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Zooxanthellae Regulation in Yellow Blotch/Band and Other Coral Diseases Contrasted with Temperature Related Bleaching: *In Situ* Destruction vs Expulsion

JAMES M. CERVINO[1*], RAYMOND HAYES[2], THOMAS J. GOREAU[3], and GARRIET W. SMITH[4]
[1]*University of South Carolina, Marine Sciences Department, Columbia, SC 29208, Email. cnidaria@earthlink.net;*
[2]*College of Medicine, Howard University, Washington, DC;*
[3]*Global Coral Reef Alliance, Cambridge, MA;*
[4]*University of South Carolina, Aiken, SC, USA*

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Abstract

Impairment and breakdown in the symbiotic relationship between the coral host and its zooxanthellae has been documented in the major Caribbean reef building coral, *Montastraea* spp., when it is infected with yellow band / blotch disease (YBD) pathogens and / or exposed to unusually high seawater temperatures. Progressive degradation of zooxanthellar cellular integrity occurs, leading to the deterioration of coral tissue. Cytoplasmic organelles were displaced and chloroplasts are reduced and marginalized which is accompanied by internal swelling, vacuolization, fragmentation, and loss of cell wall structural integrity. Changes in algae that occur in YBD-infected corals differ from changes seen in corals undergoing solely temperature-induced coral bleaching, however. In many disease-infected corals, there is no evidence of zooxanthella in the mucus, unlike in thermal bleaching, where zooxanthellae was evident in the coral surface layer. Isolated zooxanthellae

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*The author to whom correspondence should be sent.

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inoculated with YBD pathogens showed a 96% decrease in chlorophyll *a* pigments compared to controls, and a 90% decrease in mitotic cell division over 96 hours of YBD bacterial inoculation (<p=0.0016). Cytoplasmic and organelle integrity is compromised after YB infection, while host tissue remains intact. The YBD bacterial pathogens seem to target the symbiotic zooxanthellae indicating an algal infection. Isolated zooxanthellae samples subjected to higher temperatures and bacterial pathogens reveal virus-like particles (VLP) that are observed within algal cytoplasm, but their significance remains unknown and requires further investigation. The results of this study suggest that YBD and other coral disease is primarily a disease of the symbiotic zooxanthellae rather than of the coral host. These data elucidate the different cellular mechanisms between thermal bleaching and coral diseases in the Caribbean and Pacific.

Keywords: Thermal coral bleaching, Vibrionic-bleaching, coral disease, algicidal activity, necrosis, PCD

1. Introduction

Dissociation between zooxanthellae-cnidarian symbioses have been documented extensively during temperature-related coral bleaching events (Yonge and Nicholls, 1931; Jokiel and Coles, 1977; Williams and Bunkley-Williams, 1990; Glynn and D'Croz, 1990; Glynn, 1993; Goreau and Hayes, 1994; Hayes and King, 1995; Brown et al., 1996; Jones et al., 1998). Bleaching is defined as expulsion or the loss of the symbiotic zooxanthellae from corals host tissue (McCloskey et al., 1996; Gates et al., 1996; Lee-Shin Fang et al., 1998; Baghdasarian and Muscatine, 2000).

Coral disease research in the past 25 years has focused on field analysis and the quest for the identification of pathogens associated with such diseases (Antonius, 1981; Santavy and Peters, 1996; Smith et al., 1996; Bruckner and Bruckner, 1996; Richardson, 1996; Richardson, 1998; Goreau et al., 1998; Hayes et al., 1998; Cervino et al., 2001; Denner et al., 2002; Ben-Haim et al., 2002). However, very few studies focus on the physiological impairments of host coral and its symbiont that relate specifically to pathogenic stress (Peters, 1983; Glynn et al., 1985; Peters et al., 1984; Cervino et al., 2001; Bythell et al., 2002).

See figure on next page.

Figure 1. a: Early stages of a yellow-blotch lesion effecting *Montastraea* sp. developing into a band. Picture was taken in Bonaire (coral tag #2244) during Sept. 1997. b: Classic thermal bleaching of *Montastraea* sp. c: Yellow-blotch lesion developing into a band after 3 months (coral tag #2244) in Bonaire during Dec. 1997.

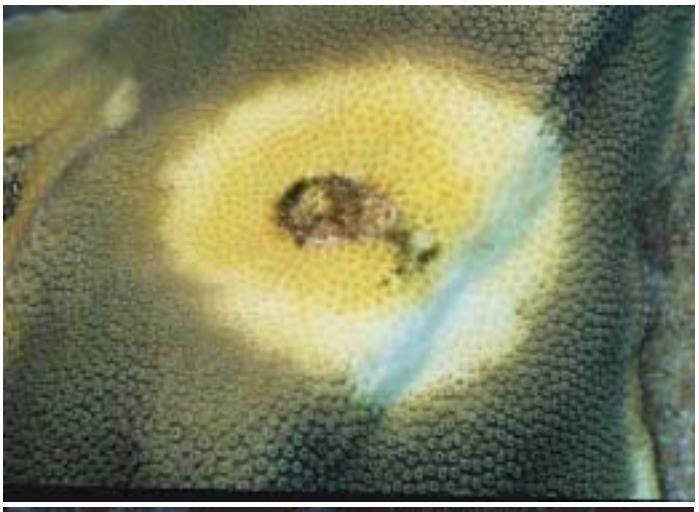

Fig. 1a.

