld et al. 2006). If lesions are persistent, development of a depression in the skeleton or tissue necrosis is possible. Even if there is no tissue loss, underlying skeletal changes may occur. These changes may include a permanent dark skeletal discoloration that is observable in longitudinal skeletal slabs (Renegar pers obs.). To date, the cause of DSS has yet to be identified.

Unlike *S. siderea*, which is affected by DSS throughout its range, *Agaricia agaricites* appears to be affected by DSS in certain locations (e.g., Colombia: Garzón-Ferreira et al. 2001; Cayman Islands: Coelho & Manfrino 2007), but not others (e.g., Bahamas: Gochfeld, pers. obs.). DSS in *A. agaricites* typically manifests as much darker brown or black patches than on *S. siderea*. It is not yet known whether DSS found on these species has a common cause or similar impacts at the cellular level. The objective of this study was to use a combination of ultrastructural and histological techniques to describe cellular characteristics and possible pathogenic microbes in DSS-affected and healthy *S. siderea* and *A. agaricites*.

Materials and Methods

Tissue samples of *S. siderea* were collected from 10 healthy and 9 diseased colonies at Lee Stocking Island, Bahamas in July 2006, and from 2 healthy and

6 diseased colonies at Little Cayman, Cayman Islands in June 2007. Samples of 4 healthy and 4 diseased colonies of *A. agaricites* were collected from Little Cayman in June 2007. Examination of the *S. siderea* samples did not indicate substantial differences among locations or years. A fragment of each healthy colony and two fragments of each diseased colony (healthy and DSS-affected tissues) were fixed in glutaraldehyde fixative solution [2 mL 70% glutaraldehyde in 68 mL cacodylic buffer (2.16 g cacodylic acid in 200 mL of 0.22 µm filtered seawater)] in the field. Samples were maintained at 4°C in the fixative for 4-6 days, then divided into three subsets: two for TEM (calcified and decalcified) and one for histological analysis.

For TEM analysis, pieces of tissue approximately 2 mm^2 in size were removed from each fixed colony and post-fixed in buffered 1% osmium tetroxide solution (5 mL 4% aqueous osmium tetroxide in 30 mL of cacodylic buffer) for 1 hour. Samples were then rinsed in buffer, and the sample subset to be decalcified was placed in a diluted decalcification solution (0.032 g potassium sodium tartrate, 0.56 g sodium tartrate, 2.8 g EDTA, 400 mL 12 N HCl in 33.6 L dH_2O). The solution was changed frequently (every 1-2 hours) until each sample was completely decalcified. Samples were dehydrated in a graded series of ethanols, embedded in Spurr resin, and ultrathin sections were cut (40 to 60 nm thick) using a Sorval MT-2 ultramicrotome fitted with a diamond knife. Sections were retrieved on nitrocellulose and carbon coated 200 mesh copper grids, stained with Reynolds lead citrate and/or 2% uranyl acetate solution, and viewed in a Phillips 300 TEM. Electron dispersive spectroscopy (EDS) was used to evaluate elemental composition. Histological samples were decalcified in a 5% HCl/EDTA solution, dehydrated and embedded in Paraplast®, sectioned at 4 µm, and stained with Movat's Modified Pentachrome or Hematoxylin & Eosin.

As mucocyte size has been demonstrated to increase under stress, (Vargas-Ańgel et al. 2007), a quantitative comparison of mucocyte size in *S. siderea* was made from sections that were 500 µm from the aboral interface in 7 healthy and 4 DSS-affected colonies from the Bahamas. The length and width of mucocytes were determined in 5 random fields of view from each section (1000X magnification). Healthy tissue on DSS-affected colonies was taken from at least 1 cm away from DSS-affected tissue. Paired t-tests were used to compare mean cell sizes between healthy and DSS-affected tissues on affected colonies, and unpaired t-tests were used for comparisons between healthy colonies and both types of tissue on affected colonies.

