Pathogens associated with diseased corals: A case study

Thesis submitted for the degree of
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in

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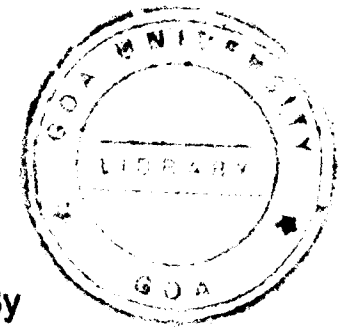
to the

GOA UNIVERSITY



By

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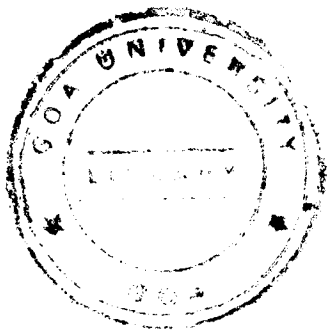
October 2002

STATEMENT

As required under the University ordinance 0.19.8(Vi), I state that the present thesis entitled "**Pathogens associated with diseased corals: a case study**", is my original contribution and the same has not been submitted on any previous occasion. To the best of my Knowledge, the present study is the first comprehensive work if its kind from the area mentioned

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.



J. Ravindran

CERTIFICATE

This is to certify that the thesis entitled **"Pathogens associated with diseased corals:a case study"**, submitted by Mr. J. Ravindran for the award of the degree of Doctor of Philosophy in Marine Sciences is based on his original studies carried out by him under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma in any universities or institutions.

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This is to certify that all the suggestions and corrections made by the viva-voce examination board have been incorporated in this thesis.

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Dedicated to
My Parents
&
Brothers

Table of Contents

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1. General introduction:

Coral reefs are renowned for their spectacular diversity and have significant aesthetic and commercial values, particularly in relation to tourism and fisheries. About half of the world's coastline is in the tropics and a third of them are coral reefs (Birkeland 1997). In terms of biodiversity, coral reefs are among earth's most diverse ecosystems and are widely recognized as the ocean's rain forest (Reaka-Kudla 1997). Their diversity is expected to be 3-5 times greater than previously recognised (Knowlton and Jackson 1994). Coral reefs are found in about 100 countries and sustain tens of millions of people in their daily protein requirement (Salvat 1992). The significance of the coral reefs fisheries is that they contribute nine million tones per year out of the total world fishery catch of 75-100 million tones (Smith 1978; Munro 1984). It has been estimated that the values of the coral reefs in the straits of Tiran and the Mollases reef off Florida Keys are \$ 1765 m^{-2} (Spurgeon 1992) and \$2833 m^{-2} (Mattson and DeFoor 1985) respectively, in terms of their potential as a source of novel and useful compounds to mankind. Any such estimation of the value of the coral reefs needs to include also the societal links of the ecosystem.

Coral reefs are being degraded on a global scale by a wide range of impacts, including mass bleaching events (Glynn 1993, 1996; Wilkinson 1998), diseases (Lessios et al. 1984; Aronson et al. 1998; Richardson 1998; Harvell et al. 1999), pollution (Pastorok & Bilyard 1985, Lapointe 1997) and several human activities (Munro 1983; Richmond 1993). Five major causes that alter the coral reefs are: 1) Storms and hurricanes, 2) Coral bleaching, 3) Diseases of reef organisms, 4) Outbreaks of coral predators like *Acanthaster*

plancii and 5) Mass mortality of the reef herbivores such as *Diadema antillarum* (Peters 1997). Natural chronic processes such as parrotfish grazing, disease and predation may result in greater cumulative mortality over a two years period than even a major hurricane impact (Bythell et al. 1993).

Disease is defined as any impairment (interruption, cessation, proliferation or other disorder) of vital body functions, systems or organs. Diseases are usually characterized either by: 1. an identifiable group of symptoms (observed anomalies indicative of disease) and/or 2. a recognizable etiologic or causal agent, and/or 3. consistent structural alterations such as developmental disorders, changes in cellular composition or morphology, and tumors (Peters 1997). Diseases can be divided into biotic and abiotic diseases. The parasitic relationship between a host and its symbionts eventually leads to the biotic disease of host. The biotic disease can be recognised when the presence of a pathogen results in a distinct deviation from normality as displayed by functional or structural deficit (Feingold 1988). When these pathogens are capable of causing or spreading disease in many individuals, they are called infectious agents. These infectious agents are either microparasites consisting bacteria, fungi, virus etc. or macroparasites like helminths and arthropods (Peters 1997). Exposure to abiotic factors beyond the tolerance of an organism can damage its body functions. This type of impairment is known as an abiotic disease (Peters and McCarty 1996). The abiotic environmental stress results in structural and functional body impairments. In any disease conditions, the biotic and abiotic factors are considered to be indistinguishable (Peters 1997).

"Diseases affect basic phenomena of life in oceans and coastal waters: for example, life span, life cycle, abundance, metabolic performance, nutritional requirements, growth, reproduction, competition, evolution, as well as organismic tolerances to natural and manmade environmental stress. In short, diseases are a major denominator of population dynamics" (Kinne 1980). Diseases can have effects that become manifested at the community level. Disease may either produce mortalities of individuals over varying periods of time (acute to chronic progressive mortalities) or alter the structure or function of an individual in such a manner so as to make it more susceptible to predation or environmental stress. The demise of even one species of coral reef may cause successional changes that alter the structure and function of a particular community and the reef ecosystem. There are several instances in which such successional changes have taken place. In coral reefs, for example, the mass mortality of the major herbivore, the *Diadema antillarum* together with hurricanes reduced the coral cover and the phase shift changed the coral dominated reef into algal dominated one (Done 1992). Replacement of *Acropora* sp. by *Agaricia* sp. in Belize (Aronson and Precht 1997) and by *Porites* sp. in Bahamas reefs (Greenstein et al. 1998) during mid 1980s to mid 1990s were not recorded in thousand year old geological cores. These results show that the diseases are recent phenomena (Harvell et al. 1999). Diseases of scleractinian corals is an important factor in changing the structure and function of coral-reef communities because loss of live tissue cover not only reduces the number of polyps producing new recruits, but also opens up new hard substratum space for settlement of sessile organisms (Peters 1997). As per the records, coral diseases have

been found in 54 different countries and 66% of the records are from Caribbean region that constitute only 8% of the world's total coral reef area (Green and Bruckner 2000). Coral diseases are a major threat to the coral reefs as the diseases are severe enough to eliminate a coral species from the ecosystem. *Acropora palmata* colonies were eliminated from the reef of the Virgin Island by the white band disease (WBD) (Gladfelter 1982). The black band disease (BBD) that spreads at an average rate of 3.5 cm day^{-1} kills corals that grow approximately 2 cm per year (Rützler et al. 1983). 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). Coral bleaching, an abiotic disease (Green and Bruckner 2000) in 1998 was the most geographically extensive and severe in recorded history (Wilkinson et al. 1999), causing significant mortality worldwide (Baird and Marshall 1998). The bleaching related mortality of some corals is likely to have been accelerated by opportunistic infections (Harvell et al. 1999). A bacterium has been believed to be an opportunistic infectious agent that could cause bleaching (Kushmaro et al. 1996). Tumours, neoplasia and hyperplasia have been recorded on corals throughout the world on several different species. 20% of the Caribbean coral colonies were reported to be having tumours (Morse et al. 1981)

Though disease related activity does not kill the entire colony, the exposure of the skeleton by the death of the polyps will increase the possibility of invasion by borers and grazers into the skeleton. Such bioeroders erode the $CaCO_3$ structures in a variety of ways and weaken the structure of the coral skeleton. The activity of these borers and grazers makes

the skeleton fragile and make them vulnerable to damages caused by simple mechanical forces (Glynn 1997). The *Acropora* corals collapsed because of the weakened skeletons that were caused by the intensified bioerosion after *Acanthaster* predation (Moran 1986; Birkland and Lucas 1990). Additionally, the tissue destruction may diminish the reproductive potential of coral colonies (Szmant-Froelich 1985) leading to diminished recruitment in already damaged reefs.

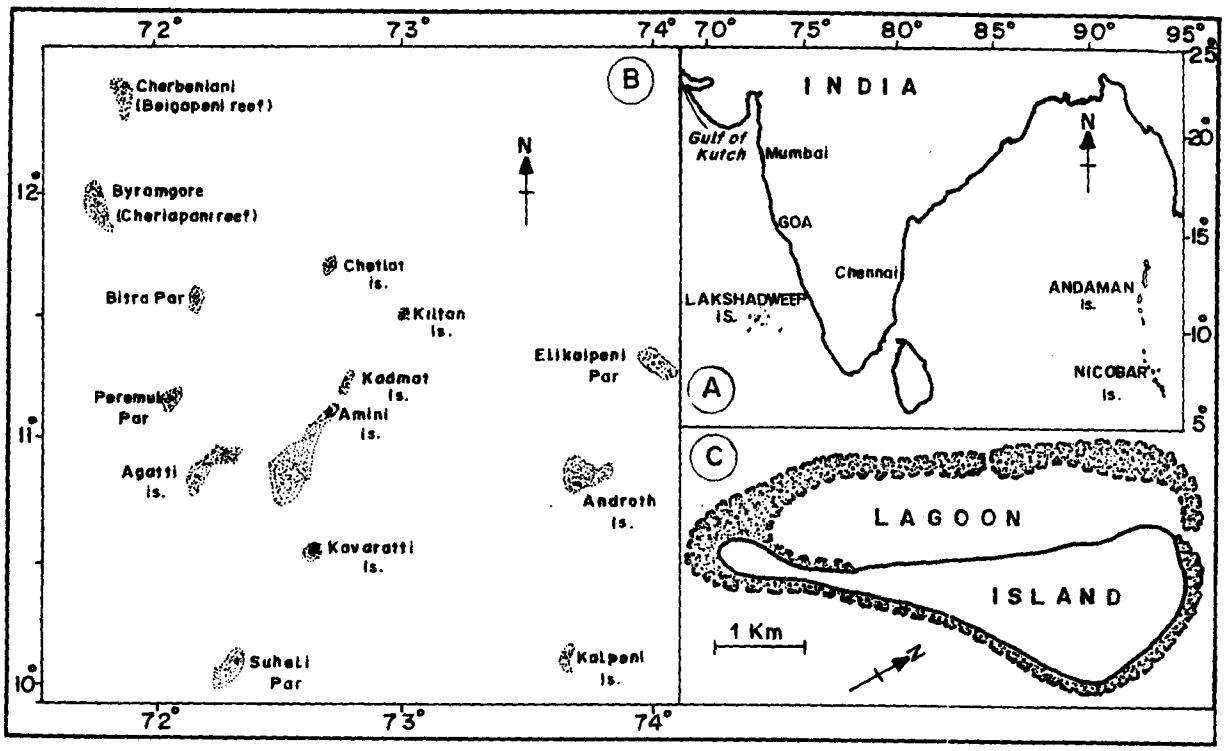
This alarming nature of diseases, if not prevented through proper management plan will significantly reduce the coral cover over a period of time. Undermining host resistance and facilitating pathogen transmission are the roles played by climate variability and human activity (Harvell et al. 1999). It is possible that the diseases or mortalities observed locally may actually signal a major epizootic in coastal habitats or even regions (Peters 1997). Therefore, understanding the epizootiology is very essential to formulate a management plan to deal with coral diseases.

