

# Pink-line syndrome, a physiological crisis in the scleractinian coral *Porites lutea*

J. Ravindran\*, Chandralata Raghukumar<sup>#</sup>

\*National Institute of Oceanography, Regional Centre, Dr. Salim Ali Road, P.B. No 1913, Cochin 682 018

<sup>#</sup>National Institute of Oceanography, Dona Paula, Goa 403 004, INDIA

\* Corresponding author E-mail: jravi@nio.org

---

**Key words:** Lakshadweep Island, Scleractinian corals, *Porites lutea*, cyanobacterium, pink-line syndrome, pCO<sub>2</sub>

**Abstract** Coral diseases are one of the major factors that alter coral cover and their diversity. We have earlier reported the “Pink-line syndrome” (PLS) in the scleractinian coral *Porites lutea* wherein a colored band appears between the dead and healthy tissue of a colony. About 20% of the *P. lutea* colonies were affected in Kavaratti of the Lakshadweep Islands in the Arabian Sea during April 1996 and the incidence increased four fold within the next four years. Fungi were associated in both PLS-affected and healthy specimens whereas the cyanobacterium *Phormidium valderianum* occurred exclusively in the PLS-affected specimens. There was increased expression of a 29 kDa protein without any significant increase in total protein content in the PLS-affected colonies. A reduced number of zooxanthellae and an increase in zooxanthellae size, mitotic index and chl *a* concentrations were some of the characteristics of the PLS-affected colonies.

PLS induction experiments conducted using selected fungi and the cyanobacterium *P. valderianum* isolated from the affected colonies and abiotic factors such as CO<sub>2</sub> enrichment and the effect of cyanobacterial photosynthesis inhibition indicated that the CO<sub>2</sub> build-up around the host tissue caused the pink coloration. We hypothesize that these physiological changes disturb the mutualism between the zooxanthellae and the host. When the symbiosis is disturbed by the external CO<sub>2</sub>, the host loses control over the zooxanthellae, causing their uncontrolled division. This process may lead to a break in photosynthate transfer to the host thereby resulting in starvation and finally leading to partial mortality. We further hypothesize that these degenerative processes are triggered by the CO<sub>2</sub> produced by *P. valderianum* through its carbon concentration mechanism. In this context, any opportunistic cyanobacteria or other agents having potential to interfere with the physiology of the host or the symbiont can cause such a physiological disorder. The mechanism of PLS formation is an early warning to protect corals as the increasing atmospheric CO<sub>2</sub> could induce PLS-like physiological disorder in corals.

## 1. Introduction

The pink line syndrome (PLS) reported in the coral *Porites lutea* (Ravindran and Raghukumar 2002) from the Lakshadweep island in the Arabian Sea has added to the growing list of diseases afflicting scleractinian corals. White band disease (WBD) virtually eliminated *Acropora palmata* from the reefs of the Virgin Islands (Gladfelter 1982) and the spread of the 'white plague II' was reported to affect reefs in a stretch of >400 km between 1995 and 1997 in Florida's reefs (Richardson 1998). About 11% disease incidence and destruction of large colonies by plague was reported from the West Indies (Nugues 2002). White syndrome was found to be affecting corals in Papua New Guinea (Sweatman et al. 2002).

Biotic and abiotic agents responsible for causing coral disease have been established for some of the coral diseases. The black band disease affecting scleractinian corals is reported to be caused by a microbial consortium containing the cyanobacterium *Phormidium corallyticum*, sulphur oxidizing and sulphur reducing bacteria (Richardson et al. 2001). Bleaching of coral *Occulina patagonica* in the Mediterranean Sea is demonstrated to be caused by *Vibrio shiloi* (Kushmaro et al. 1996). Bleaching is also caused by a plethora of abiotic factors including sedimentation, elevated sea surface temperatures, pollutants and other unknown stressors (Brown 1997; Hayes and Goreau 1998). Thus with such an array of factors affecting corals, the etiology of diseases needs to be studied in detail to evolve better management plans for preservation of coral reefs.

Several fungi and a cyanobacterium *Phormidium valderianum* were isolated from PLS-affected corals (Ravindran et al. 2001). Subsequent experiments suggested that the cyanobacterium was the etiological agent in the PLS (Ravindran and Raghukumar 2002). We investigated the extent of PLS spread in *P. lutea* in the coral lagoon at Kavaratti island in the Lakshadweep Archipelago.

Besides examining the biochemical and physiological changes in the PLS-affected colonies our objectives were to investigate whether the syndrome is in response to abiotic or biotic or a combination of these two factors. We designed experiments to observe the response of *P. lutea* to 1) inoculation with fungi and the cyanobacterium *P. valderianum* that were frequently isolated from PLS-affected colonies ; 2) elevated level of pCO<sub>2</sub> around the healthy coral polyps; 3) enhanced bacterial heterotrophic activity around healthy polyps and 4) inhibition of cyanobacterial photosynthesis.

## **2. Materials and methods**

### **2.1. Survey of PLS affected coral**

Two sampling locations in the Kavaratti Island of Lakshadweep in the Arabian Sea, one each from the north and south of the lagoon respectively, was chosen (Fig. 1). The survey was conducted twice each year between 1996 and 1999 for monitoring symptoms and extent of partial mortality of the coral *P. lutea* caused by the PLS. The number of *P. lutea* colonies affected by the PLS was surveyed following the belt transect method (English et al. 1997). After laying 2 m wide belt for 50 meters, healthy and PLS-affected *Porites lutea* colonies were counted in

the belt. Four to eight such transects were counted in each station during the survey period.

