8. Induction of Pink-line syndrome in the healthy *P. lutea* colony:

8.1. Introduction:

To portray a disease symptom as a microbial disease, the pathogenicity of the associated microbe or all the microbial components associated with the diseased coral have to be demonstrated. To demonstrate this a common procedure postulated by Koch known as Koch's postulate is followed. Robert Koch in 1870s set procedures to demonstrate presumed disease pathogen as a pathogen unequivocally (Boyd and Hoerl 1981). This procedure is called Koch's postulate. As per this 1) The microorganisms must be documented as always being found associated with a particular disease. 2) The microorganisms must be isolated from the disease state and grown in pure culture under laboratory conditions. 3) The pure culture of the microorganism must produce the disease when inoculated into or onto a healthy animal. 4) The microorganism must be related from the newly diseased animal and identified as the same microorganism as the presumptive pathogen obtained from the diseased animal.

Without the invasion of the suspected microbe, the interaction of these two partners at the cell surface level through physical interaction or chemical interaction or both may influence one or the other's cell physiology. To understand this interaction, mostly chemical interaction, the knowledge of existing literature is essential. Influence by the cyanobacterial bio-film adjacent to the coral tissue on the coral polyps may be through

1. The cyanobacterial carbon concentrating mechanism (CCM) that may create external acidic environment and elevation of pCO₂
around the polyps as described in the various photosynthetic organisms like cyanobacteria to diatom (Tchemov et al. 1997)

2. The presence of cyanobacterial film close to the polyp tissues will create competition for the dissolved oxygen between the two, causing hypoxia during the nighttime and oxidative stress during daytime due to the evolution of reactive oxygen from the cyanobacterial film by the cyanobacterial photosynthesis.

3. Extracellular toxins from the cyanobacterial film.

4. Bacterial heterotrophy is known in the coral mucus. The stress caused by the adjacent cyanobacterial bio-film on the coral polyp may induce excess mucus secretion thereby causing elevated bacterial heterotrophic activity which in turn may create hypoxia to the coral polyps.

Other possible factors causing pink line may be due to pathogenic fungi associated with the diseased tissue and the cyanobacterial bio-film formation in the dead patch may be a secondary process.

Considering all the above factors, following experiments were carried out for inducing the pink line syndrome in healthy coral polyps.

a) Inoculating healthy corals with frequently isolated fungi in PLS specimens and the cyanobacterium, *Phormidium valderianum*

b) Elevating pCO₂ around the healthy coral polyps

c) Enhancing bacterial heterotrophic activity around the healthy polyps

d) Effect of cyanobacterial photosynthesis inhibition

e) pH effect on the healthy colonies
Fig. 8.1. A hole drilled in the healthy experimental colony for inserting the inoculum. Bar = 3 cm
8.2. Methods:

The following cultures, isolated from the PLS-affected specimens were inoculated in the healthy colonies kept in aquarium tanks at Kavaratti.

a) The cyanobacterium *Phormidium valderianum*

b) The fungus *Aspergillus niger*

c) A hyaline non-sporulating fungus

d) A dark non-sporulating fungus.

8. 2. 1. Inoculation of fungi and the cyanobacterium *P. valderianum*:

Healthy-looking colonies of *Porites lutea* varying in size from 8-10 cm in diameter were collected from the lagoon. The colonies were either individual colonies or they were subsampled from a massive colony with hammer and chisel. They were maintained in 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 (Fig. 8.1). The colonies were left for two days to acclimatise under the laboratory conditions before inoculation with fungi and cyanobacteria. The tanks were cleaned everyday to remove the macroalgal and other particle deposits. The most frequently isolated fungi, a dark non-sporulating form (DNS), later identified to be *Curvularia lunata* (Wakker) Boedijn, a hyaline non-sporulating form (HNS), *Aspergillus niger* and the cyanobacterium *Phormidium valderianum* were used for inoculation. The fungal inoculum was placed in the cavities made in the coral colonies. Similarly, a loop full of the cyanobacterial mat was inoculated in the cavity. The coral colonies were maintained in triplicates for each of this inoculum. The experimental set-up was maintained for 20 days.
8.2.2. Elevation of pCO₂ around the polyps: -

To generate CO₂, molluscan shells found in the beach were collected and washed briefly with fresh water and then 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, CO₂ generated in the flask was passed on the healthy colonies through the base of a pipette tip (Fig. 8.2). Three such CO₂ generating set-ups were made to pass CO₂ on three coral colonies. Similar set-up was used to pass air from a portable aerator to a healthy colony as a control.