Coral reefs are of three types. These are the fringing reefs, barrier reefs, and atolls (Guilcher 1988). Fringing reefs are those that grow along the shore. The barrier reefs are those similar to the fringing reef that grow parallel to the shore but physically isolated from the shore. Atolls were originally fringing or barrier reefs of islands. However, as the islands submerged, the corals kept growing towards the water surface, finally appearing in the shape of a ring enclosing a lagoon within. There are four major coral reefs in India, the Andaman & Nicobar Islands, Gulf of Mannar, Gulf of Kachch and Lakshadweep group of islands. Out of these, the Andaman & Nicobar Islands, Gulf of Mannar and Gulf of Kachch are fringing reefs, while the Lakshadweep

islands are the only atolls in India (Bakus 1994). The Lakshadweep archipelago is scattered some 200-400 kilometers west off the Kerala coast, with 36 coral islands and open reefs (Fig 1.1). These islands form the smallest of the Union Territories of India, and are the country's only atolls. They lie between 10-12° N and 71°40'-74° E in the Arabian Sea covering a land area of 32 sq. km including 12 atolls of which only 10 are inhabited. The ten inhabited islands are Androtl, Amini, Agatti, Bitra, Chetlat, Kadmath, Kalpeni, Kavaratti, Kiltan and Minicoy. The main islands are Kavarrati, Minicoy, and Amini. Kavaratti is the headquarters of these islands, while Bitra is the smallest of all, with a nominal population.

Corals are classified into hexacorals and octocorals based on cycle of mesenteries. The hexacorals are those that are having six or multiples of six mesenteries and octocorals have eight mesenteries. Hexacorals are hard corals and they form reefs. They have the dinoflagellate, *Gymnodinium microadriaticum*, widely called zooxanthellae inside its gastrodermal layers as cytobiont. The zooxanthellae and the coral polyps are in mutualistic relationship. The zooxanthellae obtain nitrogen and shelter from the host and in return the host gets photosynthate from the zooxanthellae. The presence of zooxanthellae enhances the calcification thereby contributing to the reef formation. The Octocorals include many soft corals and the sea fans, the gorgonians. The octocorals do not have the zooxanthellae associations. The diseases have been reported both in hexacorals and octocorals.

During a survey in Lakshadweep islands, a variety of factors were found causing total or partial mortality in corals. Some of the factors that were affecting the corals irrespective of species were bleaching, grazing of the



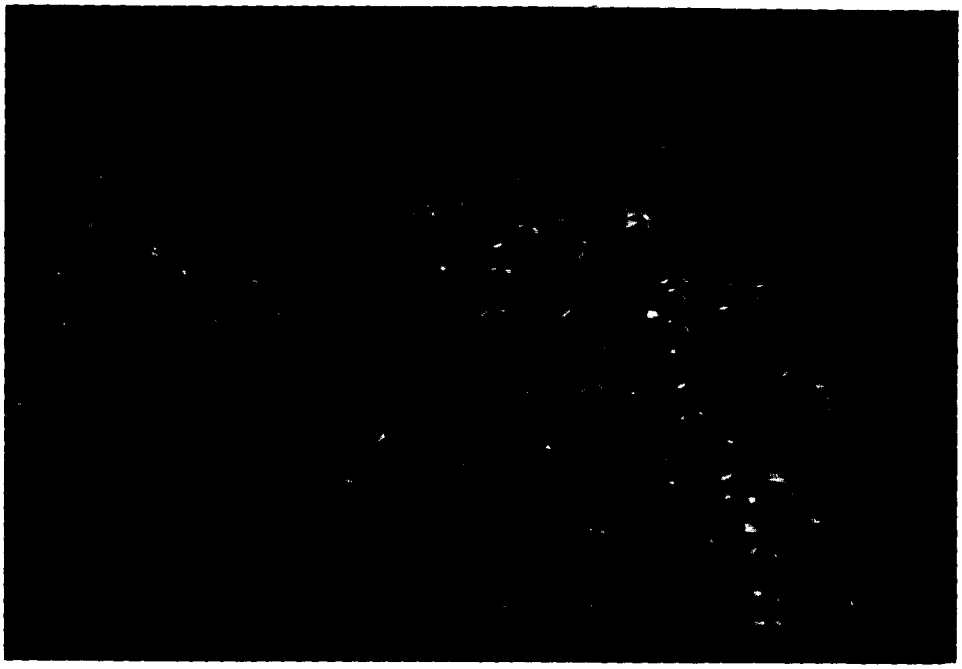
1.1. Lakshadweep group of islands showing Kavaratti Island, the study area

coral polyps by *Drupella* sp. and predation by the sea urchin, *Acanthaster plancii*, commonly called the 'crown-of-thorns'. Extensive partial mortality was observed in the dominant scleractinian coral species *Porites lutea* (Milne-Edwards & Haime). Most of these corals showing partial mortality were clearly distinguished by a pink line around the dead patch and the width of the line ranged from a few mm to a cm (Fig 1.2 & 1.3). Preliminary observations showed that the affected portions were colonised by a cyanobacterium and some fungal associations were found along with the polyps in both healthy and affected tissues.

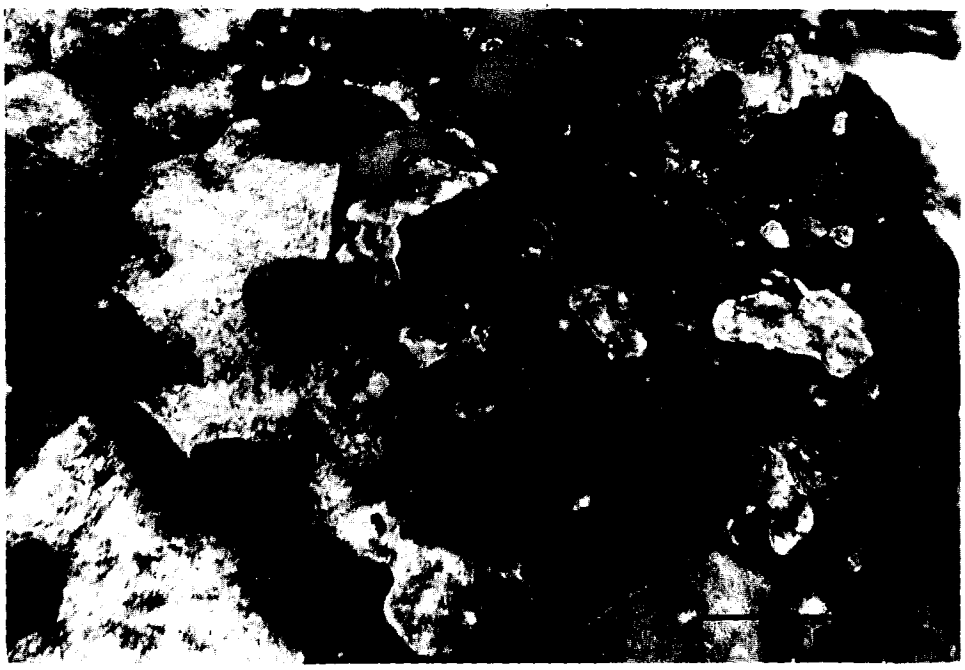
1.1. Objectives: -

The objectives of the present study were :

1. To survey the diseases in corals in the Lakshadweep Islands for a period of three years,
2. To study the etiology of the pink-line syndrome in the coral *Porites lutea* and the response of the host polyps in the pink-line affected polyps at cellular and physiological levels,
3. To explore the role of biotic and abiotic factors around the polyps in the formation of pink-line in *P. lutea* colony.



1.2. Healthy colony of *P. lutea* (bar = 30 cm)



1.3. PLS-affected *P.lutea* (bar = 30 cm)

2. Review of coral diseases:

Disease is defined as any impairment (interruption, cessation, proliferation, or other disorder) of vital body functions, systems or organs (Peters 1997). Importance of pathogens as regulators of coral populations in the tropical marine environments is poorly understood (Peters 1988). A disease not only kills the coral colony, but also exposes a substratum from the diseased corals for a new recruit (Connel and Keough 1985). Some disease causing organisms of corals have been identified and mechanisms of mortality have been studied in some diseases, such as the black band disease. However, many others remain poorly investigated. Infectious diseases in corals are different from genetic diseases found in them, such as unusual growth patterns resembling tumors, neoplasms or galls, which have been analogous to cancers (Goreau et al. 1998). Infectious diseases are not to be confused with overgrowth of other animals (Goreau et al. 1998). Often vectors spread pathogenic agents. For example, parrotfish is believed to spread the pathogens through oral mucus (Antonius 1981a). Diseases can be classified as biotic and abiotic (Peters 1997). In biotic diseases, various biological factors are responsible for the disease, while in abiotic diseases, abnormal features among environmental factors such as salinity, temperature, ultraviolet light, sedimentation or exposures to toxic chemicals may cause disorder. Biotic and abiotic factors are often interrelated. Physiological disorders often result from extreme environmental conditions. For example, corals expel zooxanthellae during times of anomaly in sea surface temperature (Bruno et al. 2001). Many coral diseases are reported from all

over the world in hexacorals and octocorals. These diseases are discussed in detail below.