## 2.2. Microscopic examination of the PLS-affected colonies

PLS-affected and healthy colonies were fixed in 3% formalin solution for 24 h followed by fixation in 10% formalin solution for 48 h. The specimens were rinsed with tap water three times and finally with 70% ethanol (Ravindran et al. 2001). These samples were preserved in 70% ethanol till further use. A few sub-samples from the primary samples were decalcified using the decalcifying solution (1:1 ratio of 20% citric acid and 50% formic acid). The decalcified, as well as whole samples were observed under an epifluorescence microscope (OLYMPUS BX-60, Tokyo, Japan) and stereo zoom microscope (OLYMPUS SZH, Tokyo, Japan) to compare the structural deviations of PLS-affected tissue from the healthy one. The decalcified tissues and the whole specimens were washed in distilled water and dehydrated in a graded acetone series. The tissues were dried critically using liquid CO<sub>2</sub> in a critical point freeze drying apparatus (Structure Probe Inc., West Chester, PA, USA). The specimens were then sputter coated with gold-palladium and examined under scanning electron microscope (JEOL, 5800 LV, Japan) to assess the abnormalities in the PLS-affected colonies in comparison to the healthy ones.

## 2.3. Gel electrophoresis

About 2 cm<sup>2</sup> pieces of frozen PLS-affected and healthy specimens were homogenized using pre-chilled mortar and pestle in a mixture of phosphate buffered saline (8.0 g NaCl, 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub> and 1.15 g Na<sub>2</sub>HPO<sub>4</sub> l<sup>-1</sup> of

distilled water, pH 7.0) with protease inhibitor cocktail containing 4-(2-aminoethyl)benzene sulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin and sodium EDTA, (1ml inhibits protease equivalent to 1 mg of USP pancreatin) and trypsin inhibitor Type I-S from Soybean (both from Sigma Chemicals, USA). The resulting mixture was centrifuged at 10,000 rpm for 10 min at 5° C. Protein content was estimated in the supernatant following the method of Lowry et al. (1951). Equal concentrations of protein from healthy and PLS-affected tissue were used for running SDS-PAGE following Laemmli's method (1970). The gels were stained with 0.3% coomasie brilliant blue R-250 in methanol: acetic acid: water at a ratio of 3:1:6 (Bythell et al. 1995).

## 2.4. Status of Zooxanthellae

### *2.4.1. Zooxanthellae numbers and mitotic index*

Sub-samples from preserved healthy and PLS-affected specimens were decalcified and homogenised in a pestle mortar with 1 ml of sterile seawater. The homogenates were centrifuged to pellet the zooxanthellae leaving the animal tissue in suspension. The pellets were resuspended in fresh sterile seawater and filtered through 60 µm mesh plankton net to remove the animal debris. Total number of zooxanthellae and dividing cells among them were counted using a Neubauer haemocytometer. Mitotic index is the % of zooxanthellae cells in doublets (McAuley and Cook 1994). Cell size of the zooxanthellae was measured under a microscope, using a calibrated ocular micrometer.

#### 2.4.2. Pigments

Pigment concentrations in the frozen healthy and PLS-affected specimens were estimated following Myers et al. (1999). The tissues from the pink line region were scraped and placed in pre-weighed tubes, containing pieces of GF/C filter paper to break the cells effectively during homogenisation. The tissues were crushed in acetone using a glass rod inside the tube and left overnight at 4° C in the dark for extraction. These contents were centrifuged at 2000 rpm for 10 minutes. Chl a, and chl c and carotenoid concentrations were calculated according to the equations of Parsons et al. (1984).

#### 2.4.3. Inducing PLS using biotic factors

The fungi *Aspergillus niger*, *Curvularia lunata* and a hyaline non-sporulating fungus isolated most frequently from the PLS-affected colonies (Ravindran et al. 2001) and the cyanobacterium *P. valderianum* found in close association with the PLS-affected colonies (Ravindran and Raghukumar 2002) were inoculated in the healthy colonies and maintained as described below. Healthy-looking colonies of *P. lutea* varying in size from 8-10 cm in diameter were maintained in the 20 litre acrylic aquarium tanks with freshly pumped unfiltered seawater from the ambient source with continuous flow. Using a sterile drill, holes of 5 mm in diameter and 4 mm deep were made in the middle of all the colonies to house the inoculum. After these physical disturbances, the colonies were left for two days to acclimatize under the simulated conditions before inoculating the fungi and the cyanobacterium. The tanks were cleaned everyday to remove the macroalgal and other particle deposits. A loop full of the selected fungi as mentioned earlier

and cyanobacterium *P. valderianum* were placed in the cavities in different colonies. The control colonies did not receive any inoculum. The coral colonies were maintained in triplicates with each of these inocula for 20 days with aeration, continuous sea water supply and 12:12 h of light and dark regime.

#### 2.4.4. Inducing PLS using abiotic factors

##### 2.4.4.1. Elevating pCO<sub>2</sub> around the healthy coral polyps

Carbon concentrating mechanism (CCM) reported in various photosynthetic organisms like cyanobacteria and diatoms (Tchernov et al. 1997) are responsible for elevated pCO<sub>2</sub>. In order to test whether a similar situation is created around coral polyps where the cyanobacterium *P. valderianum* was noticed in PLS-affected corals, we simulated such a microenvironment by passing CO<sub>2</sub> gas in the coral cavities. CO<sub>2</sub> gas was generated by treating molluscan shells with 5% HCl. Molluscan shells after washing with fresh water were put in a 500 ml conical flask containing 5% HCl. The flask was closed using a rubber cork with a vent. Using a thin intravenous tube, CO<sub>2</sub> generated in the flask was passed on the healthy colonies through the base of a pipette tip (Fig. 2). Three such CO<sub>2</sub> generating set-ups were made to pass CO<sub>2</sub> on three coral colonies. Visible bubbling in the tanks was maintained to ensure that there was a continuous flow of CO<sub>2</sub> in the experimental colonies. An aerator passing air on a healthy colony in a similar set-up was maintained as a control.

##### 2.4.4.2. Enhancing bacterial heterotrophic activity around the healthy polyps

The stress caused by the cyanobacterial bio-film around coral polyps may induce excess mucus secretion thereby causing elevated bacterial heterotrophic

activity. By placing 5 mm plugs of sterile nutrient agar (HI-MEDIA, Bombay) in cavities drilled in the experimental coral colonies we expected to increase the bacterial colonization, which in turn would increase the heterotrophic activity around the nutrient agar plugs. Colonies with out any inoculum in the cavity as described earlier remained as a control.