Results

***Siderastrea siderea*.** Healthy corals demonstrated an organized cellular configuration indicative of tissue integrity (Fig. 1A), whereas the DSS-affected tissue exhibited cellular degradation, vacuolization, and necrosis (Fig. 1B). Healthy tissue adjacent to affected tissue on DSS-affected colonies was comparable to tissue on healthy colonies. In healthy calcified tissue, normal aragonite crystals were observed directly adjacent to the calicodermis, and organic matrix was present in decalcified tissue (Fig. 1A). In contrast, aragonite crystals appeared smaller and irregular in calcified tissue from DSS-affected samples, with little organic matrix present in affected decalcified tissue. Numerous zooxanthellae in various stages of cell division were present in the healthy tissue. In DSS-affected corals, there was an apparent decrease in zooxanthellae number, and many appeared abnormal or necrotic, with internal organelle disruption and debris (Fig. 1B).

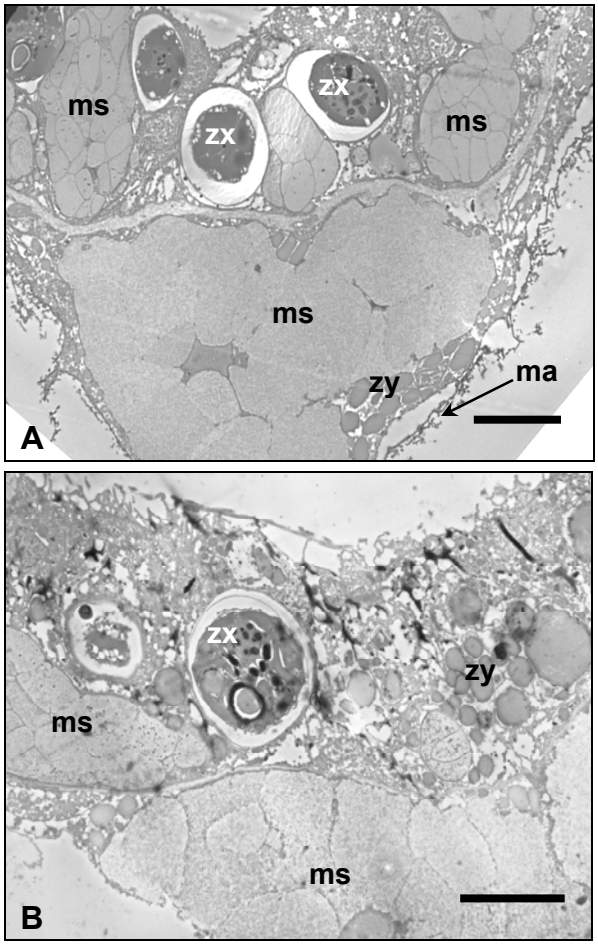

Figure 1. *Siderastrea siderea*, TEM micrographs. A) Healthy gastrodermis; B) DSS-affected gastrodermis. ma: organic matrix; ms: mucocyte; zx: zooxanthellae; zy: zymogen granules. Scale bars: A and B = 10 µm.

In all of the DSS-affected corals, numerous endolithic fungal cells were observed in close proximity to the calicodermis (Figs. 2A & 2B). These fungal cells were associated with swollen, vacuolated mucocytes and an increased concentration of acidophilic/eosinophilic, proteinaceous, electron-dense granules in the calicodermis (Fig. 2A). The morphology and staining properties indicate that these granules are zymogen granules (E. Peters pers. comm., Goldberg 2002). Fungal cells in close proximity to the tissue were not observed in any of the healthy colonies or the samples of healthy tissue from the DSS-affected colonies. Identification and characterization of these fungal cells are ongoing.