2.1. Diseases in hexacorals: -

2.1.1. Black band disease (BBD): -

Antonius (1973) first reported the black band disease (BBD) on corals in Caribbean reefs. The BBD is a major factor in decline of coral reefs in Florida reefs (Porter and Meier 1992; Peters 1993). The corals that are more susceptible are *Montastrea* sp., *Diploria* sp. and *Colpophyllia* sp. (Antonius 1981a and Rützler et al. 1983). The cyanobacterium *Oscillatoria submembranacea* (Antonius 1973) later renamed as *Phormidium corallyticum* by Rützler and Santavy in (1983) was suspected to be a pathogen. The black band disease was named based on the appearance of a black line covering the coral tissue between the polyp-depleted skeleton and the healthy portion of the colony (Antonius 1981b; Rützler et al. 1983). This band contains a microbial consortium consisting mainly *Phormidium corallyticum.* The darkness of the band is due to the presence of the dark accessory photosynthetic pigments, phycoerythrin and phycocyanin (Richardson 1996; Richardson and Carlton 1993). Thickness of the band is about 1 mm to 10 mm (Richardson 1996). It migrates at the speed of about >1 cm day^{-1}, but bright sunlight enhances the speed of the cyanobacterial movement (Rützler and Santavy 1983). As they migrate, the cyanobacterial filaments lyse the coral tissue underneath, resulting in the exposure of the skeleton (Richardson 1996). In addition to the cyanobacterium, the band consists of numerous heterotrophic bacteria (Garrett and Ducklow 1975), fungi (Ramos-Flores 1983) and sulphur-reducing bacteria like *Desulfovibrio* and the sulphur

oxidizer, *Beggiatoa* sp. (Ducklow and Mitchell 1979; Antonius 1981b). The active front end of the band is dominated by *P. corallyticum* and the rear end is dominated by *Beggiatoa sp.* (Rützler and Santavy 1983). The band contains, scavenging bacteria such as *Flexibacter, Saprospira* types, *Nitrosomonas* and ciliates such as *Philaster* and *Porpostoma* (Rützler and Santavy 1983). It was proposed that neither *P. corallyticum* nor the *Beggiatoa sp.* was the primary pathogen and their combined effect in the formation of the disease was postulated (Ducklow and Mitchell 1979). The filaments of the cyanobacterium, *P. corallyticum* migrated towards the coral tissue (Rützler et al. 1983). Widening of the band is diurnal in nature. The movement of the cyanobacterial filament in the BBD was observed to be light dependent (Richarson 1996) and proportional to the temperature, the activity being higher in the warmer months (25° C) (Antonius 1985; Rützler et al. 1983). The black band migrates at an average speed of 3.5 cm day^{-1} (Rützler et al. 1983) and the speed attains the maximum velocity of 10 cm day^{-1} depending upon the environmental conditions (Antonius 1981b). The cyanobacterial filaments showed forward movement at the front edge of the band and backward movement at the rear end. This movement facilitates more light capturing capacity for the mat (Richardson 1996). The scleractinian corals are more susceptible when the corals are suffering from acute white band disease (WBD) or when bordered by a dense growth of green algae (Antonius 1985). The occurrence of the cyanobacterium *Phormidium* among the epilithic growth of chlorophytes was believed to cause BBD in the corals having white band disease (Antonius 1985). The infection is contagious. The healthy colonies

kept 2 mm apart from the diseased colony were infected with the BBD pathogen (Rützler and Santavy 1983).

Water samples from the field did not show the presence of the cyanobacterial pathogen. Ducklow and Mitchell (1979) tried to induce the disease in corals without stirring the water so that less oxygen or anoxic condition will be created around the coral polyps. However, this experiment did not yield the disease symptom. Instead, it showed hypoxia-related syndromes and proliferation of the anaerobic bacterium, *Beggiatoa sp.* The authors (Ducklow and Mitchell 1979) concluded that the presence of the cyanobacterium *P. corallyticum* is necessary for the formation and maintenance of the black band. Rützler et al. (1983) proved that the presence of the cyanobacterium *P. corallyticum* not only maintains the band but also is the cause for the black coloration of the band and destroys the tissue. Inoculation of the BBD material from diseased corals onto healthy ones resulted in the black band. These workers removed the colored cyanobacterial filaments from heterotrophic bacterial populations in the black band mat, under a microscope. The resulting inoculum, free of the cyanobacterium did not cause the black band. The inoculum that contained few *P. corallyticum* filaments started showing migration towards the coral tissues. The artificial inoculation experiments with *P. corallyticum* proved that the cyanobacterium is the pathogen. Healthy corals in the wild expelled the inoculum of the pathogen within few hours of inoculation by their ciliary action. Stressed specimens maintained in the laboratory were affected by the cyanobacterium within 4-6 hours of inoculation.

The main cause of the coral tissue mortality covered by *P. corallyticum* is due to sulphur accumulation underneath the band where the tissue is undergoing lysis. The bottom of the band is always anoxic or sulphide enriched (Richardson and Carlton 1993). Levels of >800 µM sulphide during the night time were estimated in the band (Richardson 1996). The formation of the sulphide underneath the black band in the BBD is because of the presence of the sulphur-reducing bacteria such as the *Desulphovibrio* in the microbial consortium of the black band disease (Garrett and Ducklow 1975). This sulphide inhibits electron flow in a manner similar to the inhibition of aerobic respiration by cyanide (Richardson and Carlton 1993). Thus the sulphide accumulation inhibits the electron flow in the photosystem II of the cyanobacteria, which then revert to anaerobic photosynthesis, in which the sulphide functions as an electron donor to the photosystem I (Richardson 1996). The anoxia and the higher sulphur concentrations in the range 40-400 µM (Richardson and Carlton 1993) are harmful to the obligatorily aerobic corals, which, like other aerobic marine animals, may have less tolerance to anoxia (Vismann 1991; Llanso 1991).

2.1.2. White band disease (WBD): -

Little work has been done on the WBD (Gladfelter 1982; Peters 1984 and Antonius 1981a &b; Ritchie and Smith 1998). WBD is a sharp line of advance where the distally located brown zooxanthellae bearing coral tissue is cleanly and completely removed from skeleton, leaving a sharp white zone about 1 cm wide that grade proximally into algal successional stages (Gladfelter 1982). Zooxanthellae-bearing coral tissue peels off from the skeleton into little balls, held together with strands of mucus. These tissue

balls are carried away by the ciliary action of the corals. The white band spreads from the basal region of the colony to the tip. No environmental factors that alter the speed of the white band are known. The white band causes substantial decrease in skeletal deposition. The WBD tissues contain both gram positive and gram-negative bacteria. The disease is not transferable and it does not spread to the adjacent colony even when these colonies are fused naturally. The WBD does not respond to antibiotics as the black band does (Antonius 1985). WBD is found to occur only in the corals *Acropora palmata*, *Diploria strigosa, Montastrea annularis* and *Mycetophyllia ferox* (Antonius 1981a). This disease is considered to be the slower version of the shut down reaction (SDR) (Antonius 1981a) that is characterized by spontaneous disintegration of the coral tissues resulting in the denuded skeleton. An association of a rod shaped gram-negative bacterium was found in histological sections of WBD-affected *Acropora palmata* (Peters 1984; Santavy and Peters 1997). This bacterium was found in healthy specimens as well. But the diseased specimens had higher abundance of these bacteria than the healthy one. Two types of tissue loss, designated as type I and type II have been demonstrated in association with the WBD (Ritchie and Smith 1998). In type I, tissue loss shows active tissue necrosis along the disease line, whereas in type II, different stages of disease processes that varies from the tissue destruction to the exposure of the coral skeleton were found. Studies on the type II specimens have shown the presence of the gram-negative bacterium, *Vibrio charcharii* (Ritchie and Smith 1995, 1998) as a conspicuous one among the other bacteria. Koch's postulate experiments

have yet to prove the role of this bacterium associated with the WBD in causing the disease.

2.1.3. White plague: -

The white plague was first reported from Florida Keys (Dustan 1977) and recently also from the Puerto Rico reefs (Bruckner and Bruckner 1998). The terms 'white plague' and 'plague' are synonymous (Dustan and Halas 1987). It was found later that the disease affects many more species than previously described (Dustan and Halas 1987). The affected colonies had no visible microbial flora on the surface of the colony. Microscopic studies revealed tissue degeneration and remnants of zooxanthellae, giving a bleached effect to the diseased colonies (Richardson et al. 1998a). In north Florida Keys, 17 to 43 species were susceptible to the disease and 38% of the most susceptible species were dead within a period of 11 months (Richardson et al. 1998 a &b). Two variants of the plague epizootics were found based on the rate of tissue destruction. The plague type I spread more slowly compared to the plague type II that spreads almost a cm day^{-1} (Richardson et al. 1998b). The plague II affected a stretch of more than 400 Km of Florida reefs between 1995 and 1997 (Richardson et al.1998b). A single bacterium, *Sphingomonas* sp. was isolated from the diseased corals (Richardson et al. 1998a). The bacterium was later proved to be the pathogen through laboratory studies. This disease is transmissible and occurs seasonally (Richardson et al. 1998a).

2.1.4. Rapid wasting syndrome (RWS): -

The Rapid wasting syndrome is most prevalent in the Caribbean. The disease leaves an eroded skeleton as it spreads laterally on the colony. The

skeletal erosion may be as deep as 2 cm (Goreau et al. 1998). RWS is the synonym for the Rapid Wasting Disease (RWD) (Cervino et al. 1998). The RWS mostly affects colonies of *Montastrea annularis* and *Colpophyllia natans*. A fungus and a ciliate were found in the microscopic examination of the affected specimens (Cervino et al. 1998). The fungus is not an endolithic species and it might have been spread by parrotfish bites (Cervino et al. 1998).

2.1.5. White Syndrome: -

White syndrome is the whitening of coral tissues that is thought to be a reaction to toxic chemicals leached from antifouling paintings of marine installations (Antonius and Riegl 1997)

2.1.6. Shut down reaction: -

Stressed corals may die by even relatively mild impacts, such as a simple scratching, which will not kill healthy ones, causing a Shut Down Reaction (Antonius 1977). Shut down reaction (SDR) is a complete, spontaneous disintegration of the coral tissue, starting at the borderlines of the injury. Coenosarcal tissue sloughs off in thick strands or blobs. The disease spreads along the branches in a ramose form, leaving denuded coral skeleton without a trace of tissue. The disease advancement on the affected colony is about 10-cm hour^{-1}. The advancement is a non-intermittent process, which does not stop before killing the entire colony. SDR is transmitted by contact. A piece of sloughed tissue triggers SDR within 5-10 min after a contact with another healthy colony.

2.1.7. Yellow band disease: -

Yellow band disease (YBD) has been found in the coral *Montastrea annularis* while the colony was recovering from the bleaching event (Hayes and Bush 1990). The disease is found to appear continuously thereafter in the Caribbean (Goreau et al. 1998).