8.2.3. Elevation of heterotrophic activity: -

Sucrose agar plate was prepared by adding 10 g sucrose and 1.5 g of plain agar in 100 ml of seawater and autoclaved. The sterile sucrose agar medium was poured in Petri plates to the height of 5-mm thickness. Once solidified, agar blocks were cast by scooping the agar with a pipette tip cut to have the inner diameter of 5 mm. The cast was pushed in the coral colony by a sterile glass rod. Similarly plain agar casts were prepared and introduced in the control specimen. After introducing the agar blocks, the experimental colonies were observed for 20 days.

8.2.4. Effect of non-photoactive cyanobacteria on healthy corals: -

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 10⁻⁵M of DCMU. The inoculum was placed in the inoculum housing in the experimental coral sample.
Fig. 8.2. Schematic diagram of the CO$_2$ generation and experimental design
8.2.5. Effect of acidic pH on the healthy corals: -

The cyanobacterial CCM that was discussed earlier could create acidic environment around the polyp where the cyanobacteria interact with them. To test its role in the formation of the pink coloration in the healthy specimens, the coral nubbins (nubbins = replicates of the same colony) were prepared by breaking the healthy colonies into small (~ 1-1.5 cm$^2$ each) pieces. Another set of nubbins were prepared for the control. About 25 pieces were stuck on plastic coated cardboard with quick fix. The preparation of the nubbins was completed within 10 minutes from the time the colony was exposed to prepare the nubbins. They were kept in a 2-litter container with seawater under illumination with the photoperiod of 8 h for acclimatization for two days. After two days, the container of one set of the nubbins was filled with seawater that was acidified using hydrochloric acid and adjusted to the pH 5. The acidified seawater was changed every six hours. The seawater of the control nubbins also was changed every six hours. Both the containers were aerated with a small aquarium pump.

8.3. Results: -

During the incubation period of 20 days, there were no signs of fungal inoculum spreading across the healthy colonies. They remained in the inoculum cavity till the end of the experiment. They did not cause any response from the host by their presence along with coral polyps. But the cyanobacterial inoculum in the healthy colonies started spreading at an average speed of 4-6 mm day$^{-1}$. By third day, all the colonies with the cyanobacterial inoculum turned pink around the cyanobacterial inoculum
Figs. 8.3 & 8.4. Response of *Porites lutea* colonies to inoculum with *Phormidium valderianum*
(Figs. 8.3 & 8.4). The cyanobacterial inoculum was visibly found spreading on the healthy colonies, which already turned pink. Within a week, the entire colony turned pink. At the end of the experiment, the entire colony was covered with the cyanobacterium, *Phormidium valderianum*.

Elevating pCO₂ around the coral polyps by passing CO₂ yielded some interesting result. After two weeks, the polyps adjacent to the CO₂ passage showed pink color in all the three experimental coral colonies. The thickness of the pink line was about 1 mm. The pink tissue was characteristically similar to the natural pink colored tissue in PLS-affected specimens. But other characters on skeletal abnormalities were not observed, as the development of pink color was limited within 1 mm width.

Agar plugs enriched with 10% sucrose increased heterotrophic activity. Mucus sheets were formed around the inoculum cavity and no other response was observed. The non-photoactive cyanobacteria treated with DCMU did not spread across the healthy colony. There was no other response observed from the healthy colonies by the presence of the cyanobacterial inoculum treated with DCMU.

Acidification of the seawater did not yield any symptom of PLS. The nubbins in the second day started becoming pale and at the end of the seventh day, all the nubbins became almost white. No mortality of the nubbins occurred in control and those treated with acidified seawater.
### Results of experiments to induce pink color in the coral *P. lutea* by different factors

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8.4. Discussion: -

The result proves that there is a correlation between the pCO₂ elevation around the polyps and the formation of the pink line. The negative results rules out the role of bacterial heterotrophic activity and the role of the fungi in the formation of the pink line in the PLS affected corals. The mechanism that may trigger the formation of the pink line due to the increased pCO₂ and the sequence of events that follows are described below.
The presence of the cyanobacteria adjacent to the coral polyp influences them by many ways. They can be mechanical or chemical interaction. The mechanical interaction will elicit mere physical changes in the coral skeleton as the borers do and it may eventually trigger the allorecognition as observed in the coral *Stylophora pistillata* (Frank et al. 1997). The chemical interaction between the cyanobacteria and the coral polyp will create cellular responses that could cause accumulation of the pink colored compound.