#### 2.4.4.3. Effect of inhibiting cyanobacterial photosynthesis

The proximity of the cyanobacterial biofilm to coral polyp might trigger competition for the dissolved oxygen between the two, causing hypoxia during the night and oxidative stress during the day due to the evolution of reactive nascent oxygen by the cyanobacterial photosynthesis. Therefore, in order to stop the photosynthetic processes in the cyanobacterial cells, the photosynthetic inhibitor, 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU) was used as described by Marshall (1996). An axenic culture of the cyanobacterium, *P. valderianum* in its exponential growth was treated with  $10^{-5}$ M of DCMU and placed in the inoculum cavity of the experimental coral sample. The experiment using the cyanobacterium, as a biotic factor to induce PLS was the control.

### **3. Results**

#### 3.1. Survey of PLS-affected corals

The incidence of the PLS in the northern and southern stations increased from 20% in 1996 to 80% in 1999 (Fig. 3). The incidence of PLS remained higher during survey in southern than the northern part of the lagoon.

### 3.2. Microscopic examination of the PLS-affected colonies

A cyanobacterial mat consisting, apparently of one species, covered the dead patches in the PLS-affected colonies. Observations through the stereo zoom microscope showed that the wall of the corallite in PLS-affected region was thin and fragile (Fig. 4). Decalcified tissue of the PLS-affected specimen showed that the cyanobacterial filaments were in close association with the host tissue (Fig. 5, sorry for the poor quality of the picture). Microscopic observations of the PLS-affected polyps did not reveal any distinct heterotrophic bacterial associations. Fungi but not bacteria or cyanobacteria were constantly associated with the polyps in the healthy specimens. The dissolution of the septa changed the architecture of the corallites in PLS-affected corals. The coenosarcial tissue was degenerated in the PLS-affected colony, exposing the skeleton intermittently. The decalcified PLS-affected specimens showed that the polyp tissue and not the skeleton had turned pink (Fig. 6). The PLS-affected and not the healthy tissue when excited by blue light (400-440 nm excitation with 475 nm barrier filter) under an epifluorescence microscope showed bluish green fluorescence (Figs. 7, 8). Scanning electron microscopic (SEM) observation of the PLS-affected tissue showed expelled zooxanthellae, on the surface of the PLS-affected tissue (Fig. 9).

### 3.3. SDS-PAGE of proteins

The difference in total protein concentrations between healthy and PLS-affected colonies was not significant. However, the healthy specimens showed fewer protein bands than the PLS-affected specimens in SDS-PAGE (Figs. 10, 11).

The protein bands in the healthy specimens are approximately in the range of 3, 14, 20 and 43 kDa. The PLS-affected colonies expressed all of the above proteins in addition to a 29 kDa protein. The 14 kDa band in PLS-affected specimen appears to be more concentrated than in the healthy specimens.

#### 3.4. Status of zooxanthellae

There was a significant variation in the mitotic index (MI) of zooxanthellae, their density and size in whole polyp preparations between healthy and PLS-affected corals (Table 1). The number of zooxanthellae in the PLS-affected specimens was reduced in spite of having higher MI. This could be the result of zooxanthellae expulsion from the PLS-affected tissue (Fig. 9). Chl a concentration was significantly higher in the PLS-affected colony than in the healthy. Concentrations of chl c and carotenoid did not vary significantly in the two conditions (Table 1).

#### 3.5. Induction of PLS using biotic and abiotic factors

The results of PLS-induction using biotic and abiotic factors are summarised in Table 2. There were no signs of fungal inoculum spreading across the healthy colonies. They remained in the inoculum cavity till the end of the experiment and did not cause any visible response on the coral colonies. However, the cyanobacterial inoculum in the healthy colonies spread at an average rate of 4-6 mm day<sup>-1</sup>. By third day, all the colonies turned pink around the cyanobacterial inoculum (Fig. 12). Within a week, the entire colony turned pink. At the end of the experiment (after 2 weeks) the entire colony was covered with the cyanobacterium.

After two weeks, the polyps adjacent to the elevated CO<sub>2</sub> microenvironment showed pink color in all the three experimental coral colonies. The width of the pink line was about 1 mm. The pink tissue resembled morphologically to the pink colored tissue in PLS-affected specimens found in the lagoon (Fig. 13). However, no skeletal abnormality was observed, as the development of pink color was observed to be limited to 1 mm width for the duration of the experiment (2 wks after CO<sub>2</sub> purging). The status of zooxanthellae in the PLS-induced colonies could not be determined due to lack of enough material. Besides pink line we also noticed pink lesions on *P.lutea* in natural samples (Fig. 14).

Nutrient agar plugs showed bacterial colonization and thus it was assumed to have increased the heterotrophic activity around the plugs. Mucus sheets with bacterial colonies were formed around the inoculum cavity but no pink coloration of the tissue was noticed. The non-photoactive cyanobacteria treated with DCMU did not spread across the healthy colony. There was no other response observed from the healthy colonies to the presence of the cyanobacterial inoculum treated with DCMU.

#### **4. Discussion**

The pink line-syndrome discussed here seems to be caused by the cyanobacterium *P. valderianum*, as borne out by the observations that it was always present in PLS-affected colonies of *P. lutea* and inoculation with the cyanobacterium but not with the fungi caused the PLS (Ravindran and Raghukumar 2002). The most well-known cyanobacterial disease of corals is the

black band disease (Antonius 1981). Cyanobacterial pathogen in BBD is believed to be promoted by excess nutrients, elevated temperatures and pollution (Rützler et al. 1983; Edmunds 1991). We believe that the PLS is also related to such factors. The incidence of PLS was higher in summer months (April 1997, 1998 and 1999) when the seawater temperature reaches its maximum. PLS was higher in the southern part of the lagoon in Kavaratti (Fig. 2) which is shallower than the northern part of the lagoon. Additionally, the faster tidal currents in the northern part of the lagoon (Chandramohan et al. 1996) where it opens through a channel into the sea may flush out the resident pathogen from the lagoon and lower its chances of settling on coral colonies more frequently than the southern part of the lagoon which is more stagnant.