Fig. 1b.

Fig. 1c.

Figure 1. See legend on previous page.

An understanding of the stability between coral host and its symbiotic zooxanthellae is germane to understanding homeostasis within this dynamic relationship (Pardy, 1974; Drew, 1972; Trench, 1987; Muscatine et al., 1981; 1989; Taylor et al., 1989). Integrating the links between thermal expulsion and coral diseases may provide a better understanding of cellular mechanisms that induce the breakdown of coral the host and its symbiotic partner. This research attempts to address the unique differences between thermal coral bleaching and pathogenic-related coral death, which has been classified as "coral disease bleaching" and Vibrionic-bleaching, because it is caused by *Vibrio* spp. (Kushmaro et al., 1996; Ben-Haim et al., 2000; Kushmaro et al., 2001). Vibrionic-bleaching, which sometimes results in white or pale appearance can be readily confused with thermally related bleaching (Figs. 1 a–c).

Temperature stress or exposure to chemicals such as copper (Jones, 1997) and excess ammonium (Hoegh-Guldberg et al., 1989) or sodium cyanide can cause zooxanthellar expulsion and bleaching (Jones and Stevens, 1997; Cervino et al., 2003). An increase in mitotic cell division occurs when corals are exposed to higher temperatures and cyanide (Cervino, 1996; Cervino et al., 2003). However, during pathogenic stress the rate of cell division and densities decreases dramatically (Cervino et al., 2001). Although the rate of cell division increases in coral exposed to higher temperatures or to chemicals, the rate of zooxanthellar cell density decreases because the symbionts are being expelled from the tissue as described in Gates et al. (1992) outlining the five mechanisms of the release of zooxanthellae from host coral tissue, including detachment. It is imperative to distinguish between the mechanisms of coral bleaching and coral disease infection for accurate field assessments of reef health.

During temperature or chemical stress, density-dependent factors regulating cell division may be impaired (Jones and Yellowlees, 1997). Coral-host cells synthesize heat shock protein at a lower temperature compared to the zooxanthellae, indicating that the alga could be more sensitive to heat and may trigger the zooxanthellae release process (Suharsono and Brown, 1992; Fang et al., 1998).

The % Mitotic Index (MI%) equation has been used before in vitro as a method to determine stress (Wilkerson et al., 1988; Jones, 1997). Mitotic Index measures cell division of zooxanthellae in culture in a known population density of cells. Chlorophyll *a* measurements have also been used as a method in the determination of algal pigment concentration (Porter et al., 1989; Coles and Jokiel, 1977; Kleppel et al., 1989; Jones, 1997), in stressed corals. PAM Chlorophyll electron fluorescence analysis *in vivo* displays large changes in fluorescence yield during different times of the diurnal cycle. Careful measurements during medium-term controlled experiments are mandatory when measuring chlorophyll fluorescence in symbiotic algae infected with

pathogens. PAM fluorometry can provide notable information about the functionality of photosystems 1 and 2 during photosynthesis and about the state of the symbiotic zooxanthellae during temperature and pathogenic stress, however as noted by Warner et al. (1996) and Fitt et al. (2001), caution must be taken when extrapolating fluorescence data, as there can be numerous biochemical explanations for the fluorescence patterns recorded during lab experiments.

This paper clarifies the differences between symbiotic zooxanthellae expulsion induced by temperature-related bleaching, or loss of symbiotic zooxanthellae in corals that are stressed by pathogen-induced diseases. This paper highlights the physiological process when corals are exposed to higher temperatures and YB pathogens and compares this to temperature induced bleaching stress. We stress that YBD bacterium primarily target the zooxanthellae and not the host. We also document, for the first time the discovery of viral-like particles (VLPs) found in symbiotic algae in *Montastraea* spp. with YBD.

2. Materials and Methods

Isolation of bacterial cultures and suspected pathogens

Bacterial cultures were taken from corals in the Florida Keys and Bonaire showing classic signs of yellow blotch/band disease that were evident on colonies of *Montastraea faviolata*. Mucus samples were vacuumed lightly in the center of the yellow lesion using a 10 cc syringe with no needle attached. Control samples were also vacuumed from corals that were healthy at both study sites. Three corals were selected in the FL Keys and three corals were selected from Bonaire. Bacterial isolates were grown on glycerol artificial seawater medium (GASW) (Smith et al., 1982). Isolated colonies were selected from culture plates and chosen based on distinct morphological and growth characteristics such as color and shape. Those isolates were extracted then re-streaked to obtain pure cultures.

In total, 143 pure cultures were isolated, 35 originating from control samples (the healthy samples) and 108 from diseased coral samples. After 48 hours a culture of each isolate was then subjected to Carbon Source Utilization Pattern (CSUP) analysis (Ritchie et al., in press). This was accomplished using BIOLOG GN1 96-well MicroPlates (BIOLOG Inc., Hayward, CA, USA; Bochner, 1989). All Florida samples were first grown on Tryptic Soy Agar/Salt (TCBS) media then transferred to GASW. *Bacillus* sp. and *E. coli* were inoculated and tested as controls. YBD pathogens were chosen according to the Biolog metabolic fingerprint analysis. Four chosen suspected pathogenic

bacteria were grown again on GASW, then diluted with 3 ml of sterile filtered seawater. Aliquots of the diluted material were used as the inoculate into cultures of symbiotic zooxanthellae isolated from *Montastraea* sp.