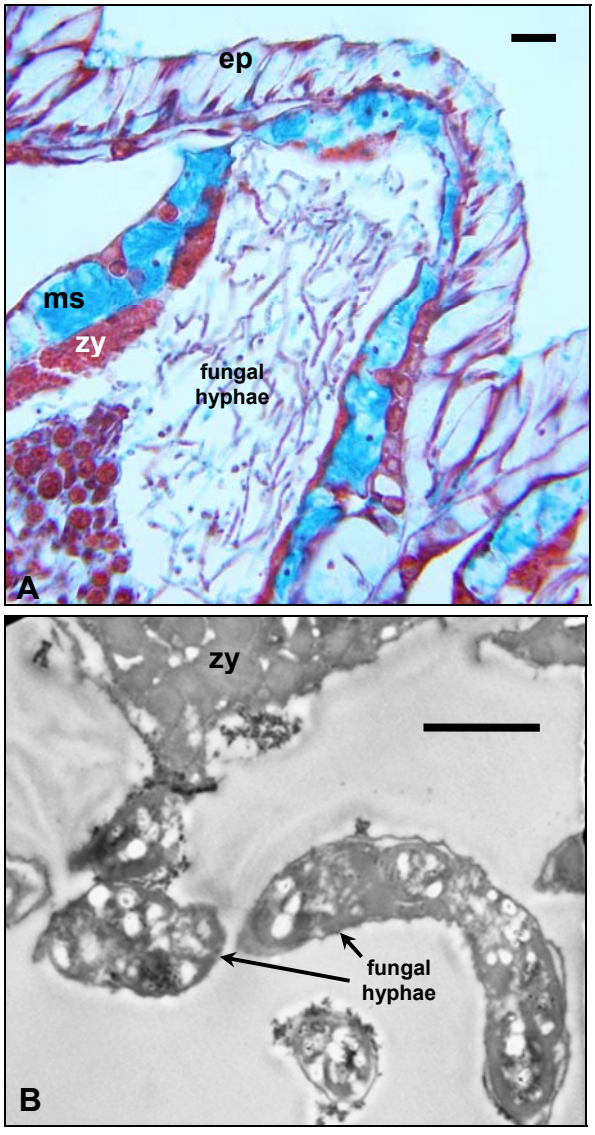

Figure 2. *Siderastrea siderea*. A) Histological micrograph, DSS-affected coral and endolithic fungi; B) TEM micrograph, decalcified DSS-affected coral tissue and fungal cells. ep: epidermis; ms: mucocyte; zy: zymogen granules. Scale bars: A = 20 µm and B = 3 µm

Mucocytes were significantly larger in DSS-affected tissues than in healthy tissues on either healthy (unpaired t=-4.78, df=9, P=0.001) or affected colonies (paired t=3.3, df=3, P=0.046; Table 1). There was no significant difference in the size of mucocytes between healthy tissues on healthy or affected colonies (unpaired t=0.23, df=9, P=0.82).

Table 1. *Siderastrea siderea.* Size of mucocytes (μm^2) in healthy colonies and healthy and DSS-affected tissue on affected colonies.

Tissue type	Mucocyte size (n = cells/colony)
Healthy coral (n=7)	10.3 ± 1.3 (94-236)
DSS-affected (n=4)	26.6 ± 4.1 (62-132)
Healthy tissue on DSS-affected coral (n=4)	9.8 ± 1.5 (150-190)

Agaricia agaricites. Healthy colonies of *A. agaricites* also demonstrated normal cellular configuration (Fig. 3A). The DSS-affected tissue exhibited moderate cellular degradation and vacuolization, particularly of the calicodermis (Fig. 3B). Healthy tissue adjacent to affected tissue on DSS-affected colonies was similar to tissue on healthy colonies. Organic matrix was evident in healthy decalcified tissue. Little organic matrix was present in the DSS-affected decalcified tissue, and aragonite crystals appeared irregular in the affected calcified tissue.

As in *S. siderea*, the most notable difference between affected and healthy tissue in *A. agaricites* was the accumulation of acidophilic electron dense zymogen cells and dispersed granules in the calicodermis and adjacent gastrodermis of DSS-affected tissue (Fig. 3B). Zooxanthellae density appeared to be similar between healthy and affected tissue, and zooxanthellae were observed in various stages of cell division in all of the samples. Zooxanthellae in healthy corals appeared to have a moderately greater intracellular accumulation of starch compared to zooxanthellae in affected corals. This may indicate greater energy demands placed on the zooxanthellae by the coral host in affected corals.