2.1.8. Coral bleaching: -

Coral reefs have been projected in the report of the intergovernmental panel on climate change, to be among the most sensitive ecosystem to long-term climate change (IPCC 1998). When physiologically stressed, the critical balance that maintains their symbiotic relationship with algae is lost. The corals may lose some or most of their algae, a major source of nutrition and color. In this condition, corals are referred to as "bleached". Bleaching is defined as the paling of the host due to the loss of zooxanthellae (Hoegh-Guldberg and Smith 1989; Fitt and Warner 1995; Lesser et al. 1990) or loss of photosynthetic pigments from zooxanthellae (Sharp 1995; Fitt and Warner 1995). In-depth investigations on the bleaching mechanisms show that there are several mechanisms involved in the expulsion of zooxanthellae during the bleaching process. The primary mechanism involved in the coral bleaching is the host cell detachment of zooxanthellae (Brown 1997b). The zooxanthellae number in the tissue is reduced by the release of the zooxanthellae into the coelenteron or by the release of the detached gastrodermal cells into the coelenteron (Gates et al. 1992). In addition to the reduction of zooxanthellae in the bleached tissue, chlorophyll content per zooxanthellae has also been observed to be reduced (Brown et al. 1995), reaching near zero levels, although the carotenoid levels did not change (Lee-Shing Fang et al. 1995).

Chlorophyll *c* content was 35 times lower in the bleached corals than in healthy specimens (Kleppel et al. 1989). Zooxanthellae are degenerated or are released from the damaged gastrodermal cells or the gastrodermal cells themselves are released along with the zooxanthellae. Tissue growth is halted in the affected species and skeletal accretion is stopped (Goreau and Macfarlane 1990, Leder et al. 1991), while sexual reproduction is suspended (Szmant and Grassman 1990). Corals survive if the stress is brief, but will die if it is prolonged (Wilkinson, et al. 1999; Glynn 1996). However, even a sublethal stress may make corals highly susceptible to infection by a variety of opportunistic pathogens. Disease outbreaks (epizootics) may result in significant coral mortality (Hayes and Goreau 1998). Once mortality occurs, the coral's soft tissue becomes a food source for scavengers, making the increasingly bare skeleton a site of attachment for rapidly growing seaweed and other opportunistic organisms (Hayes and Goreau 1998). Coral bleaching is most often associated with a significant rise in sea surface temperatures (Brown 1997b; Glynn 1996; Goreau et al. 1993; Glynn 1991; Cook et al. 1990; Gates 1990; Jockiel and Coles 1990; Hoegh-Guldberg and Smith, 1989; Jaap 1985; Fankboner and Reid 1981). On site observations and National Oceanic and Atmospheric Administration (NOAA) satellite-derived sea surface temperature records from North Atlantic and Caribbean reef locations show a significant correlation between all large scale bleaching events and high sea surface temperatures (Strong et al. 1998; Gleeson and Strong 1995; Goreau et al. 1993). Water temperatures of even one degree Celsius above normal summer maxima lasting for at least two or three days appear to provide a potentially useful predictor of consequent bleaching (Goreau and Hayes

1994). While there are differences in response among species and populations, most corals are likely to bleach but survive if temperature anomalies persist for less than a month, enabling corals to recover. However, the chronic stress of sustained high temperatures can cause physiological damage that may be irreversible (Wilkinson et al. 1999). Stress related bleaching may also be induced if corals are subjected to a reduction of salinity, intense solar radiation (especially ultraviolet wavelengths), exposure to the air (by low tides or low sea level), sedimentation, or xenobiotics such as copper, herbicides, and oil (Brown 1997a; Glynn 1996). Often, these conditions are at least an indirect consequence of extremes in weather (such as hurricanes and typhoons) which may be produced by or occur concurrently with elevated sea surface temperatures. As a consequence, multiple factors may act in concert to cause bleaching. High solar irradiance (particularly ultraviolet wavelengths) is thought to be especially stressful to corals when coupled with elevated sea surface temperatures (Glynn 1996). The recent 1997-98 El-Nino warming event is considered the strongest on record by some measures (Chavez et al. 1999; Mc Phaden and Xuri Yu 1999; Enfield 2001). The El-Nino of 1997-98 was estimated to have caused $33 billion damage and 23,000 deaths world wide (Kerr 1999). The mass coral bleaching of 1997-98, coincident with the 1997-98 El-Nino, is believed to be the most severe on record of International Society for Reef Studies (ISRS 1998). Coral reefs in the Indian Ocean were severely affected with shallow reef coral mortalities of up to 90% (Wilkinson et al. 1999) It has been suggested that large colonies have more energy available for regeneration (Loya 1976; Bak 1983). This is due to the nutrient translocation from healthy tissues adjoining

the bleached ones (Mascarelli and Bunkley-Williams 1999). It was recently reported that bleaching of the coral *Occulina patagonica* from the Mediterranean Sea is the result of a bacterial infection (Kushmaro et al. 1996, 1997, 1998; Rosenberg et al. 1998). The causative agent, *Vibrio shiloi* was shown to infect the host at elevated temperatures (Kushmaro et al. 1998). Temperature affects the adhesion of the bacteria to the coral to a ß-galactoside containing receptor (Warner et al. 1999). These bacteria were shown to multiply in the coral tissue but were unculturable in the normal routinely used media (Banin et al. 2000).

2.2. Diseases of Octocorals: -

The octocorals, similar to the scleractinian hexacorals, serve as hosts for numerous commensals, symbionts and parasites and also provide refuge for reef fish (Bayer 1961). Among the octocorals, the diseases affected the gorgonians. Causes that result in the loss of tissue in gorgonians are detachment, fracture of the skeleton and overgrowth by fouling organisms (Yoshioka & Yoshioka 1991). There are a few reports of disease-related mortality in gorgonians and other octocorals. Some of the diseases of gorgonians are described below.

2.2.1. Black band disease (BBD): -

BBD is known in the scleractinian corals caused by the cyanobacterium *Phormidium corallyticum* (Rützler and Santavy 1983). The same pathogen, *P. corallyticum* is also causing black band disease in the gorgonia *Pseudopterogorgia acerosa* and *P. americana* (Feingold 1988) and the mode of tissue loss in the gorgonians are similar to the BBD in the scleractinians corals.

2.2.2. Red band disease (RBD): -

The RBD was reported to affect the octocoral *Gorgonia ventalina* in Belize (Rützler and Santavy 1983). RBD contains a cyanobacterium from the genus *Oscillatoria* (Richardson 1992) in addition to other cyanobacteria. There is no particular cyanobacterium observed to be associated with the diseased corals, and different species have been thought to be responsible in different locations (Santavy and Peters 1996). RBD is similar to the BBD in its development of a microbial consortium in the mat, containing other cyanobacteria, the sulphur-oxidizing bacterium (*Beggiatoa*), heterotrophic bacteria and the nematode, *Araeolaimus* (Santavy and Peters 1996).

2.2.3. Aspergillosis: -

The fungal disease in a gorgonian is the first coral disease in which the complete processes such as entry and spread of the pathogen in the coral reef ecosystem and the role of global change in the disease propagation have been studied. There has been a correlation between the decline in the Caribbean coral reef and sharp increase in the transport of the African dust over the western Atlantic (Shinn et al. 2000). It is hypothesised that the prolonged drought in the highly grazed grasslands of the Sahel in Africa and the desiccation of the water bodies resulted in abundant fungal spores that are transported through the wind to the western Atlantic Ocean (Shinn et al. 2000). This finding was further supported by the study that shows that there are no spores in the clear air (Weir et al. 2000) and, therefore, the African wind was established as an effective carrier of fungal spores from African deserts to the western Atlantic region. *Gorgonia ventalina* and *G. flabellam* in the Caribbean suffer by the recession of rind tissues called coenenchyme,

which is the outer organic rich matrix containing the living polyps (Smith et al. 1996). Only one species of fungus was found common to all the affected colonies. The fungus was identified to be *Aspergillus* sp. The 18S ribosomal RNA analysis showed that the fungus may be *A. fumigatus* (Smith et al. 1996). The fungus was later identified as *Aspergillus sydowii* (Geiser et al. 1998). Weir et al. (2000) successfully established Koch's postulates by inoculating the *A. sydowii* cultured from the spores collected from the African dust.

2.3. Summary of the diseased states of the corals:

Name of the diseased states	**Hexa/Octocoral**	**Etiological agent**	**References**
Black band disease (BBD)	Hexacorals & Octocorals	The cyanobacterium *Phormidium corallyticum*	Porter and Meier 1992; Peters 1993; Feingold 1988
White band disease	Hexacorals	Not known	Gladfelter 1982; Peters 1984; Ritchie and Smith 1998
White plague Type 1 & 2	Hexacorals	The bacterium *Sphingomonas* sp.	Richardson et al. 1998 a &b
Rapid wasting syndrome	Hexacorals	Not known	Cervino et al. 1998

White syndrome	Hexacorals	Toxic chemicals	Antonius and Riegl 1997
Shut down reaction	Hexacorals	Not known	Antonius 1977
Yellow band disease	Hexacorals	Not known	Hayes and Bush 1990; Goreau et al. 1998
Coral bleaching	Hexacorals	Abiotic factors: Elevated sea surface temperature Biotic factors: *Vibrio shiloi*	Wilkinson et al. 1999; Kushmaro et al. 1998
Red band disease	Octocorals	Not known	Santavy and Peters 1996
Aspergillogis	Octocorals	The fungus *Aspergillus sydowii*	Smith et al. 1996; Weir et al. 2000; Shinn et al. 2000

3. Survey and characteristics of the pink-line syndrome (PLS) in *Porites lutea*:

3.1 Introduction: -

The extent of coral colonies affected by diseases needs to be monitored to understand the role of diseases in reef decline in association with or independent of other factors. Such a survey can provide clues regarding measures to be taken to arrest disease propagation. During the present work, the extent of colonies of the scleractinian coral, *Porites lutea* affecGted with pink line was surveyed. The survey was carried out in two seasons, one in the pre-monsoon period of April-May and another in the post monsoon period of Nov-Dec. The work was aimed at providing an estimation of the number of pink line affected specimens and its spread over a period during the investigation.

3.2. Method: -

3.2.1. Transect survey: -

Two stations were selected in the Kavaratti island, one each from the north and the south of the lagoon respectively (Fig. 3.1). These sites were surveyed wice in a year, during April-May and in Nov-Dec for monitoring symptoms and extent of partial mortality of the coral *Porites lutea* caused by the pink-line syndrome from the year 1996 to 1999. In order to survey the intensity of PLS spread among coral colonies, a modified belt transect method was used (Eylish et al. 1997). After laying a line of 50 meters, healthy and PLS-affected colonies of the *Porites lutea* colonies within the swath of one meter from the line were counted. Several such transects were made in each station in all the seasons.