Comparisons of mucus from thermally bleached corals and corals showing disease lesions of the same species

Mucus samples using a 10 cc sterile syringe were collected using SCUBA from corals in-between depths of 2–5 meters in Papua New Guinea and the Caribbean. For Pacific samples mucus was collected during a mild temperature related bleaching event. Winter water temperatures ranged between 31°C and 32°C. Caribbean samples were also collected during a temperature related bleaching event which occurred in the spring and was characterized by temperatures ranging between 29°C and 30°C during the spring for that region. The syringe samples were brought to the lab fixed in 10% glutaraldehyde solution and centrifuged at 5,000 rpm for three minutes for cytological examination of the symbiotic zooxanthellae.

Collection and isolation of symbiotic zooxanthellae

Ten 5-cm diameter samples of healthy corals were collected from 10–12 meters depth using SCUBA. The samples were placed in polyethylene bottles containing filtered seawater (FSW) and immediately brought back to an out-door flow through seawater aquaria system at Mote Marine Lab Florida Keys where they were monitored for 30 days before zooxanthellae inoculation experiments. This was designed to see if the YBD bacteria from infected corals can induce the infection in the symbiotic zooxanthellae that we cultured similar to YBD in the field.

Selected healthy corals were chosen for zooxanthellae isolation and culturing for infection experiments. Host coral tissue was removed from all specimens using a Water Pik following the methods of Johannes and Wiebe, (1970). The liquid extract containing tissue as well as the zooxanthellae were homogenized and centrifuged at 5,000 rpm for 5 minutes, in order to separate host tissue from zooxanthellae as shown in Gates et al. (1992). The liquid extract was discarded and pellet was re-suspended in FSW.

Zooxanthellae inoculation experiment

Twenty-one 1.5 ml culture tubes were filled with 1 ml of F2 culture medium for infection experiments. Each tube was then filled with 0.5 ml of freshly isolated zooxanthellae pellet. Finally, 250 µl of diluted bacterial culture was added to

the culture medium. The 250 µl had been extracted from a 3 ml bacterial culture including *Bacillus* sp. and *E. coli*. Three culture tubes were set up for each of the exposure time of 24, 48 and 96 hours. Corresponding controls were prepared to mimic bacterial infection experiments using an inoculation of fresh sterile seawater. Replicate experiments and methodology were conducted for Chlorophyll *a* and c_2 analysis following the Spectrophotometric equations of Jeffery and Humphrey (1976).

At the end of the exposure time, samples for mitotic indices (Wilkerson et al., 1988; Jones, 1997) were preserved in 10% glutaraldehyde seawater solution, which had an original glutaraldehyde concentration of 90% and immediately counted. The liquid extract was discarded and pellet was re-suspended in a solution of FSW / 10% gluteraldehyde and re-centrifuged for zooxanthellar cell counts. Zooxanthellae abundance and mitotic index were determined by direct examination under a phase contrast microscope at 400× and 1000× magnification, and counted using a Neubauer ruling hemocytometer.

Histology and TEM

Samples from zooxanthellae inoculation experiments were extracted from the glutaraldehyde solution and placed in a 10% phosphate buffered solution (PBS) for TEM analysis and cytological examinations. All whole coral tissues that were examined using light microscopic histology were fixed in a 3%-solution of glutaraldehyde in FSW. Samples of intact coral tissue displaying macroscopic features of yellow blotch / band disease were extirpated, immersed and fixed in glutaraldehyde, and post-fixed in 1.0% osmium tetroxide in phosphate buffer. These tissues were processed for examination with both optical and electron microscopy. Fixation lasted for three days, and the tissues were then transferred to a 70% ethanol solution. The tissues were left in alcohol for four more days and then decalcified in 70% alcohol mixed with a solution containing ethylene diamine tetra acetic acid (EDTA), tannic acid and hydrochloric acid. Decalcification was continued until release of gaseous carbon dioxide was no longer released. The tissues were then dehydrated through a graded series of alcohols to absolute ethanol. After gradual transfer to propylene oxide, tissues were embedded in low viscosity Spurr plastic resin. The plastic blocks were cured at 60°C for 48 hours before the specimens were trimmed and prepared for sectioning. One-to-two micron thick sections were cut on an LKB microtome using a diamond knife. The sections were adhered to clean glass microscope slides, stained with aqueous Toluidine blue. A cover slip was mounted over the section before photomicroscopy. Pictures were taken on a B&L Balplan microscope with 35-mm photographic attachment. Fuji print film (ASA 100) was used to generate the images shown.