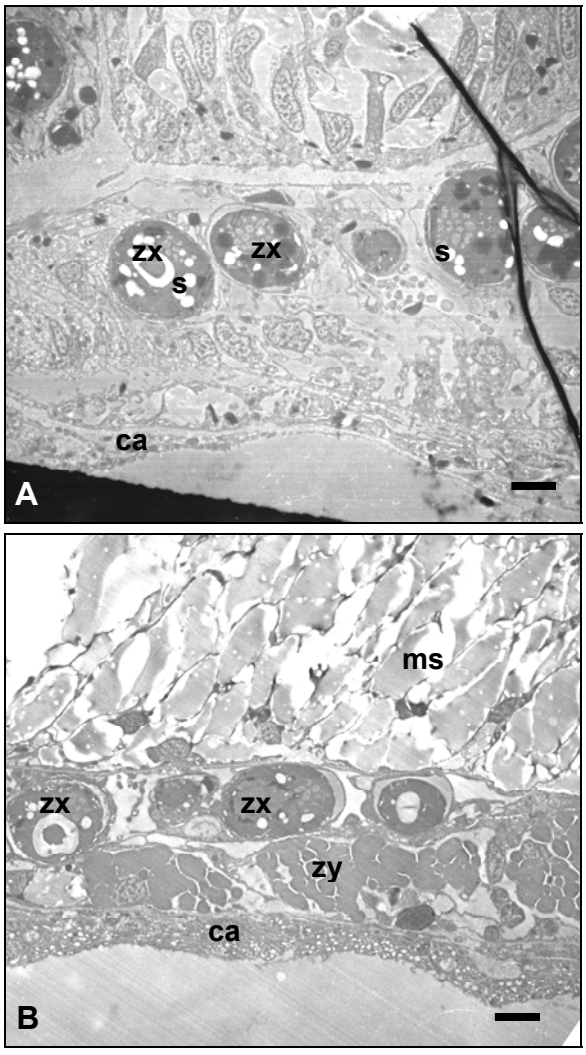

Figure 3. *Agaricia agaricites*. TEM micrographs. A) Decalcified healthy gastrodermis and calicodermis; B) decalcified DSS-affected gastrodermis and calicodermis. ca: calicodermis; ms: mucocyte; s: starch; zx: zooxanthellae; zy: zymogen granules. Scale bars: A and B = 5 µm.

In all of the DSS-affected tissues, numerous protein-staining, unciliated cells were present in close proximity to the calicodermis (Fig. 4). These cells were not observed in any of the healthy corals or in the healthy tissues on DSS-affected colonies. These cells have not yet been identified, although assessment of spatial relationships as well as identification and characterization of these cells is ongoing using fluorescent in-situ hybridization.