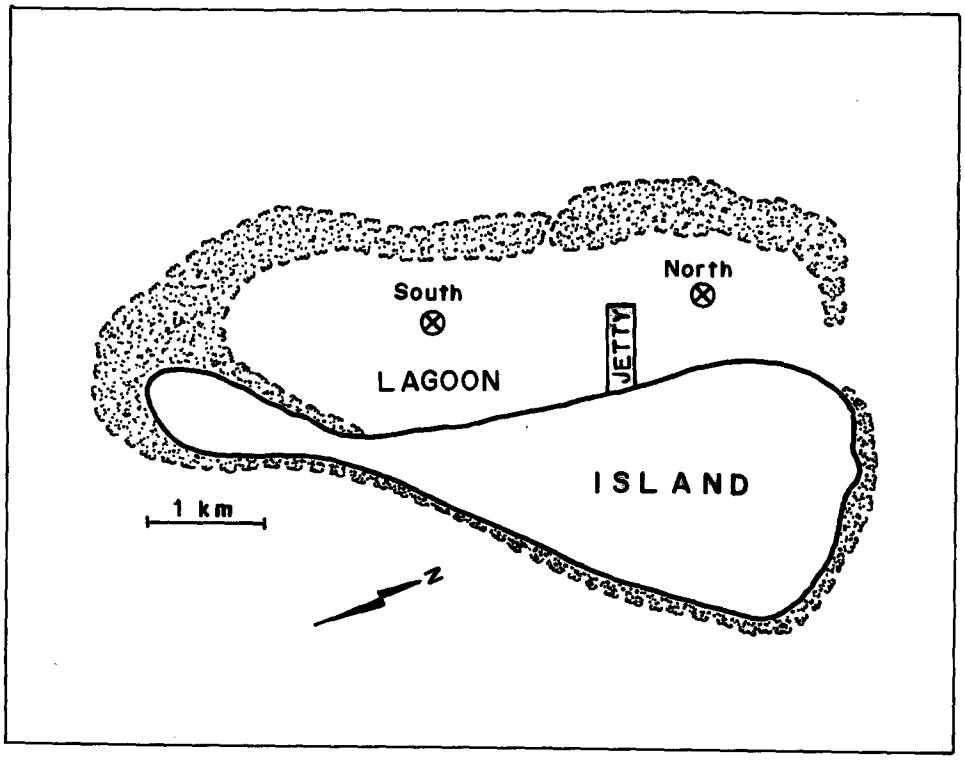


Fig. 3.1. North and South sampling stations in the Kavaratti lagoon

3.2.2. Microscopic examination of the PLS-affected colonies: -

PLS-affected and healthy samples 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 stored in 70% ethanol and transported to the lab. A few sub-samples of 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). 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_2 in a critical point freeze drying apparatus (Structure Probe Inc., West Chester, PA, USA). These critically dried specimens were sputter coated with gold-palladium and examined under scanning electron microscope (model 5800 LV, JEOL, Akishima, Japan).

3.3. Results: -

In the PLS-affected specimens, a pink line appeared as an interface between the dead patch and the healthy living portion of the colony. The width of the pink line varied from 2 mm to 10 mm. A cyanobacterial mat consisting, apparently of one species covered the dead patch. Observations through the stereo zoom microscope showed that the wall of the corallite in PLS-affected region was thin and fragile (Fig. 3.2) and the decalcified tissue of the PLS-affected specimen showed that the cyanobacterial filaments were in close association with the tissue (Fig. 3.3). Whole polyps removed after careful

Fig. 3.2. Fragile corallite (F) wall of the PLS-affected zone. Notice the normal (N) corallite (bar = 1 mm)

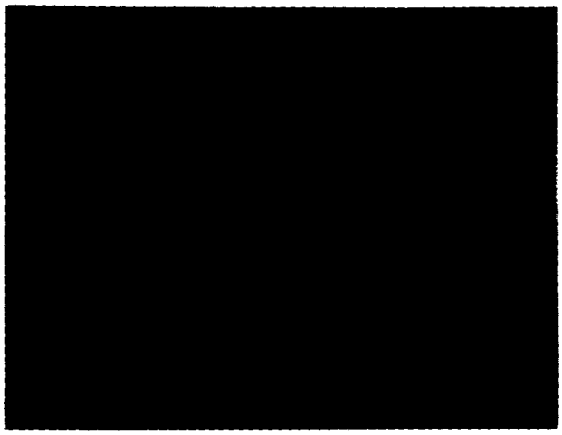


Fig. 3.3. Cyanobacteria associated with the PLS-affected specimens fluorescing red with green excitation under epifluorescence microscope (bar = 10 μm)

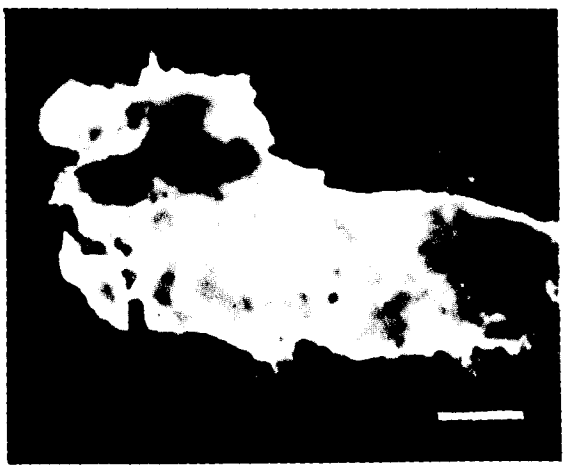


Fig. 3.4. Fungal hyphae around a polyp picked up from the pink-line. Bar = 370 μm

decalcification of surface layer of coral in the pink-line zone also revealed presence of fungal hyphae around periphery of the polyps (Fig. 3.4). Microscopic observations revealed that the PLS-affected polyps were not having any distinct bacterial associations other than cyanobacterial and fungal associations. The healthy colonies had fungal association but there were neither bacteria not the cyanobacteria found with the healthy polyps. The dissolution of the septa changed the architecture of the corallites. The coenosarcal tissue was degenerated in the PLS-affected colony, exposing the skeleton intermittently and the tissue was pink. The decalcified tissue of the PLS specimens was pink and it differed from the healthy looking portion of the tissue that was away from the pink line (Fig. 3.5). The PLS affected tissue excited by the blue light (400-440 nm excitation with 475 nm barrier filter) under the epifluorescence microscope showed bluish green fluorescence whereas the healthy tissues did not show this characteristic (Fig. 3.6, 3.7). Scanning electron microscopic observation of the PLS-affected tissue showed expulsion of the zooxanthellae, the cytobiont of the corals, in the surface of the PLS-affected tissue (Fig. 3.8).

The incidence of the pink line syndrome in the North and South stations showed an increasing trend from 1996 to 1999. This increase was four times in the fourth year, from 20% to 80% in the fourth year (Fig. 3.9). In the south, the PLS incidence was of the same intensity as of the north station. Only during Nov 97, a sharp decrease in PLS incidence was noticed at both the sites and the percentage of PLS-affected specimens during this period of survey was the same as in the initial period (May 96).

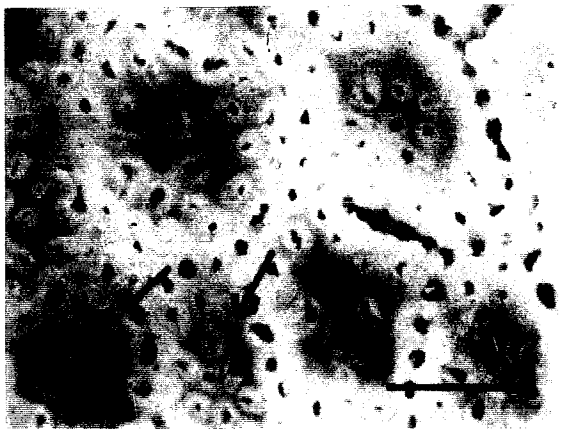


Fig. 3.5. Pink Coloration of the polyps (arrow) in the PLS-affected specimens (bar = 0.5 mm)

Fig. 3.6. Fluorescence around oral disk in PLS-affected polyp (bar = 50 μm)

Fig. 3.7. Absence of Fluorescence Around oral disk In healthy Specimens (bar = 50 μm)