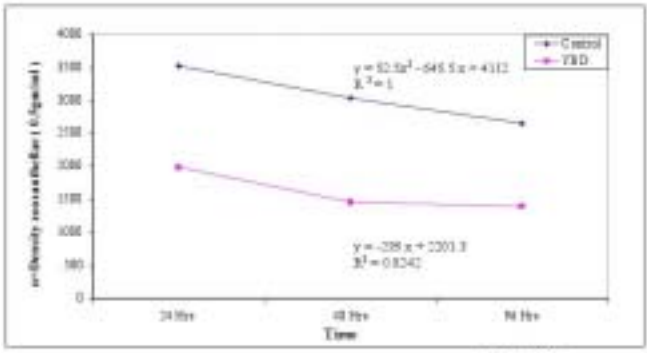

Figure 2a

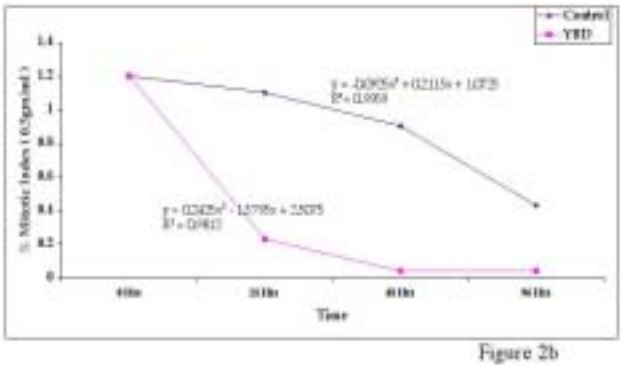

Figure 2b

Figure 2. a–d: M,STDEV, Pairwise Sample T-Test for P values. a: Cultured zooxanthellae with YBD pathogens show a decline in cell density of 44% when compared to controls at 24 hours, at 48 hours a further decline of 52% is shown, and at 96 hours a significant drop in densities to 94% is shown (<p=0.013). b: The rate of % MI between within 24 hours, show a decrease 81% when compared to controls, after 48 hours there is a 95% drop and at 96 hours it remains constant compared to non-pathogenic bacteria at about 97% (<p=0016). Zooxanthellae inoculated with *E. coli* and *Bacillus* spp. show no significant change in algal density, however the decline in % Mitotic Index may be due to the alga outside of its symbiotic relationship from the host animal (c, d).

For TEM; tissues was removed from slides and fixed for two hours at 4°C in a solution containing 4.5 volumes of 0.18 M Sorensen phosphate buffer, pH 7.4, 4.5 volumes of 0.15 M sucrose and 1 volume of 10% glutaraldehyde. Fixed tissue protocol followed preparation by Muscatine et al. (1997). Tissues were then

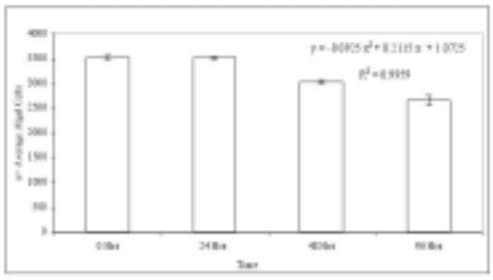

Figure 2c.

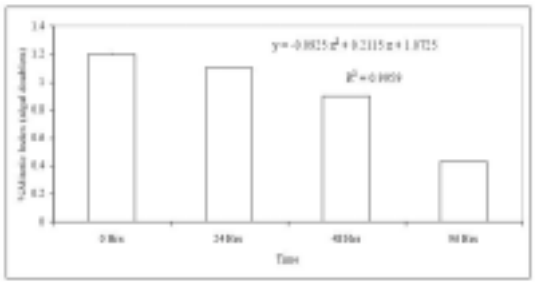

Figure 2d.

post-fixed for 1.5 h at room temperature in 1% osmium tetroxide in 0.18 M phosphate buffer, stained in uranyl acetate in 70% ethanol, and then embedded in Epon resin. For initial orientation, 1 ml-thick sections were cut with a diamond knife, stained in toluidine blue, and viewed under light microscopy. For EM, ultra-thin sections were cut with a diamond knife.

3. Results

Cell densities, Mitotic Indexes and chlorophyll a and c_2 analysis (M, STDEV; Pairwise Sample T-Test for P values)

Symbiotic zooxanthellae from healthy corals that were cultured in a F2 medium showed no significant decline in algal density (3521 to 3519) after 24 hours. However, zooxanthellae cultures that were inoculated with the YB

pathogens showed a 44% decline after 24h, and a 52% decline after 48 h. The sharpest decline was evident after 96 h. Zooxanthellae density declined by 94% (<p=0.013) (Fig. 2a).

Mitotic Index did not decline significantly (from 1.2 to 1.1%) in healthy algal cultures after 24 h. In comparison, mitotic index in algal cultures inoculated with YB bacteria declined by 81% (from 1.2% to 0.23%) after 24 h. After 48 h, mitotic index in YB-inoculated algal cultures declined by 95% (to 0.04%), compared to a decline of only 25% in healthy algal cultures. After 96 h, inoculated cultures showed a 97% decline in MI (dropping to 0.04%), compared to a 64% decline in healthy algal cultures (<p=0.0016) (Fig. 2b). Symbiotic zooxanthellae inoculated with *E. coli* showed no significant change in algal density or % MI, similar to controls. Zooxanthellae infected with *E. coli* and *Bacillus* sp. showed no significant changes in cell density morphology however, changes in cell division may be due to the alga being out-side of the host animal symbiotic association (Figs. 2c and d).

Chlorophyll *a* declined 96% after 96 hours that were inoculated with YBD pathogens compared to healthy symbiotic zooxanthellae. A decline of only 10% of C2 was observed after 96 hours of exposure to YBD pathogens (Fig. 3).

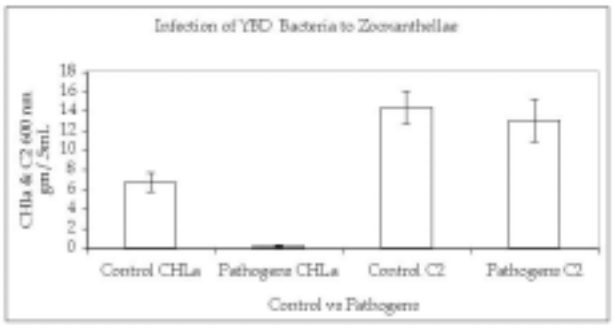

Figure 3: Changes in Chlorophyll a and c_2 concentrations in YBD infected corals and controls.