The PLS-affected colonies expressed a protein of 29 kDa in addition to the high expression of 14 kDa band. This indicates that the cells in the PLS-affected colonies responded to the diseased conditions. Insignificant changes in protein concentration and difference in the protein expression between the PLS-affected and healthy specimens indicate that the cells undergo degenerative processes without synthesizing new protein. The protein content of the PLS-affected specimens are the same as in healthy one and therefore the profile changes may be due to the breakage of a large protein by proteases as described in plants by Vierstra (1993) when the cell is under nitrogen starvation. Vierstra (1993) proposed that an increase in the protease in plant cell might be related to recovery of nitrogen from the non-essential proteins. Alternatively, the expression

of 29 kDa in PLS-affected specimen may be a small heat shock protein (Hsp) that is produced in cells in response to stress (Ussel et al. 1999).

The MI could have been higher in the PLS-affected polyps than what was observed but this was probably not detected due to the heavy loss of the zooxanthellae from the tissue through expulsion (Fig. 9). Increased chl a concentrations in the PLS-affected polyps might have been due to the enhanced doubling of the zooxanthellae. However, the observed density of zooxanthellae was not high and we assume that this might be due to the preferred expulsion of the doubling zooxanthellae from the host tissue. Suharsono and Brown (1992) noticed similar preferred expulsion of dividing zooxanthellae in *Anemonia viiridis* under temperature stress. They further suggest that once zooxanthellae are released into the coelenteron, they divide, unrestricted by host influence. Increased size of the zooxanthellae in the PLS-affected tissue might have contributed to increased chl a concentrations to a limited extent (Table 1). Since the whole live colony of polyps was extracted for chlorophyll, the expelled zooxanthellae in the coelenteron might have been the major contributor to the increased chlorophyll a content.

The major symptoms that are associated with PLS are increase in zooxanthellae size, MI values and chl a concentration, decrease in zooxanthellae density accompanied by skeletal erosion, pink line formation and partial mortality. By considering these observed parameters and establishment of a successful induction of PLS using the cyanobacterium and gaseous CO<sub>2</sub> lead us to hypothesize that the development of the PLS might be related to one single

mechanism namely the cyanobacterial carbon concentrating mechanism (CCM). We hypothesize that the processes described below could destabilise the symbiosis in *P. lutea* and lead to pink-line syndrome.

Many aquatic photosynthetic microorganisms including cyanobacteria possess an inorganic carbon concentrating mechanism that raises the CO<sub>2</sub> concentration at the intracellular carboxylation sites (Badger et al. 2002) thus compensating for the relatively low affinity of the carboxylating enzymes for its substrates. The energy dependent CCM in cyanobacteria, accumulates carbon largely in the form of HCO<sub>3</sub><sup>-</sup> in the cytoplasm and CO<sub>2</sub> is generated by carbonic anhydrase (CA) which is in close proximity to the carboxylation sites (Tchernov et al. 1997). In the cyanobacteria *Synechococcus* sp., *Nannochloropsis* sp and in the diatom *Thalassiosira weissflogii*, CO<sub>2</sub> efflux was reported during the photosynthesis (Tchernov et al. 1997) and this was generalized to be common in all the cyanobacterial cells (Badger et al. 2002). The occurrence of a similar efflux in the cyanobacterium *Phormidium valderianum* associated with PLS-affected colonies is assumed to be disturbing the physiology of the polyps. A lowering of the pH will then be created during the efflux of the CO<sub>2</sub>, and this, in turn, will dissolve the skeleton of the coral as observed in the PLS-affected corals. The elevation of the pCO<sub>2</sub> due to the CCM of the cyanobacteria will enhance the availability of the inorganic carbon (C<sub>i</sub>) to the zooxanthellae as it was shown by Goiran et al. (1996) that the zooxanthellae isolated from the coral have the capacity to actively take up the CO<sub>2</sub>. This will prompt the carbon limited endosymbiotic zooxanthellae (Weis 1993) to divide and increase in numbers.

The subsequent increase in photosynthesis by the increased number of symbionts possibly causes oxidative stress to the host cell. The host is induced to produce defense enzyme such as superoxide dismutase, catalase and peroxidase (Lesser et al. 1990). This, in turn, may drain the host energy pool as the host cell may not receive photosynthate from the highly dividing cells of zooxanthellae because this is used up for their biomass build-up through faster division. The stress experienced by the host in the above-mentioned way will cause many cellular reactions that may lead to the pink colour formation and finally the tissue death. The fact that CO<sub>2</sub> also produces the PLS, indicates a correlation between the pCO<sub>2</sub> elevation around the polyps and the formation of the pink line. Muscatine et al. (1998) observed that the environmentally induced zooxanthellae population reduced the calcification in corals. The reduction in calcification was observed in PLS-affected specimens.

Absence of any response by the DCMU-treated, non-photoactive cyanobacterium in the healthy colonies indicates that in absence of the cyanobacterial photosynthesis, there is no CCM-related destabilisation of symbiosis with the host as explained earlier.

We conclude that PLS type of disease need not have to be caused by specific pathogens. Any opportunistic cyanobacteria or other agents having potential to interfere with the host or symbiont's physiology can cause physiological disorders. The possible increase of this kind of mortality in corals could be due to eutrophication in the coral reefs. As bleaching related mass

mortality of corals create more substratum for the algal succession, prevention of eutrophication will control the algal biomass build-up in the coral reefs,

Future of the corals in response to the global climate change needs to be understood thoroughly as the possibility of PLS-related mortality in corals is likely. Atmospheric carbon dioxide is expected to reach double of the pre-industrial levels by the year 2065 (Houghton et al. 1996) that will increase the  $p\text{CO}_2$  in seawater. Acid formation during the dissolution of  $\text{CO}_2$  lowers the pH of seawater. It is predicted that the seawater pH will decrease by 0.25 unit by 2100 (Gattuso et al. 1999). Increase in the  $p\text{CO}_2$  and acidic pH in seawater due to increasing atmospheric  $\text{CO}_2$  affects corals. Lowering of pH as low as 0.1 unit reduces phosphofructokinase activity, a crucial enzyme in the glycolytic pathway by 10-20 fold (Trivedi and Danforth 1966). Host supplies  $\text{C}_i$  to the symbionts in the form of  $\text{CO}_2$  by dehydrating bicarbonate. Increased supply of  $\text{CO}_2$  to the symbionts by direct diffusion from ambient waters besides the supply from the host will simulate the condition that is prevailing in PLS specimens.