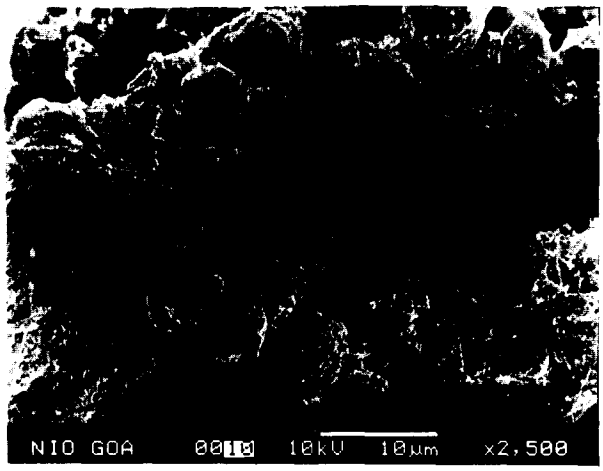


Fig. 3.8. Expelled zooxanthellae (arrow) in the PLS-affected specimens

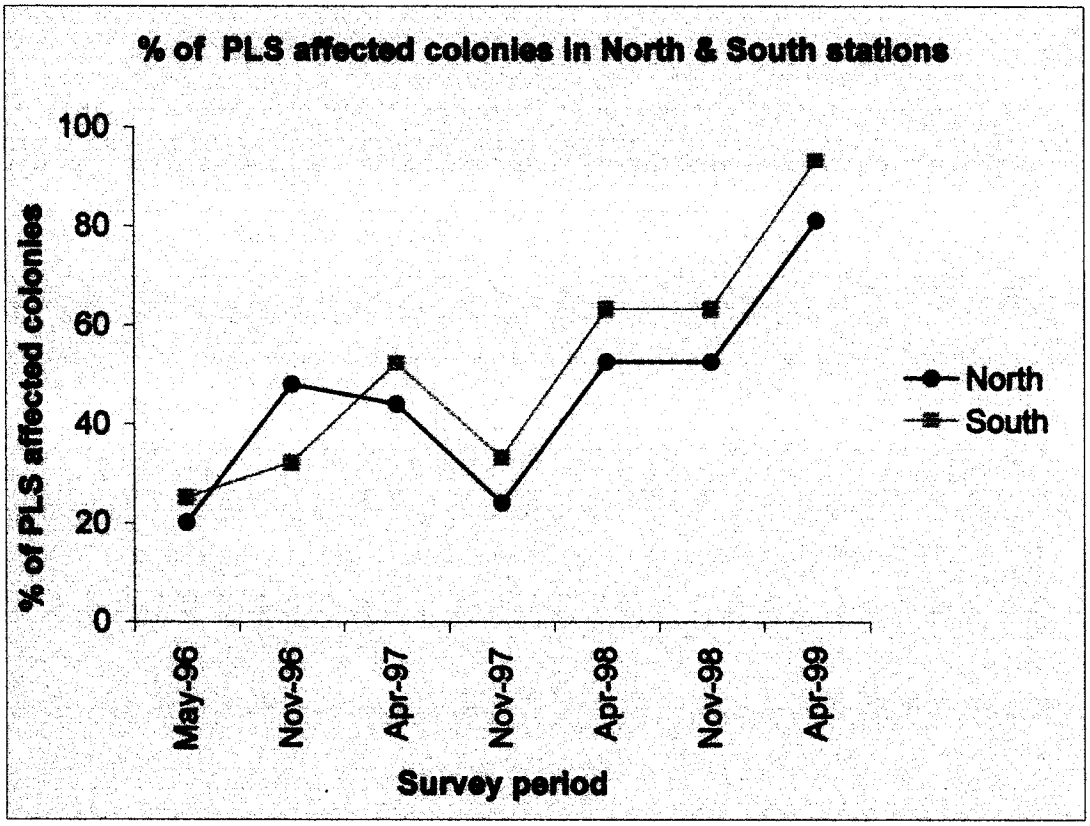


Fig. 3.9. Incidence of PLS in *Porites lutea* in the north and south stations during the study period 1996-99

3.4. Discussion: -

Syndrome is the collection of all the disease symptoms and the term 'disease' is the ill condition for which the etiology is well established. The pink line syndrome is called so, as no etiological agent was established at least during the early part of this work that causes the pink colour in the affected colonies. The pink coloration of the decalcified PLS-affected specimens confirms that the pink coloration is of the tissue rather than that of the skeleton or from any associated organisms as in the case of black band disease (Richardson 1996). The pink colour appears to be a tissue reaction, in response to the presence of microbial associations found around the pink

line (Ravindran et al. 2001). The acidic environment that is generated by the presence of the microbial community adjacent to the pink line could be the reason for the formation of the fragile coral skeleton in the PLS-affected colonies and subsequent skeletal dissolution.

The stressed polyps in the PLS-affected zone that fluoresce green when excited with blue light might be similar to the green fluorescent protein (GFP). This protein has not been reported in *Porites lutea.* The jellyfish *Aequorea victoria* fluoresce green when excited with blue light because of the presence of the green fluorescence protein (Ikawa et al. 1995). The main role of the GFP in many coelenterates is to transfer the energy by producing green light (Chalfie 1995). The exact mechanism in the production and the role of GFP in PLS-affected polyps is not known.

The PLS incidence was higher in summer than the post monsoon in many seasons. This pattern matches with the earlier report where infection of the black band disease on corals increases in late summers when the seawater temperature reaches its maximum (Edmunds 1991; Rützler et al. 1983). The dead surface of corals creates new substrata and these may be colonised by algae (Wellington & Victor 1985; Peters 1997). Reduction of herbivorous fishes due to the increased fishing activity and other anthropogenic activities in the lagoon, favours the cyanobacterial spread (Thacker et al. 2001). The algae on a partially dead coral colony pose serious threat to the adjacent host polyp for space. This triggers the host response against the algae. The north station that lies close to the channel through which the seawater enters during tidal fluctuation may flush out or dilute the nutrient. The role of the current in carrying the pathogens from one spot to

another is well known (Bruckner et al. 1997). The intensity of tidal current in the north station where the lagoon opens into the sea through a channel is reasonably higher than the south station (Chandramohan et al. 1996). The speed of the current may be inversely proportional to the pathogenesis thereby minimising the host-pathogen interaction time which otherwise may eventually lead to the settlement of the pathogen on the host that causes disease. The pathogenesis in the case of the PLS appears to be following this concept, as there is high mortality in the southern lagoon than in the north lagoon where the speed of the current is relatively faster. The possibility of the sedimentation as a mode of pathogen transport may be ruled out, since there is no appreciable level of sedimentation in the southern lagoon (Chandramohan et al. 1996). In the northern lagoon, the silt is carried away from the coral rich area towards the sea, minimising the possibility of siltation in this part (Chandramohan et al. 1996). More study is needed to understand the role of water current and frequency of seawater renewal in the north and south stations and its effect on the PLS incidence. The pathogen that causes PLS may be transported through water rather than through siltation or sedimentation, as there is no sedimentation or siltation in the southern lagoon bed or on reef.

Bacteria were observed to be present in association with the BBD (Garrett and Ducklow 1975; Ducklow and Mitchell 1979 and Antonius 1981b). Dustan (1977) reported an association of flexi-bacteria and gram-negative bacteria with white plague. Histological studies on WBD by Peters et al. (1983) showed dense packets of gram-negative bacteria. In the present study on PLS-affected polyps, no such bacterial associations were found in the

microscopic observation of tissues. Since cyanobacterial and fungal filaments were found in close associations with the PLS-affected polyps, these organisms were investigated in detail for their putative role in causing pink-line.

4. Fungal association with healthy and PLS-affected colonies:

4.1. Introduction: -

Direct coral fungal interactions are reported from geographically diverse parts and from taxonomically different species of corals. Thus, fungal endoliths, acting as opportunistic pathogens, may play a greater role in the ecology of coral reef system than previously recognised (Bentis et al. 2000). Very little is known about the fungal pathogenicity in lower aquatic invertebrates and their invasion is associated with obvious pathogenic changes in these tissues (Kendrick et al. 1982, Ramos-Flores 1983). Boring fungi are wide spread in modern corals. Twelve anamorph genera and two unidentifiable sterile mycelial penetration of shore rocks are reported (LeCampion-Alsumard 1979). In naturally bored corals, two major classes of microborings are reported. They are wide and narrow and are respectively by algae and fungi. Some of the boring fungi are from common terrestrial conidial (anamorphic) fungi. Many of these anamorph-species such as those of *Aspergillus*, *Penicillium* and *Cladosporium* are omnipresent and have no difficulty in coping with the salinity of the marine habitat. Members of these genera are among the most osmotolerant organisms known, often being found in habitats of very high osmotic pressure, such as jams and jellies (Pitt 1981). The regularity with which such "terrestrial" fungi occur within corals in widely separated geomorphic areas, suggests that some of these fungi are even more omnipresent and ecologically important than has been realised (LeCampion-Alsumard 1979). Low light intensities (Halldal 1968) and low and fluctuating oxygen pressures (Shashar & Stambler 1992) within coral skeleton render this environment ecologically extreme, which only a few specialised

organisms can endure. Fungi in corals are saprophytes that exploit dead organic matter incorporated in coral skeletons by the coral or produced by endolithic algae and cyanobacteria (Kendrick et al. 1982). Bak and Laane (1987) reported dark discoloration and banding inside coral skeleton caused by fungi. They also drew attention to a possible active interaction between corals and fungi. Fungal hyphae penetrated independent of the boring green alga *Ostreobium queketti,* and even attacked the algae and continued to grow. LeCampion et al. (1995a) reported that the stromatolite-like layering around growing tip of boring fungi arrested the advancement of the fungal boring towards polyps. These fungal hyphae did not enter the pore space or the polyps. The aim of the present work was to isolate, enumerate and identify fungi associated with PLS-affected and healthy colonies of *Porites lutea*, with a view to examine their possible pathogenicity to corals. Further, it was necessary to estimate the biomass of fungi inside *P. lutea* and distribution of fungal hyphae within the depth of the coral colony.

4.2. Methods: -

4.2.1. Isolation of fungi from corals: -

Small pieces from the surface of healthy-looking colonies of *P. lutea* and those showing PLS were chiseled and collected in sterile plastic bags under water. The samples were stored in ice and processed within 2 h of collection. The coral samples were broken further into smaller pieces inside the same plastic bags, thereby avoiding exposure of the broken surface to air to avoid contamination by fungal spores in the air. The broken pieces ranging in size from 1-3 mm^3 were directly plated on 1/5 diluted corn meal agar medium (HI-MEDIA, Mumbai, India) prepared with half-strength sea water and

fortified with streptomycin (0.5 g L^{-1} medium) and penicillin (100, 000 Units L^{-1} medium), to inhibit bacterial growth. A check for contamination by air-borne spores of terrestrial fungi at the experimental site was carried out by exposing 3-5 Petri plates containing corn meal agar medium (CMA) to air for 10 sec during the isolation procedure. Subsequent comparison of these plates with experimental plates facilitated in detecting aerial contamination in isolation plates. Fungal hyphae emerging out of coral pieces after 5-7 d of incubation in the dark were identified up to generic level. Frequency of different fungi isolated from corals is expressed as percentage.

4.2.2. Estimation of fungal biomass: -

Coral samples from the polyp zone (up to 5 mm below surface) were weighed and decalcified in a decalcification solution (Ravindran et al. 2001) (1:1 ratio of 20% citric acid and 50% formic acid). The resulting material was homogenized in a VirTis homogenizer (VirTis Co., Gardiner, NY, USA) at 5,000 rpm for 15 sec and filtered over a 0.22 μm pore size Nuclepore polycarbonate filter paper under vacuum. The filter paper was stained with 0.01% Calcofluor, an optical brightener that stains chitin and cellulose (Sigma, St Louis, MO, USA) for 5-10 sec (Mueller & Sengbusch 1983). After washing the excess stain with distilled water, the filter was mounted with a drop of non-fluorescent immersion oil and a cover slip applied.

The slides were observed under epifluorescence microscope using a blue excitation filter (Olympus BX-60, Japan). The images of fungal hyphae (Fig. 4.1) were captured using a CCD camera (Optronics Engineering, CA, USA) and digitized using a frame-grabber card (Coreco, Oculus TCX). Since some coral detritus also showed fluorescence, images were manually traced

in conjunction with Optimas 6.0 (Optimas Corporation, Bothell, WA, USA) image analysis software calibrated to the particular objective. Total length and average width of the mycelia for the total area were estimated using the software to calculate fungal biovolume. Mycelial fungal biomass was determined by assuming a mass density of 0.2 g cm^{-3} (Newell et al. 1986) and expressed as mg fungal biomass per g wet weight of coral tissue. About 3-10 coral pieces were examined as replicates for this study.