Comparative analysis of zooxanthellae in mucus from thermally bleached corals and diseased corals of the same species

Mucus samples extracted from bleached Pacific corals *Porites lutea*, *Acropora tortuosa*, *Acropora turaki*, *Acropora muricata*, and *Acropora valenciennesi* contained more than 100+ algal cells per 10 ml mucus extracted from the surface of the stressed colonies. Whole and fragmented symbiotic zooxanthellae were

seen in all thermally bleached corals. Since mucus does not normally contain large numbers of zooxanthellae, this indicates that the zooxanthellae are being expelled. Samples of the *Acropora* sp. showing signs of white-band disease (WBD) contained no evidence of intact algae, but occasional fragments of degenerative zooxanthellae were present (20 or less, algal cells per 10 ml). *Porites* sp. pocked by pink swollen nodules are present when infected by the trematode *Podocotyloides stenometra* (recent observation Aeby, 2003) contained no zooxanthellae in the mucus. Mucus samples from Caribbean corals (*Montastraea* sp., *Acropora* sp., and *Stephanocenia michillini*) suffering from YBD, white plague, white band, and dark spots diseases showed no signs of containing intact symbiotic zooxanthellae. However, mucus samples from the same coral species suffering from temperature-related bleaching (but not from disease) showed the classic signs of expulsion of both intact and fragmented symbiotic zooxanthellae (Table 1).

Table 1. Sampling of mucus to determine expulsion of whole intact algae and fragments during temperature related bleaching events and disease out-breaks. + or – of mucus samples containing zooxanthellae in bleached and diseased corals. Twenty samples of each were tested.

Species	Coral bleaching	Coral diseases
Porites lutea PLD Pacific	+	–
Acropora tortuosa WBD Pacific	+	–
Acropora turaki WBD Pacific	+	–
Acropora muricata WBD Pacific	+	–
Acropora valenciennesi WBD Pacific	+	–
Montastraea YBBD Caribbean	+	–
Montastraea WP Caribbean	+	–
Acropora palmata Caribbean	+	–
Acropora cervicornis Caribbean	+	–
Stephanocenia michilinni DSD Caribbean	+	–

Microscopy

Light microscopy show that symbiotic zooxanthellae inoculated with YB pathogens induce algcidal activity, exhibiting fragmented degenerate algal cells (Figs. 4a–e). Electron micrographs showed re-distribution and degeneration of algal organelles, vacuolization, fragmentation, swelling, and displacement of algal cell features were evident. Degeneration of nuclear material can be seen (Figs. 5a and b).