Natural stressors like UV radiation, temperature anomalies and bacterial infections are known to uncouple the algal-host symbiosis resulting in whitening of colonies (Brown 1997; Hayes and Goreau 1998; Kushmaro et al. 1996). However these factors do not elicit pink coloration and therefore PLS appears to be specific in response to  $p\text{CO}_2$ . The presence of pink lesions in natural samples (Fig. 14) where no biotic factors were found to be associated further strengthens our hypothesis regarding association of  $p\text{CO}_2$  with pink coloration.

## **5. Conclusion**

The sequence of most possible events that is proposed to lead to the formation of the pink line in the coral is as follows. 1) The cyanobacterium *P. valderianum* settles on the coral colony; 2) higher CO<sub>2</sub> concentration adjacent to the polyps created possibly through the cyanobacterial CCM becomes the source of C<sub>i</sub> required for the carbon limited zooxanthellae in the host cell; 3) zooxanthellae grow and proliferate utilising photosynthate they generated; 4) host loses its share of photosynthate from the zooxanthellae as this is diverted for the division of zooxanthellae; 5) calcification and growth of the host polyps are retarded. 6) the acidic environment created by the release of CO<sub>2</sub> may cause mortality of coral polyps and skeletal erosion and 7) the synergistic effects of CO<sub>2</sub> influx and acidic environment around the polyps might induce the pink coloration in the host.

## **Acknowledgements**

The first author thanks Council for Scientific and Industrial Research (CSIR) for award of senior research fellowship. The second author acknowledges Department of Ocean Development and Ministry of Environment and Forests, New Delhi for financial grants. We acknowledge Dr. Ismail Koya, Deputy Director, Department of Science and Technology, Union Territory of the Lakshadweep for logistic support in the island. We thank Dr. S. Raghukumar and Dr. M. V. M. Wafar for critical review of the manuscript. We also thank anonymous referees for the useful comments for the improvement of this manuscript.

## References

- Antonius A (1981) Coral reef pathology: a review. Proceedings of the fourth international coral reef symposium. 2:3-6
- Badger MR, Hanson D, Price GD (2002) Evolution and diversity of CO<sub>2</sub> concentration mechanism in cyanobacteria. *Funct Plant Biol* 29:161-173
- Brown BE (1997) Coral bleaching: causes and consequences. *Coral Reefs* 16: 129-138
- Bythell JC, Sharp VA, Miller D, Brown BE (1995) A novel environmentally-regulated 33 kDa protein from tropical and temperate cnidarian zooxanthellae. *J. Therm. Biol.* 20: 15-22
- Chandramohan P, Sanil Kumar V, Jaya Kumar S (1996) Dredge spoil disposal off Kavaratti island, Lakshadweep. *Indian. J Mar Sci* 25:67-70
- Edmunds PJ (1991) Extent and effect of Black Band Disease on a Caribbean reef. *Coral Reefs* 10:161-165
- English S, Wilkinson C, Baker C (1997) Survey manual for tropical Marine resources. Australian Institute of Marine Science, Townsville
- Gattuso JP, Allemand D, Frankignoule M (1999) Photosynthesis and calcification at cellular, organismal and community levels in coral reefs: A review on interaction and control by carbonate chemistry. *Amer Zool* 39: 60-183
- Gladfelter WB (1982) White band disease in *Acropora palmata*: implications for the structure and growth of shallow reefs. *Bull Mar Sci* 32:639-643
- Goiran C, Al-Moghrabi S, Allemand D, Jaubert J (1996) Inorganic carbon uptake for photosynthesis by the symbiotic coral/dinoflagellate association I.

- Photosynthetic performance of symbionts and dependence on sea water bicarbonate. *J Exp Mar Biol Ecol* 199:207-225
- Hayes RL, Goreau NI (1998) The significance of emerging diseases in the tropical coral reef ecosystem. *Rev Biol Trop* 46:173-186
- Houghton JT, Meira-Filho LG, Callander BA, Harris N, Kattenberg A, Maskell K (eds.) (1996) *Climate change 1995 - the science of climate change*, Cambridge University Press, Cambridge –UK
- Kushmaro A, Loya Y, Fine M, Rosenberg E (1996) Bacterial infection and coral bleaching. *Nature* 380: 396
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680
- Lesser MP, Stochaj WR, Tapley DW, Shick JM (1990) Bleaching in coral reef anthozoans: Effects of irradiance, ultraviolet radiation and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs* 8: 225-232
- Lowry OH, Rosebrough NJ, Farr AL, Randell RH (1951) Protein measurement with the Folin-phenol reagent. *J Biol Chem* 193:265-275
- Marshall AT (1996) Calcification in the hermatypic and ahermatypic corals. *Science* 271:637-639
- McAuley PJ, Cook CB (1994) Effects of host feeding and dissolved ammonium on cell division and nitrogen status of zooxanthellae in the hydroid *Myrionema amboinense*. *Mar Biol* 121:343-348