4.2.3. Vertical distribution of fungi in corals: -

In order to determine the depth of fungal abundance in coral colonies, their vertical distribution was studied in PLS-affected and healthy-looking colonies of *P. lutea*. The coral pieces were cut into 1-cm thick sections using an electric rock cutting saw. These sections were washed with a jet of water to remove free particles and stained with 0.01% Calcofluor solution for 2-3 min. After washing the excess stain, they were examined under an epifluorescence microscope (OLYMPUS BX 60, Japan), using a blue excitation filter for the presence of fungi. Dark melanized non-sporulating fungi did not stain with Calcofluor but could nonetheless be detected directly. The results are qualitatively expressed as presence or absence of fungi at various depths (from surface up to 50 mm depth) in the coral sections.

4.2.4. Scanning electron microscopy of the PLS-affected and healthy specimens: -

Healthy-looking specimens of *P. lutea* and those showing pink-line syndrome collected from 1-3 m depth in the lagoon were fixed and preserved according to Ravindran et al. (2001). The collected specimens were fixed immediately with 3% formalin prepared with seawater for 24 h. They were

further fixed with 10% formalin solution for 48 h. The fixed samples were rinsed thrice with tap water and were preserved in 70% ethanol until use. Fixed samples were later decalcified (as described above) and washed twice in distilled water and dehydrated in a graded acetone series (from 20 to 100 % with an increment of 20%). The samples were dried critically using liquid CO_2 in a Critical Point Freeze Drying apparatus (Structure Probe Inc., PA, USA). The whole specimens (containing tissue and skeleton) were prepared in a similar manner without the decalcification step for SEM studies. Critically dried specimens were sputter coated with gold-palladium and examined under a scanning electron microscope (model 5800 LV, JEOL, Japan) to observe the fungi.

4.2.5. Immunofluorescence detection of selected fungi in *P. lutea*: -

In order to confirm the presence of fungi obtained by isolation procedures, attempts were made to detect their presence directly in the coral colonies by using immunofluorescent probes. Antibodies were raised commercially (Bangalore Genei Pvt. Ltd. Bangalore, India) for two of the most frequently isolated fungi, namely a dark mycelial fungus (isolate #98 N-28), *Curvularia lunata* (Wakker) Boedijn) and a hyaline non-sporulating, unidentified isolate (Isolate # 98 N-18) obtained from partially dead and PLS-affected coral respectively. The technique followed for raising antibodies was as follows (Bangalore Genei Pvt. Ltd. pers. Comm.). About 2 mg of fungal pellet was crushed in 2 ml 0.15 M NaCl, centrifuged and 1ml supernatant was emulsified with 1 ml Freund's adjuvant. This was injected subcutaneously at multiple sites on the back of New Zealand male white rabbits (2 rabbits for each fungus). The first booster injection was given after 6 weeks of the

primary injection and the second booster was given after a further 4 weeks. The antibody titre was monitored by Dot ELISA after 10 d of the booster injection and yielded a titer value of 1:2000 for isolate # 98 N-18 and 1:5000 for isolate # 98 N-28. In the laboratory, the antibodies thus obtained were diluted four times in phosphate buffer saline (see Annexure) (Mendoza et al. 1995). The various coral specimens were crushed into a coarse powder and incubated with 50 µl of the diluted antibody solution in an Microfuge tube for 1 h at 25°C. The mixture was vortexed after diluting it to 1000 µl with phophate buffered saline (PBS) solution. The slurry was centrifuged at 4000 rpm for 1 min. After decanting the supernatant, the coral debris was similarly washed three times with 1000 µl of PBS. The samples were incubated in 50 µl of the secondary antibody, the goat anti-rabbit anti-serum tagged with fluroscein isothiocyanate (FITC) (Bangalore Genei Pvt. Ltd, Bangalore, India) for 1 h at 25° C. The excess stain was washed with PBS buffer as described. The coral debris was transferred on to a slide and viewed under blue excitation (excitation wave length 400-440 nm and barrier filter # 475) for the presence of fungi and photographed. Specificity of the antiserum was confirmed by the absence of immunofluorescence staining by 4 species of *Aspergillus, Cladosporium sp.* and mycelial yeast isolated from *Porites lutea*. Staining of coral sections with pre-immune sera (obtained from rabbit) did not show any immunofluorescence.

4. 3. Results: -

Table 4.1. Fungi isolated from the polyp zone of *Porites lutea* collected from the lagoon in Kavaratti, Lakshadweep Island.

Frequency of fungal presence (%)				
Fungal species	Healthy		PLS-affected	
Year of isolation	1998	1999	1998	1999
Dark no sporulating forms	22	0	0	25
Orange non-sporulating forms	6	0	0	0
Hyaline non-sporulating forms	0	36	25	0
Acremonium sp.	0	0	0	0
Fusarium sp.	11	0	0	0
Aspergillus sp.	17	7	25	15
Cladosporium sp.	0	7	8	5
Mycelial yeasts	0	21	0	0
Labyrinthula sp.	0	0	0	5
Unidentified	0	0	0	5
Chaetomium sp.	0	0	0	10
Aureobascidium sp.	0	0	0	5
Total	56	71	58	70

Several fungi from PLS-affected corals as well as from healthy ones were isolated from the surface of the coral up to 5 mm depth (Table 4.1). There were no differences in the frequencies of fungi occurring in healthy and PLS-affected colonies of *P. lutea*. Most of the identified species of fungi belonged to terrestrial genera. Dark non-sporulating forms (DNS) and hyaline non-sporulating forms (HNS) were the most dominant in all the healthy-looking and the pink line specimens (Table 4.1). The other dominant and frequently isolated fungi from these corals were various species of

Fig. 4.1. Fluorescing fungal hyphae stained with the optical brightner calcofluor under UV excitation (bar = 20 μm)

Fig. 4.2. Fungal hyphae (fine filaments) and algal filaments (broad filaments) from the polyp zone visible after decalcification of the surface layer (bar = 50 μm)

Fig. 4.3. Septate hyphae of a dark non-sporulating fungus seen after decalcification of the surface layer (bar = 10 μm)

Fig. 4.4. Detection of the hyaline non-sporulating fungus strain 98 N-18 with an immunofluorescent probe

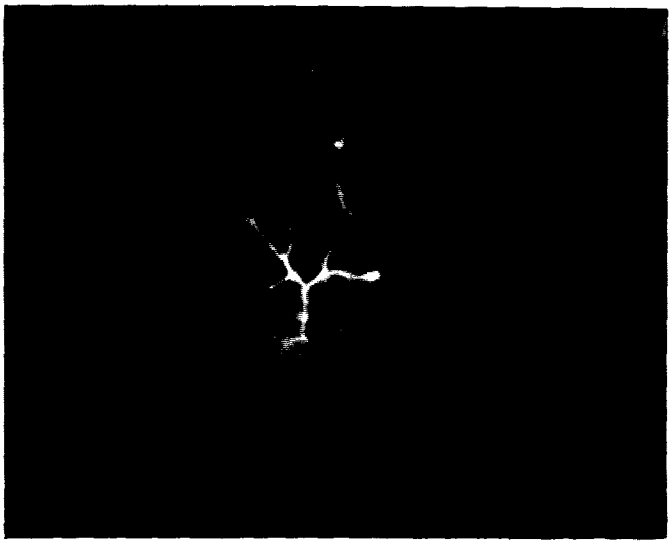


Fig. 4.5. Detection of the dark non-sporulating fungus strain 98 N-28 *Curvularia lunata* inside coral skeleton with an immunofluorescence probe

Aspergillus, a species of *Cladosporium* and mycelial yeast. We did not attempt to isolate fungi from other coral species. Therefore we are not sure weather these fungi are specific to the coral *Porites lutea*. On decalcification of the top 5 mm layer, black septate fungal mycelia interspersed with thin hyaline fungal mycelia, cyanobacterial and algal filaments were observed (Figs. 4.2, 4.3).

The HNS fungi (# 98 N-18) as well as *Curvularia lunata* (# 98 N-28) were detected in corals using the immunofluorescence probes (Fig. 4.4, 4.5). Branched mycelia of both the fungi adhered closely to calcium carbonate particles and often appeared to be present within the carbonate skeleton (Fig 4.4, 4.5). Frequencies of occurrence of *Curvularia lunata* (isolate # 98 N-28) were similar in healthy, and PLS-affected corals (about 50% frequency). The HNS form (isolate # 98 N-18) was found to be more frequent in the healthy (60% frequency) than in PLS affected corals with 50% frequency (Fig. 4.6).

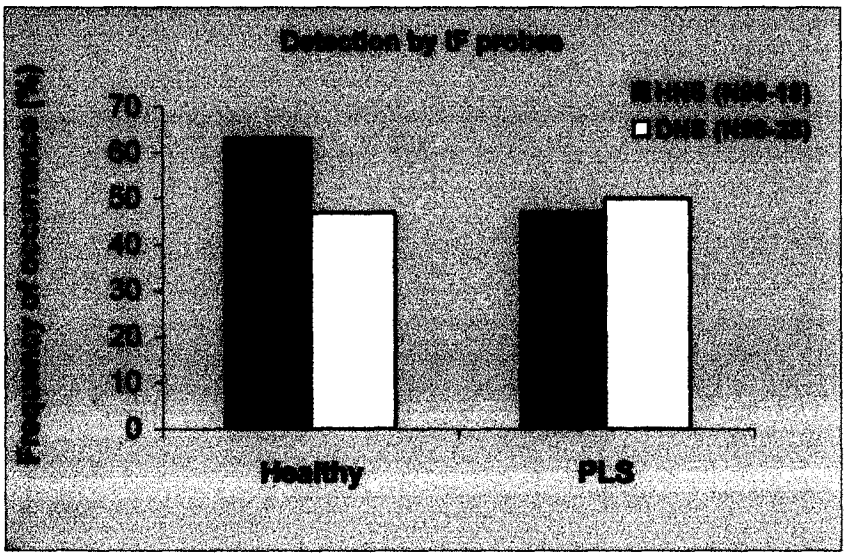


Fig. 4.6. Percentage frequency occurrence of the hyaline non-sporulating (HNS) strain 98 N-18 and dark non-sporulating (DNS) strain 98 N-28 in the healthy and PLS-affected corals of *Porites lutea*, detected by immunofluorescence probes.