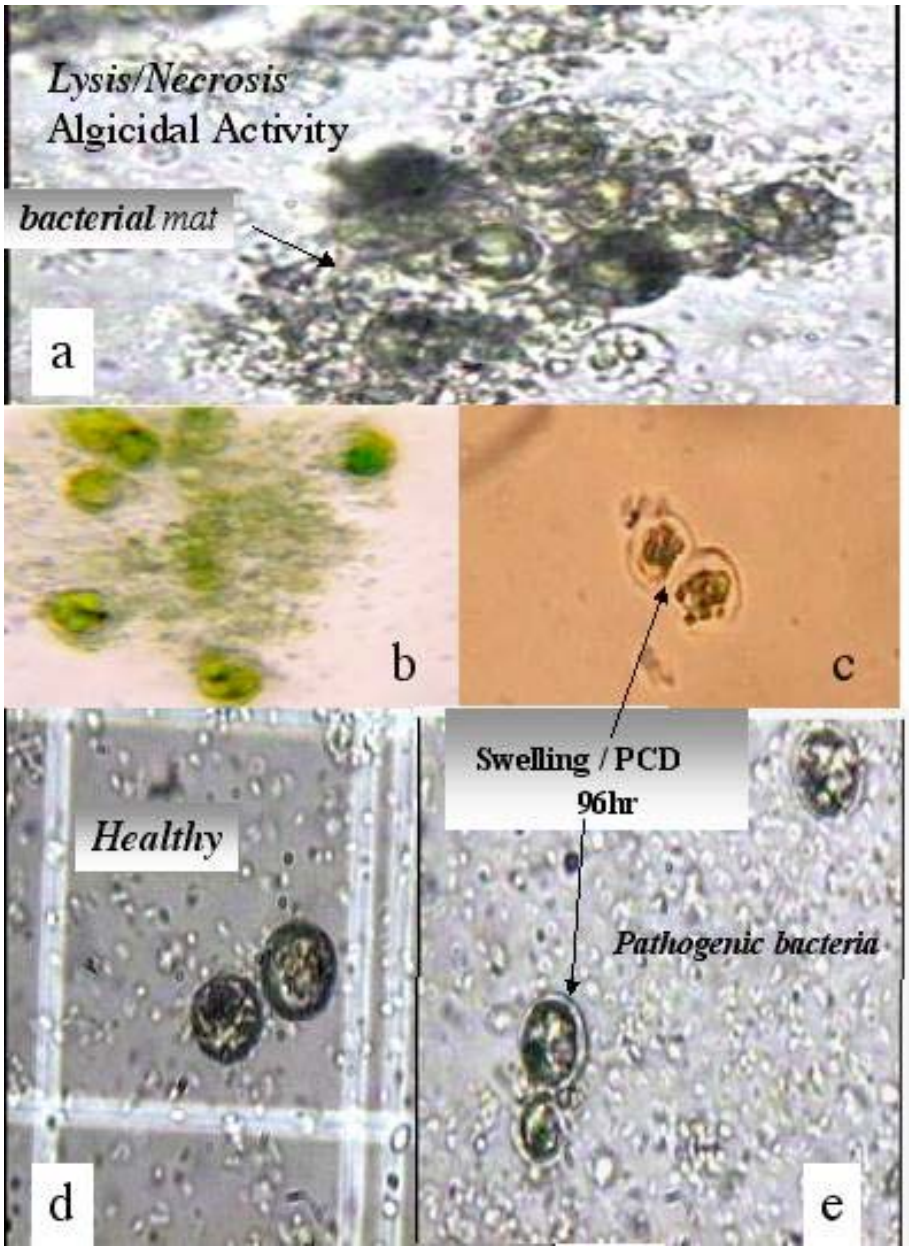

Figure 4. See legend on next page.

Fragmentation of the pyrenoid was apparent in YB bacterial treatments and in others is seems normal. Virus-like particles (VLP) were observed in the cytoplasm of zooxanthellae (Fig. 6). These VLPs resemble PhycoDNAviruses. The chloroplasts are still visibly intact, but, the granum and thylakoid membranes were disorganized in YBD specimens. Field samples of mucus taken from thermally bleached corals contain zooxanthellae expelled from the oral cavity, as has been confirmed through histological sections clearly showing expulsion from the gastrodermal tissue.

4. Discussion

This research has shown that the loss of the symbiotic zooxanthellae occurs via a different mechanism in corals suffering from diseases than in corals stressed from thermal bleaching. The results of this study also indicate that physiological mechanisms of coral reef bleaching are distinct from those of coral diseases. Our lab and field examinations of corals suffering from pathogenic stress such as YBD show no loss of symbiotic zooxanthellae by mode of expulsion. During temperature-related bleaching events, copious numbers of wholly intact symbiotic zooxanthellae are seen residing in the coral surface mucus, and few degenerate alga are present (personal observation). Corals showing signs of pathogenic stress show only scattered remnants of algal fragments residing on the surfaces, compared to during temperature- or chemical-related bleaching. This may be due to cellular necrosis or (PCD) programmed cell death during the early stages of YB infection as the mode of zooxanthellae loss (Dunn et al., 2002) internally during early stages of the disease progression. *In situ* examinations of Caribbean and Pacific diseases show small fragmented remnants of degenerate alga residing in the mucus (Table 1). Cytoplasmic condensation, algal cell-wall degradation, and overall internal cell shrinkage was seen in corals exposed to high temperatures and YBD pathogens. Degenerate endosymbiotic algae have been shown before (Steel and Goreau, 1977; Titlyanov et al., 1996; Cervino et al., 2001; Cervino et al., 2003).

See figure on previous page.

Figure 4. a–e: Photographs were taken through the 100× objective lens with a film magnification of 300× of infected zooxanthellae with YBD bacteria. Symbiotic zooxanthellae degeneration and lysis is evident surrounded by bacterial mats. a: Vacuolation and swelling is extreme and also showing evidence of algicidal activity of alga (b). PCD like symptoms can be seen in approximately 30% of all zooxanthellae that are infected (c–d). e: healthy symbiotic zooxanthellae.