- Muscatine L, Ferrier-Pages C, Blackburn A, Gates RD, Baghdasarian G, Allemand D (1998) Cell specific density of symbiotic dinoflagellates in tropical anthozoans. *Coral Reefs* 17: 329-337
- Myers MR, Hardy JT, Mazel CH, Dustas P (1999) Optical spectra and pigmentation of Caribbean reef corals and microalgae. *Coral Reefs* 18: 179-186
- Nugues MM (2002) Impact of a coral disease outbreak on coral communities in St. Lucia: What and how much has been lost?. *Mar Ecol Prog Ser* 229: 61-71
- Parsons TR, Maita Y, Lalli CM (1984) Determination of Chlorophylls and total carotenoids: Spectrophotometric method. *In* A manual of chemical and biological methods for seawater analysis. 1st ed. 101-104.
- Ravindran J, Raghukumar C, Raghukumar S (2001) Fungi in *Porites lutea*: association with healthy and diseased corals. *Dis Aquat Organ* 47:219-228
- Ravindran J, Raghukumar C (2002) Pink line syndrome (PLS) in the scleractinian coral *Porites lutea*. *Coral Reefs* 21: 252.
- Richardson LL (1998) Coral diseases: What is really known? *Trends Ecol Evol* 13: 438-443
- Richardson LL, Smith GW, Ritchie KB, Carlton RG (2001) Integrating microbiological, microsensor, molecular, and physiologic techniques in the study of coral disease pathogenesis. *Hydrobiologia* 460: 71-89

- Rützler K, Santavy DL, Antonius A (1983) The black band disease of Atlantic reef corals, III. Distribution, ecology and development. *PSZNI Mar Ecol* 4:329-358
- Suharsono S, Brown BE (1992) Comparative measurement of mitotic index in zooxanthellae from a symbiotic cnidarian subject to temperature increase. *J Exp Mar Biol Ecol* 158:179-188
- Sweatman HK, Osborne Smith L, Grubba T, Kinch J, Jones G, Rai V (2002) Status of coral reefs of the world, , p. 163-180. In Wilkerson C (ed), Status of coral reefs of Australia and Papua New Guinea. Australia AIMS Townsville.
- Tchernov D, Hassidim M, Luz Boaz, Sukenik A, Reinhold L, Kaplan A (1997) Sustained net CO<sub>2</sub> evolution during photosynthesis by marine microorganism. *Curr Biol* 7:723-728
- Trivedi B, Danforth WH (1966) Effect of pH on the kinetics of frog muscle phosphofructokinase. *J Biol Chem* 241: 4110-4112
- Ussel P, Van den D, Norman G, Quinlan RA (1999) Molecular chaperones: Small heat shock proteins in the limelight. *Curr Biol* 9:103-105
- Vierstra RD (1993) Protein degradation in plants. *Ann Rev Plant Physiol Plant Mol Biol* 44:385-410
- Weis VM (1993) Effect of dissolved inorganic carbon concentration on the photosynthesis of the symbiotic sea anemone *Aiptasia pulchella* Carlgren: role of carbonic anhydrase. *J Exp Mar Biol Ecol* 174:209-225

Table 1. Zooxanthellae status in healthy and PLS-affected *P. lutea* and single factor analysis of variance (ANOVA) for various parameters between the two.

Parameters	Healthy	PLS-affected	Significance (P)	df	F-value	F-critical
Zooxanthellae number cm <sup>2</sup> of the colony	2.7x10 <sup>6</sup> ± 1.4x10 <sup>6</sup>	0.7x10 <sup>6</sup> ± 0.6x10 <sup>6</sup>	2.2 X 10 <sup>-9</sup> ***	1, 58	50	12
Mitotic Index (MI)	3.93 ± 2.9	5.75 ± 2.2	0.04*	1, 58	4.6	4
Zooxanthellae size in whole tissue preparation	7.45 ± 0.76	8.75 ± 1.4	1.3 X 10 <sup>-7</sup> ***	1, 98	32.3	11.5
Chl <u>a</u> (µg cm <sup>-2</sup> )	13.8	54.8	6 X 10 <sup>-6</sup> ***	1, 18	39.9	15.4
Chl <u>c</u> (µg cm <sup>-2</sup> )	10.2	7.2	0.32	1, 18	1.04 <sup>NS</sup>	4.4
Carotenoids (µg cm <sup>-2</sup> )	0.009	0.011	0.72	1, 18	0.1 <sup>NS</sup>	8.3

Note: df= degree of freedom, Significant at \* = 0.05, \*\* = 0.01, \*\*\* = 0.001  
NS = non-significant.

**Table 2. Results of the experiments to induce PLS in *P. lutea* by different factors.**

<b>PLS Inducing factors</b>	<b>PLS induction</b>
Inoculation with fungi	-
Inoculation with the cyanobacterium <i>P. valderianum</i>	+
Elevation of pCO <sub>2</sub> around polyps	+
Elevation of heterotrophic activity	-
Effect of non-photoactive cyanobacteria	-

+ indicates induction of PLS; - indicates absence of PLS induction

## Figure Legends

Fig. 1. (A) Location of the Lakshadweep group of islands; (B) various islands in the Archipelago; (C) sampling location in the lagoon at Kavaratti showing the south (S) and north (N) stations.

Fig. 2. Experimental set up for CO<sub>2</sub> enrichment around coral polyps

Fig. 3. Percentage of PLS-affected specimens observed in the lagoon at Kavaratti island, Lakshadweep during the study period.

Fig. 4. Eroded corallite wall in *P. lutea* affected with PLS. N=normal; F=fragile

Fig. 5. Cyanobacterial filaments (arrow) in close association with PLS-affected coral tissue (sorry for the poor quality of the picture).

Fig. 6. Pink coloration of tissue from PLS-affected colony seen after decalcification

Fig. 7. Presence of fluorescence in PLS-affected tissue

Fig. 8. Absence of fluorescence in healthy tissue

Fig. 9. Expelled zooxanthellae in PLS-affected tissue

Fig. 10. Protein profile in healthy tissue of *P. lutea*

Fig. 11. Protein profile of PLS-affected tissue of *P. lutea*

Fig. 12. Effect of *P. valderianum* on the healthy colony of *P.lutea*.

Fig. 13. PLS-affected coral from natural field sample.

Fig. 14. *P. lutea* colony showing pink lesions where no associated biotic agent was found.