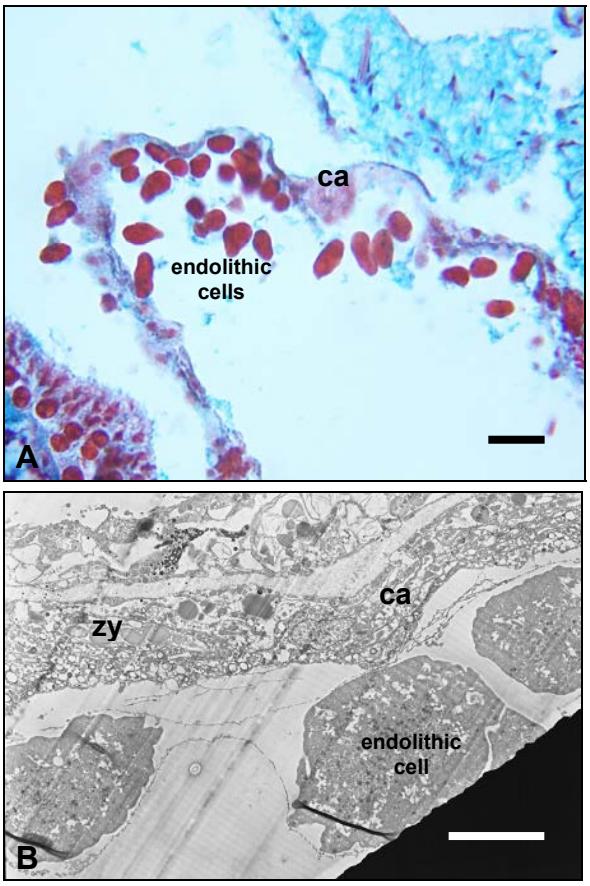

Figure 4. *Agaricia agaricites*. A) Histological micrograph, DSS-affected coral and endolithic cells; B) TEM micrograph, decalcified DSS-affected coral and endolithic cells ca: calicodermis; zy: zymogen granules. Scale bars: A = 20 µm and B = 10 µm.

Discussion

Dark Spot Syndrome causes cellular degradation, vacuolization, and necrosis in affected tissues of *S. siderea* and *A. agaricites*. A concomitant decrease in organic matrix suggests cessation of calcification in affected areas.

An increase in the concentration of proteinaceous, electron-dense inclusions was observed in the calicodermis by histological (Miller et al. 2006) and ultrastructural analysis. The morphology and staining properties indicate that these are zymogen granules. Zymogen granules are normally observed within secretory epithelial cells (granular gland cells), and are reported to function as an aid in feeding. The granules can be released into the gastrovascular cavity for extracellular digestion (Galloway et al. 2007, Goldberg 2002). In the DSS-affected tissues, large zymogen cells were observed in the calicodermis and zymogen granules were often observed free within the calicodermis and adjacent gastrodermis. Galloway et al. (2007) also observed dispersed acidophilic granules associated with endolithic mycosis.

Numerous endolithic fungal cells were observed directly adjacent to the calicodermis in DSS-affected *S. siderea*. Fungal hyphae have recently been observed in association with DSS lesions in several species of Pacific corals (Work et al. 2008) and in *S. siderea* from Florida and Puerto Rico (Galloway et al. 2007). Fungal endoliths are well known from corals and may be pathogenic to their hosts (Le Campion-Alsumard et al. 1995, Bentis et al. 2000, McClanahan et al. 2004). In gorgonians, the fungus *Aspergillus sydowii* causes the disease Aspergillosis, which causes purpling and necrosis of the gorgonian tissue (Smith & Weil 2004). The fungal cells in *S. siderea* are morphologically consistent with the genus *Aspergillus* (Moore et al. 2003); however, their identity has not yet been ascertained.

In contrast, DSS-affected tissues of *A. agaricites* did not contain fungal hyphae. However, a large number of unidentified endolithic cells were observed directly adjacent to the calicodermis. Thus, although DSS-affected tissues in both species exhibit tissue degradation and increased zymogen concentration, the difference in the type of endolithic cells present suggests that the causal agent of DSS in these two coral species may be different. In fact, Weil (2004) suggests that there may be multiple types of DSS (=DSD), and our observations, in concert with differences in zooxanthellar responses in two DSS-affected species (Cervino et al. 2001), suggest that DSS may represent a suite of different conditions.

The increased size of mucocytes along the calicoblastic epithelium in DSS-affected tissues of *S. siderea* suggests the presence of an inflammation response, possibly due to the presence of fungal endoliths. The absence of this response in healthy tissues on affected colonies suggests that inflammation may be localized. The presence of these endolithic cells, coupled with the increased concentration of zymogen granules in adjacent tissues generates questions: is the coral using this digestive enzyme as a defensive mechanism, and does increased concentration of these granules inhibit the endolithic cells or just prevent active invasion of the coral tissue? Although it not yet clear whether the endoliths observed are the causal agents of DSS, our observations suggest that *S. siderea* and *A. agaricites* may utilize a digestive enzyme as a defensive mechanism against endolithic cellular invasion.