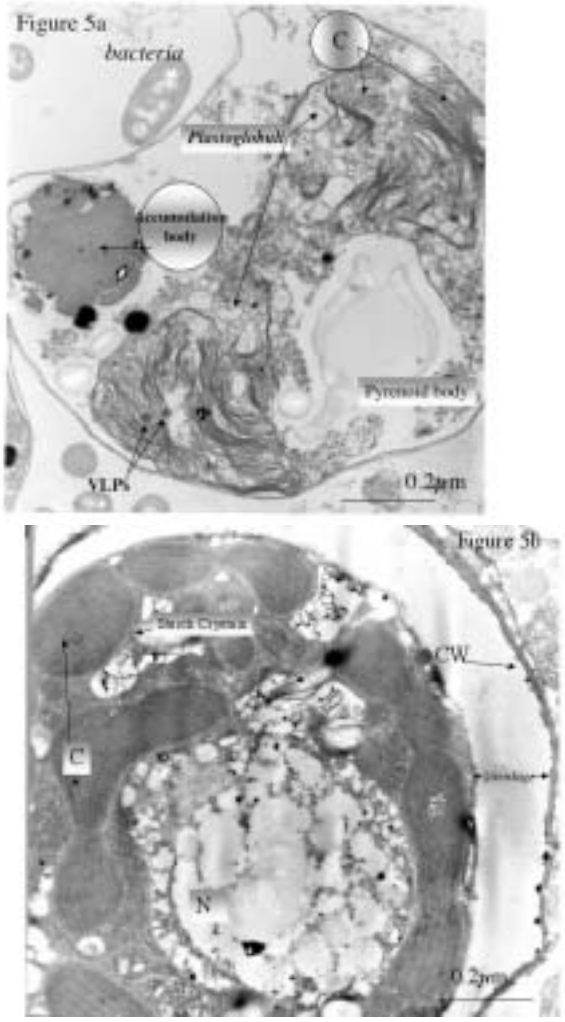

Figure 5. a–b: Shows a transmission electron micrograph (TEM) image of symbiotic exposed to YBD bacteria. TEMs of zooxanthellae *in situ* indicate the same general morphology as seen in light microscopy, but reveal far greater detail. a: Image shows degradation of cell wall that appears to be evident along with vacuolation and cytoplasmic shrinkage. The chloroplasts are disorganized to the outer margin of the cell; however, they appear intact. There are numerous non-membrane bound vacuoles that appear within the nucleus and cytoplasm of the zooxanthellae. Some of these are filled with an electron dense substance, but many are empty or filled with fluid. b: The core of the algal cells is severely vacuolated. The pyrenoid body is apparent and normal in structure. However, in some cells, the pyrenoid is fragmented into separate segments. Algal swelling is primarily attributable to the expansion of the accumulation bodies separation of the algal surface membranes from the peripheral cytoplasm containing chloroplasts. N=nucleus, C=chloroplasts, S=starch crystals, CW=cell-wall.

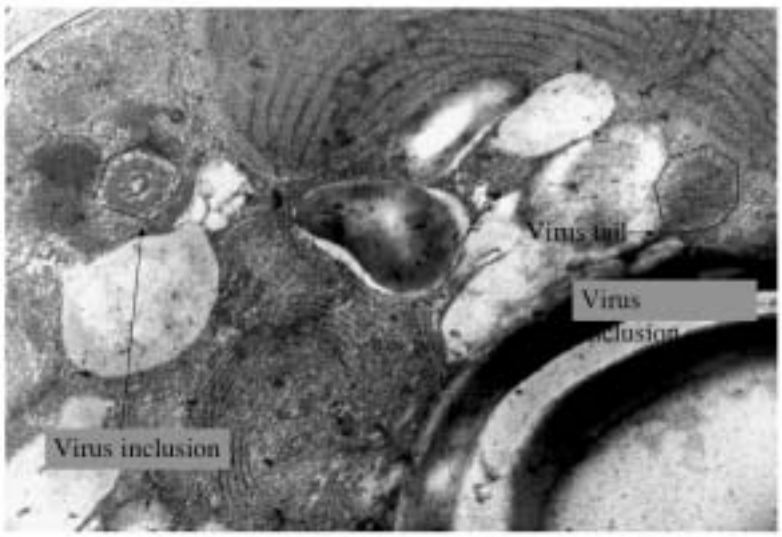

Figure 6. Viral-like particles (VLPs) are observed in several algal cells. These particles are approximately 0.10–0.15 µM in diameter. They have a central dense core and a peripheral envelope or coating. They resemble published profiles of immature and mature virons. Their contribution to the disease process is not established. However, the presence of VLPs may indicate that the algae are subjected to genomic or metabolic stress from yet another unanticipated source.

It has been shown that the rate of algal cell division increases when corals are exposed to higher temperatures, thereby releasing copious amounts of mucus and zooxanthellae *in situ* and *in vitro* and leaving the tissue devoid of much of its pigments (Cervino, 1996). With YB infection % MI decreases (Fig. 2b) whereas during thermal bleaching, % MI increases (Cervino, 1996). It is important to note that densities of zooxanthellae decline when corals are thermally stressed and infected with YB bacterium, yet the mechanism of the zooxanthellae loss is different: with temperature stress the zooxanthellae are expelled (Cervino, 1996) whereas during combined pathogenic and temperature stress the alga become senescent within the host tissue and are not expelled (Fig. 7). This may be due to the slow-down in algal cell division.

It has been shown during *in vitro* experiments that symbiotic algae are less heat tolerant than the coral host itself (Iglesias-Prieto et al., 1992; Fitt et al., 1993; Iglesias-Prieto and Trench, 1997). Heat-shock protein (Hsp) is expressed in zooxanthellae exposed to temperature stress (Hayes et al., 1995). Sharp et al. (1997) found that when zooxanthellae was exposed to temperatures of 30°C,