The possible mechanism of the formation of the pink coloration, skeletal erosion and the higher mitotic index in the PLS affected coral may be explained based on the cyanobacterial carbon concentrating mechanism. Many aquatic photosynthetic microorganisms including cyanobacteria possess an inorganic carbon concentrating mechanism that raises the CO$_2$ concentration at the intracellular carboxylation sites (Badger et al. 2002) thus compensating for the relatively low affinity of the caboxylating enzymes for its substrates. In cyanobacteria, the CCM involves the energy dependent influx of inorganic carbon, the accumulation if this carbon is largely in the form of HCO$_3^-$ in the cytoplasm and generation of CO$_2$ at carbonic anhydrase sites in close proximity to the carboxylation sites (Tchernov et al. 1997). In many cyanobacteria like *Syneccococcus* sp., *Nannochloropsis* sp and in the diatom *Thalassiosira weissflogii*, the CO$_2$ efflux were reported during the photosynthesis (Tchernov et al. 1997) and this was generalized for all the cyanobacterial cells by Badger et al. (2002). The occurrence of a similar efflux in the cyanobacterium *Phormidium valderianum* associated with PLS affected colonies will disturb the physiology of the polyps. A lowering of the
pH will then be created during the efflux of the CO₂, and this, in turn, will dissolve the skeleton of the coral as observed in the PLS affected corals. The elevation of the pCO₂ due to the CCM of the cyanobacteria will enhance the availability of the inorganic carbon (Cᵢ) for 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 CO₂. In this condition, the lower pH around the polyps causes the skeletal dissolution that may probably enhance the availability of the Cᵢ for the zooxanthellae in the PLS affected specimen further. The increased zooxanthellae density would reduce the calcification in the PLS-affected corals. Muscatine et al. (1998) observed that the environmentally induced zooxanthellae population reduced the calcification in corals. The reduction of the calcification by the host might lead to the accumulation of calcium in the gastrodermal cells.

In the case of acidification of the ambient water, the polyps did not show any symptoms of the PLS. The acidic ambient medium created around the polyps may not be sufficient to create the CO₂ gradient that could cross the two cell layers of the polyp to reach the zooxanthellae in the gastrodermal layer as envisaged by Goiran et al. (1996). It is the intimacy of the cyanobacterium *P. valderianum*, that could supply enough gradient to reach the zooxanthellae.

The increased availability of the carbon source thorough the interaction of the cyanobacterial filaments with the polyps makes the zooxanthellae to increase its photosynthesis. This shift in the increased photosynthesis, in addition to minimizing the translocated food to the host may also cause oxidative stress to the host cell. This oxidative stress will induce the host cell
to produce the defense enzymes like super oxide dismutase (SOD), catalase and peroxidase. It has been shown by Shick et al. (1995) that the oxidative stress to the host is reducing with increasing depth where photosynthesis reduces proportionately.

From the experiments to induce the PLS in the healthy colonies, the interaction of the cyanobacterial filaments are confirmed to be the reason for the formation of the PLS. The inhibition of the photosynthesis of the cyanobacterium *P. valderianum* in the Koch's postulate did not cause any change in the experimental coral specimen. This shows that the cyanobacterial CCM that increases the inorganic carbon availability to the mutualisitic zooxanthellae. This increase in the availability of the inorganic carbon triggers the carbon limitation thereby reducing the photosynthate to the host. This increased photosynthesis causes oxidative stress to the host cell. The host that produces defense enzyme such as SOD, catalase and peroxidase photosynthesize. This, in turn, drains the host energy pool as the host cell already is not receiving the photosynthate from the highly dividing cell of zooxanthellae. The stress experienced by the host in the above-mentioned way causes many cellular reactions that leads to the pink color formation and finally the tissue death.