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References

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

Borger J (2005) Dark spot syndrome: a scleractinian coral disease or a general stress response? Coral Reefs 24:139-144

Cervino JM, Goreau TJ, Nagelkerken I, Smith GW, Hayes R (2001) Yellow band and dark spot syndromes in Caribbean corals: distribution, rate of spread, cytology, and effects on abundance and division rate of zooxanthellae. Hydrobiol 460:53–63

Coelho VR, Manfrino C (2007) Coral community decline at a remote Caribbean island: marine no-take reserves are not enough. Aquat Conserv: Mar Freshw Ecosyst 16:1-20

Galloway SB, Work TM, Bochsler VS, Harley RA, Kramarsky-Winter E, McLaughlin SM, Meteyer CU, Morado JF, Nicholson JH, Parnell PG, Peters EC, Reynolds TL, Rotstein DS, Sileo L, Woodley CM (2007) Coral disease and health workshop: coral histopathology II. NOAA Technical Memorandums NOS NCCOS 56 & CRCP 4. National Oceanic & Atmospheric Administration, Silver Spring MD

Garzón-Ferreira J, Gil-Agudelo DL, Barrios LM, Zea S (2001) Stony coral diseases observed in southwestern Caribbean reefs. Hydrobiol 460:65–69

Gil-Agudelo DL, Smith GW, Garzón-Ferreira J, Weil E, Petersen D (2004) Dark spots disease and yellow band disease, two poorly known coral diseases with high incidence in Caribbean reefs. in Rosenberg E, Loya Y (eds) Coral health and disease. Springer, Berlin, pp 337–350

Gochfeld, DJ, Olson JB, Slattery M (2006) Colony versus population variation in susceptibility and resistance to Dark Spot Syndrome in the Caribbean coral *Siderastrea siderea*. Dis Aquat Org 69:53-65

Goldberg WM (2002) Gastrodermal structure and feeding responses in the scleractinian *Mycetophyllia reesi*, a coral with novel digestive filaments. Tissue & Cell 34:246-261

Le Campion-Alsumard T, Golubic S, Priess K (1995) Fungi in corals: symbiosis or disease? Interaction between poyps and fungi causes pearl-like skeleton biomineralization. Mar Ecol Prog Ser 117:137-147

McClanahan TR, McLaughlin SM, Davy JE, Wilson WH, Peters EC, Price KL, Maina J (2004) Observations of a new source of coral mortality along the Kenyan coast. Hydrobiol 530/531:469-479

Miller JD, Olson JB, Gochfeld DJ (2006) Histological examination of *Siderastera siderea* affected by Dark Spot Syndrome, EOS Trans AGU, 87(36), Ocean Sci Meet Suppl, Abstract OS26G-14

Moore CW, McKoy J, Del Valle R, Armstrong D, Bernard EM, Katz N, Gordon RE (2003) Fungal cell wall septation and cytokinesis are inhibited by bleomycins. Antimic Agents Chemother 47:3281-3289

Rosenberg E, Loya Y (2004) Coral health and disease. Springer, Berlin

Smith GW, Weil W (2004) Aspergillosis of gorgonians. in Rosenberg E, Loya Y (eds) Coral health and disease. Springer, Berlin, pp 279-287

Vargas-Ańgel B, Peters EC, Kramarsky-Winter E, Gilliam, DS, Dodge RE (2007) Cellular reactions to sedimentation and temperature stress in the Caribbean coral *Montastraea cavernosa*. J Invert Path 95:140-145

Weil E (2004) Coral reef diseases in the wider Caribbean. in Rosenberg E, Loya Y (eds) Coral health and disease. Springer, Berlin, pp 35–68

Work TM, Aeby GS, Stanton FG, Fenner D (2008) Overgrowth of fungi (endolithic hypermycosis) associated with multifocal to diffuse distinct amorphous dark discoloration of corals in the Indo-Pacific. Coral Reefs 27:663