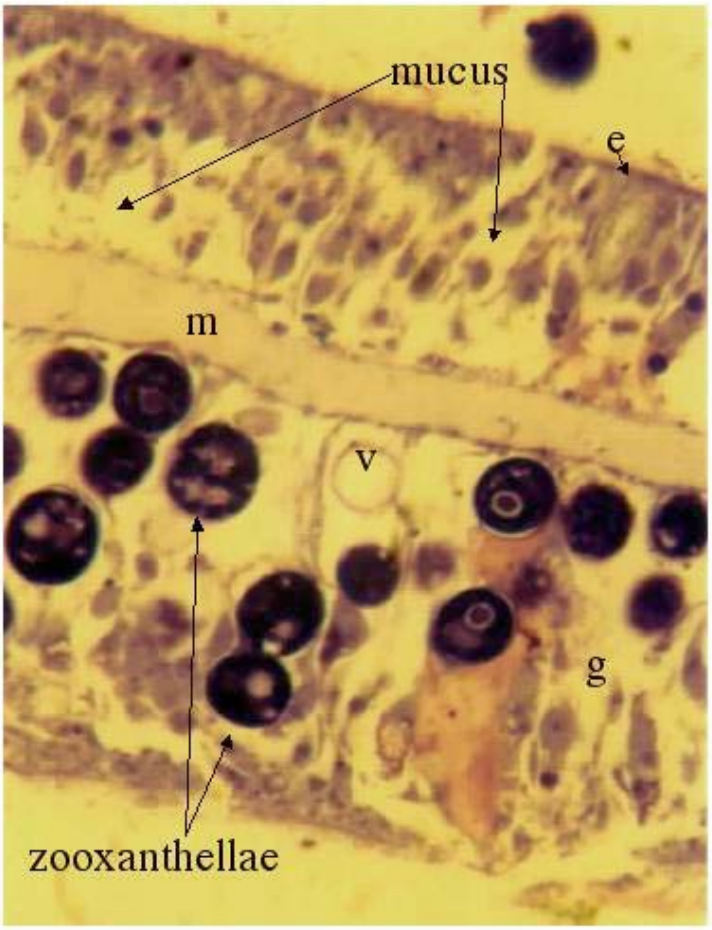

Figure 7. a–b: Histology of *Montastraea* sp. exposed to YB pathogens at 32°C. Vacuolation and swollen alga can be seen. However, host structural features are evident indicating early breakdown and pockets of copious mucus, however, symbiotic zooxanthellae remain inside the host gastroderm. b: Histological section of healthy Montastraea sp at 25°C, early signs of stress are evident due to a build-up in mucocytes. E=epidermis, V=vacuole, M=mesogleoa, G=gastroderm and zoox= symbiotic algae.

Hsp was immediately triggered, while in the host animal, it took two hours of exposure at 33°C for Hsp to be expressed. This research and our *in situ* and *in vitro* analyses of coral diseases and thermal bleaching suggest that the

symbiotic alga are more sensitive to temperature and pathogens combined than to temperature stress alone. Tropical reef-building corals that have suffered temperature stress will regain their symbionts when temperatures return to their normal range and the corals recover (Goreau and Hayes, 1994). However, the results of this study have shown that corals impacted by a coupling of both temperature and pathogenic stress are unlikely to recover when temperatures return to normal. During thermal bleaching the tissue may still be alive, but during YBD infection, the tissue dies back after the alga are senescent. Therefore it is highly possible that corals impacted by both temperature and pathogenic stress are unlikely to recover when temperatures return to normal.

Several coral diseases/syndromes result in the coral turning white, and a decrease in chlorophyll concentration due to the loss of the pigmented zooxanthellae as in localized Vibrionic-bleaching (Kushmaro et al., 2001). This is why diseased corals are often confused with thermally-bleached corals. With the exception of black band (BBD) and white plague (WPD) diseases, which appeared to attack the host tissue, all of the coral diseases examined in this study seem to be attacking the symbiotic zooxanthellae inside of the gastrodermal tissue, which evidently died not as a result of expulsion from the gastroderm. Our data on YBD and WBD seem to suggest that *in situ* destruction of the algae occurred, rather than an attack on both algae and host tissue. Primary pathogens effecting the zooxanthellae can be considered a zooxanthellae disease if the host is not harmed. Bacterial infection of *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus* was recently found in controlled lab experiments to lyse the symbiotic algae and was characterized as "bleaching" (Kushmaro et al., 2001; Ben-Haim et al., 2003). However, classifying this infection and lysing as bleaching is ambiguous, because the observations don't match the traditional mechanisms of thermal bleaching as outlined in the field and in controlled lab experiments, therefore Vibrionic-bleaching may be a more appropriate term that can be used. These data more appropriately support the hypothesis that many known coral diseases are infections of the symbiotic zooxanthellae, which are the primary target of the pathogen, and should not be related to mass coral reef bleaching due to thermal stress.

Viruses and algicidal attack

The presence of virus-like particles (VLPs) in corals that were undergoing thermal bleaching stress) has been documented (Wilson et al., 2001). We documented the presence of VLPs in zooxanthellae of all corals inoculated with the YBD-associated bacteria; however, not at the high densities seen by Wilson et al. (2001). The results of this study suggest that YBD-infected corals

experience the induction of VLPs during elevated water temperatures. The role of the viruses is not fully understood and warrants further investigation to determine whether they trigger, or exacerbate, coral stress. Infection of dinoflagellates by the bacterium *Pseudoalteromonas* has been documented during brown tides (Lovejoy, 1989; Doucette et al., 1999). *Pseudoalteromonas* exhibit potent algicidal properties that are harmful to algal bloom species including *Gymnodinium* (Lovejoy, 1989). During this analysis, we observed the identical algicidal attack on the symbiotic zooxanthellae in coral host tissue (Figs. 4a and b), making this condition clearly different from that of thermal bleaching. A complete understanding of specific density dependant release factors by the host to symbiont during stress of the symbiotic zooxanthellae requires further investigation as well as the role of host lysosomes during cell necrosis or PCD. During pathogenic stress, these symbiotic zooxanthellae are dying by way of necrosis (bacterial lysing of the algae, and possibly PCD. It remains unconfirmed whether host lysosomes are digesting these infected algae or if the bacterial toxins are causing necrosis. Zooxanthellae normally resist digestion by the host lysosomes (Muscatine et al., 1989). Further work is needed using electron microscopy and specific stains to determine if the degenerative fragmented algae seen in YBD-infected host tissue are being digested by host lysosomes. The pattern of zooxanthellae cell loss is evident during temperature and pathogenic stress. However, regulatory mechanisms remain unclear and warrant intense investigation in understanding the symbiotic relationship between coral host and the symbiotic zooxanthellae.

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