**Figures: 1-14**

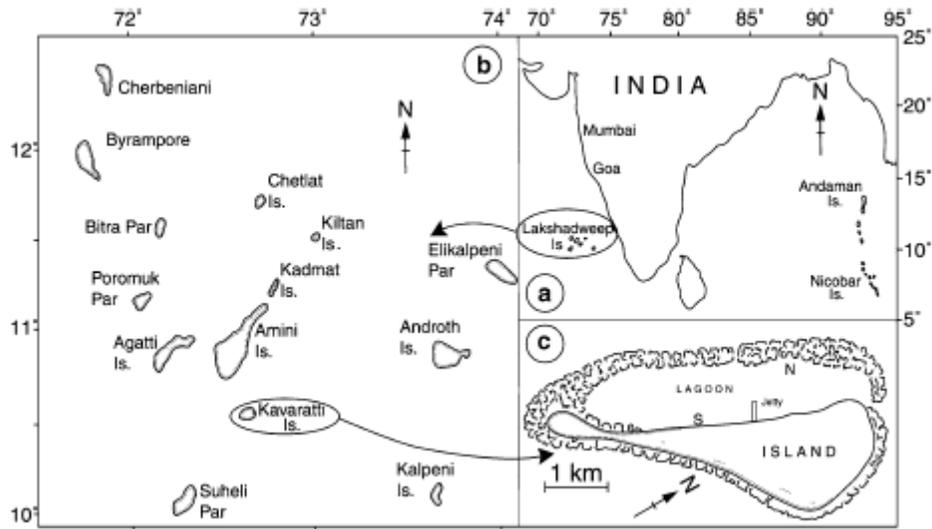


Fig. 1. (A) Location of the Lakshadweep group of islands; (B) various islands in the Archipelago; (C) sampling location in the lagoon at Kavaratti showing the south (S) and north (N) stations.

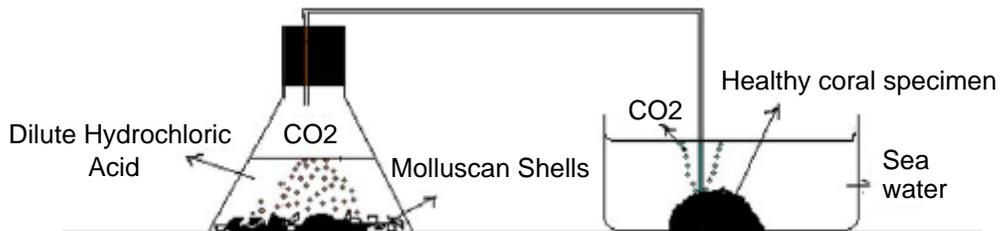


Fig. 2. Experimental set up for CO<sub>2</sub> enrichment around coral polyps

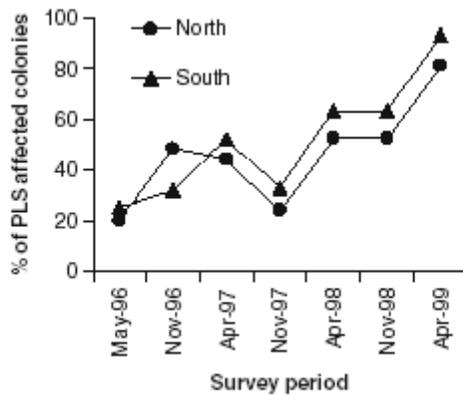


Fig. 3. Percentage of PLS-affected specimens observed in the lagoon at Kavaratti Island, Lakshadweep during the study period.

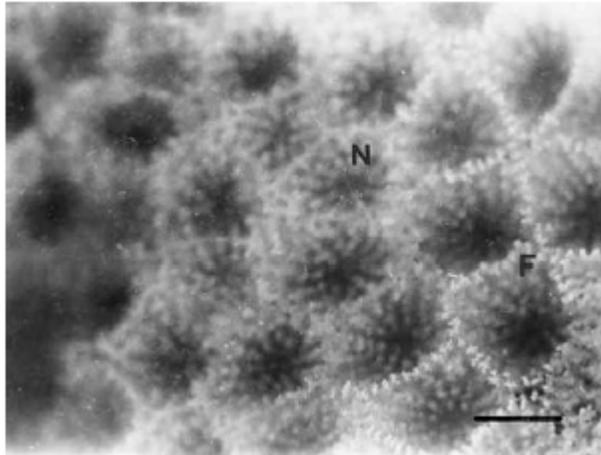


Fig. 4. Eroded corallite wall in *P. lutea* affected with PLS. N=normal; F=fragile

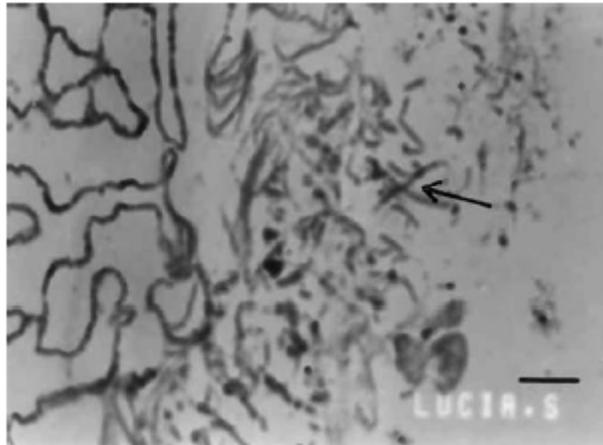


Fig. 5. Cyanobacterial filaments (arrow) in close association with PLS-affected coral tissue (sorry for the poor quality of the picture).



Fig. 6. Pink coloration of tissue from PLS-affected colony seen after decalcification

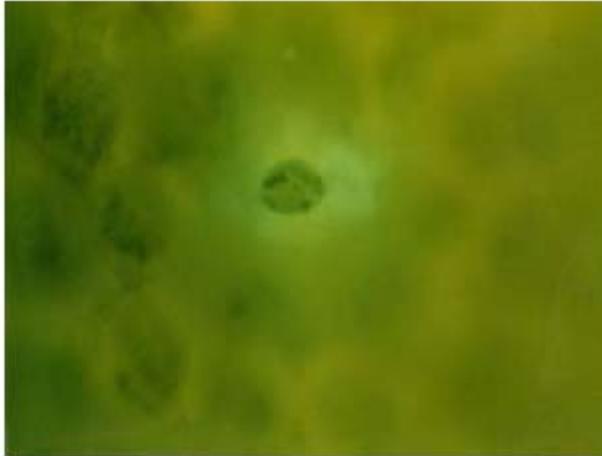


Fig. 7. Presence of fluorescence in PLS-affected tissue

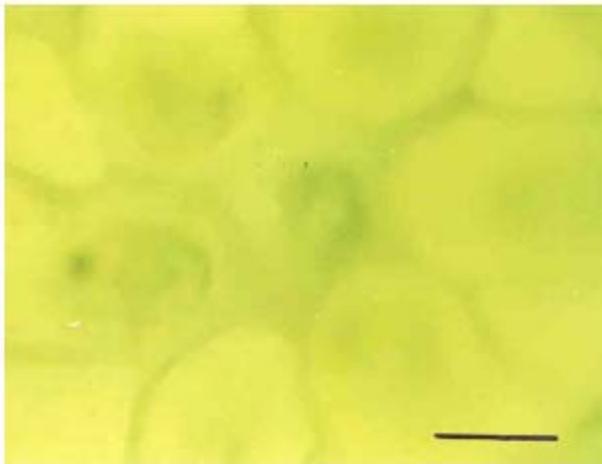


Fig. 8. Absence of fluorescence in healthy tissue

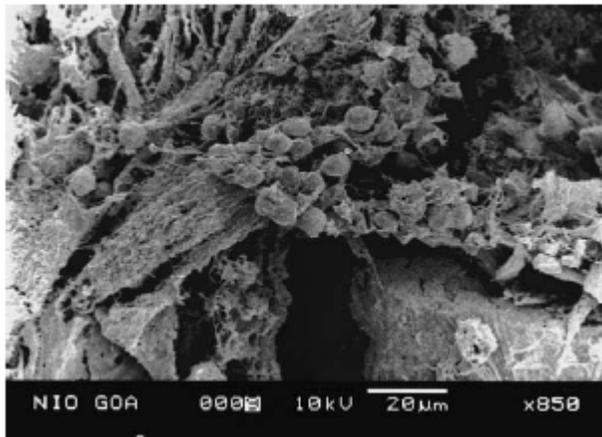


Fig. 9. Expelled zooxanthellae in PLS-affected tissue

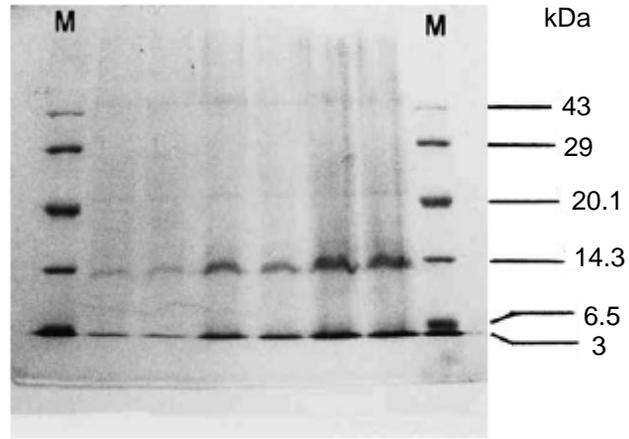


Fig. 10. Protein profile in healthy tissue of *P. lutea*

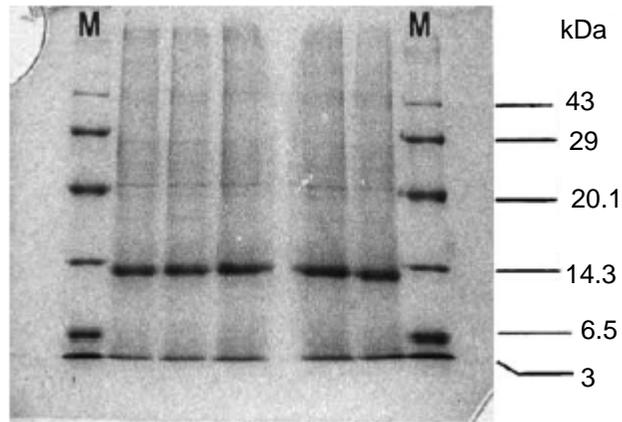


Fig. 11. Protein profile of PLS-affected tissue of *P. lutea*



Fig. 12. Effect of *P. valderianum* on the healthy colony of *P. lutea*.



Fig. 13. PLS-affected coral from natural field sample.

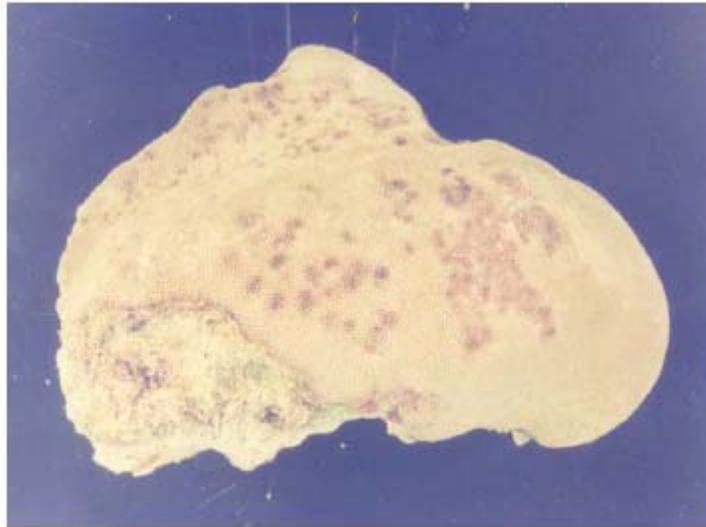


Fig. 14. *P. lutea* colony showing pink lesions where no associated biotic agent was found.