Sections of corals stained with Calcofluor revealed the presence of fungi as bright blue septate filaments under an epifluorescence microscope in contrast to bright orange colored cyanobacteria or red colored chlorophycean algal filaments. The biomass of fungi in the healthy-looking colonies was 0.4 mg g^{-1} wet weight of the colony, but was much less in the PLS specimens. Examination of the depth-wise distribution of the hyaline and dematiaceous fungi revealed that they pervaded the PLS-affected corals more deeply than the healthy ones (Fig. 4.7).

Scanning electron microscopy of the healthy and the PLS-affected corals showed that fungi perforated the coral skeleton (Figs. 4.8, 4.9, 4.10). The hyphae showed distinct swellings at regular intervals (Figs. 4.8, 4.9). Fungal hyphae showed basal swellings on emergence from the carbonate skeleton (Figs. 4.10, 4.11). Ramifying fungal hyphae emerged from the coral skeleton and spread around the polyps (Figs. 4.12, 4.13, and 4.14). The fungal hyphae were covered by remineralized carbonate precipitate (Figs. 4.8, 4.11, 4.14) and most often fresh accretion of amorphous repair carbonate around fungal hyphae was noticed (Figs. 4.10, 4.11). Sections of polyps from healthy and PLS-affected corals, stained with basic fuchsin showed abundant fungal hyphae around the polyps (Fig. 4.15).

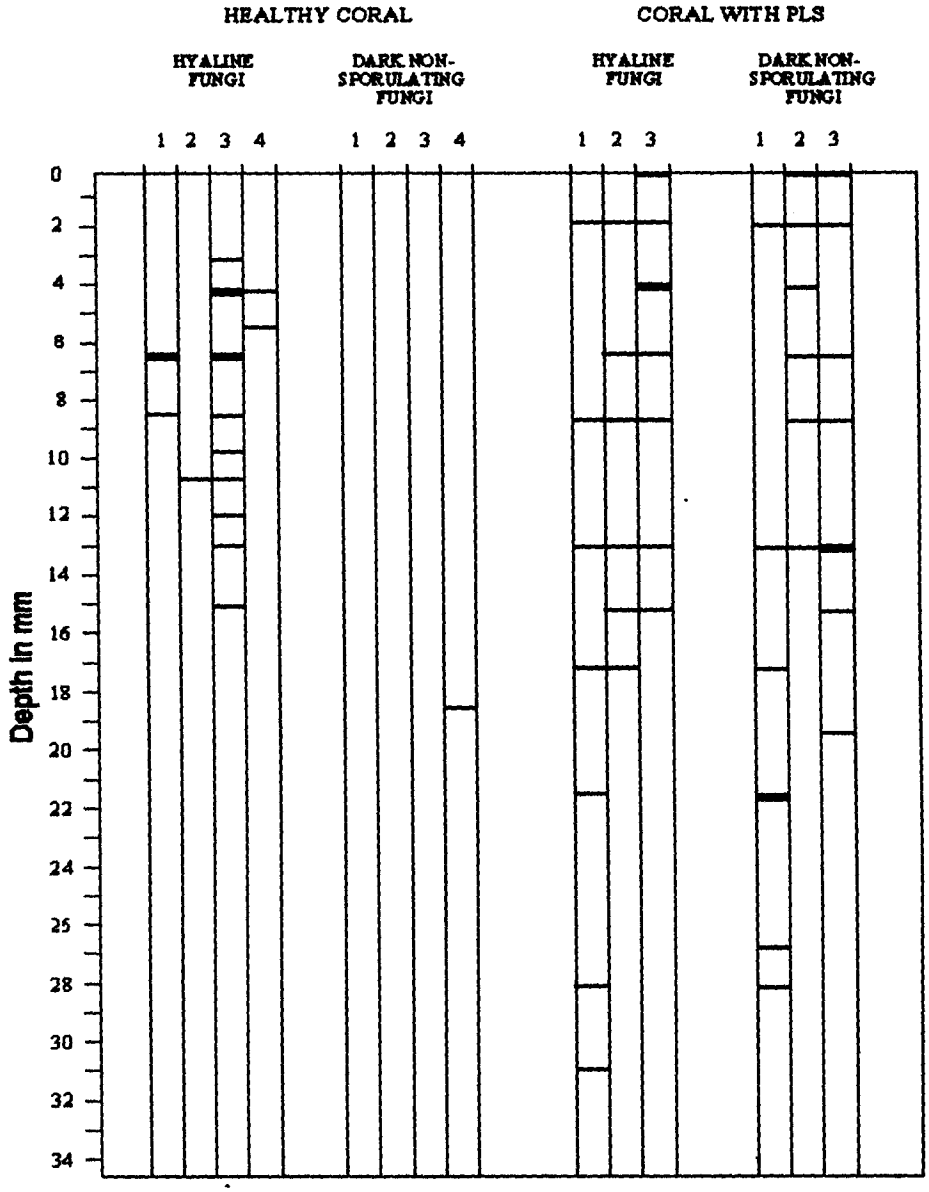


Fig 4.7. Vertical distribution of fungi within healthy and PLS-affected corals. The horizontal bars represent fungal presence detected by calcofluor staining (as described in the 'methods') at a particular depth in corals. The dark bars represent detection of fungi in more than 2 transects in a vertical section of the coral. Numbers 1 to 4 represent replicate coral colonies examined.

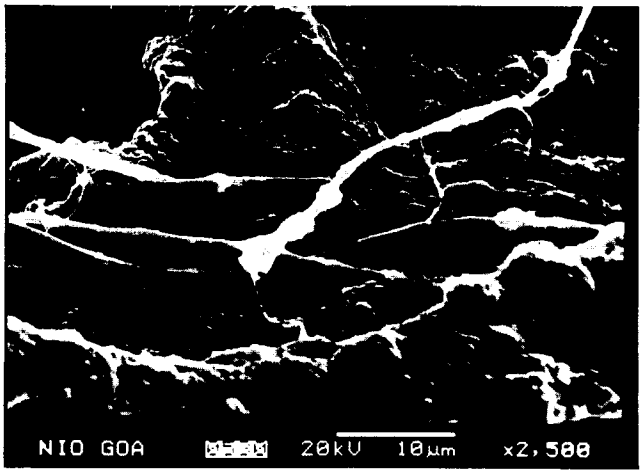


Fig. 4.8.

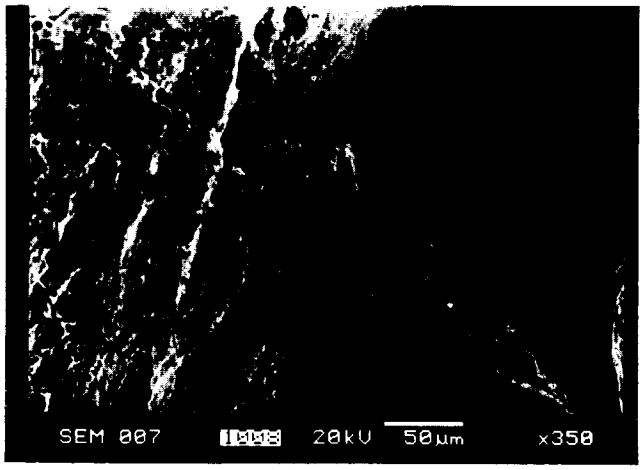


Fig. 4.9

Figs. 4.8 & 4.9. Fungal hyphae with swellings ramifying within coral skeleton as observed under a scanning microscope

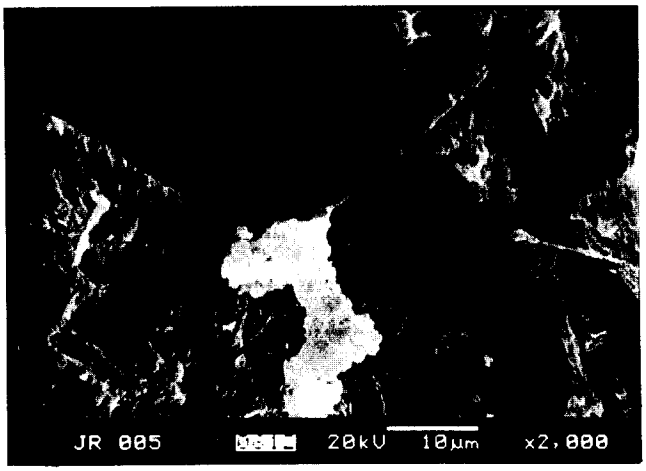


Fig. 4.10. Deposition of amorphous repair carbonate around the invading fungal hypha (arrow)

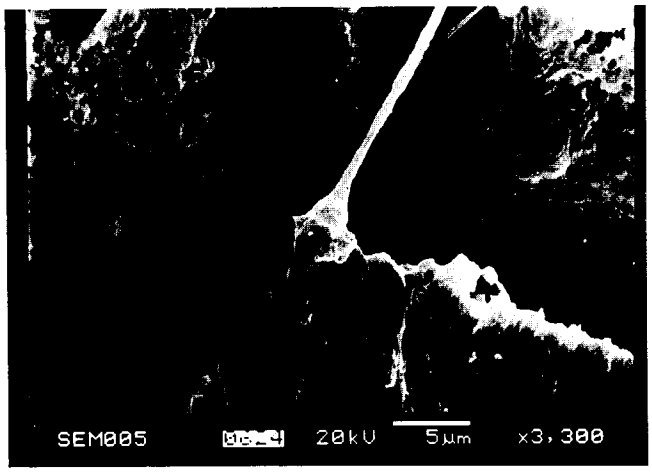


Fig. 4.11. A fungal hypha showing basal swelling at the site of emergence from the carbonate skeleton

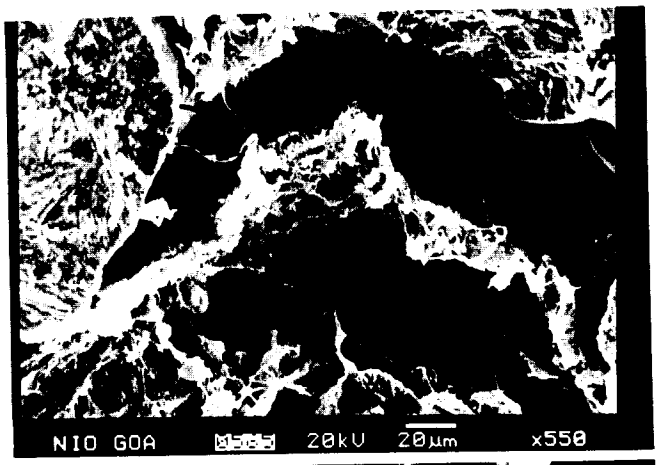


Fig. 4.12

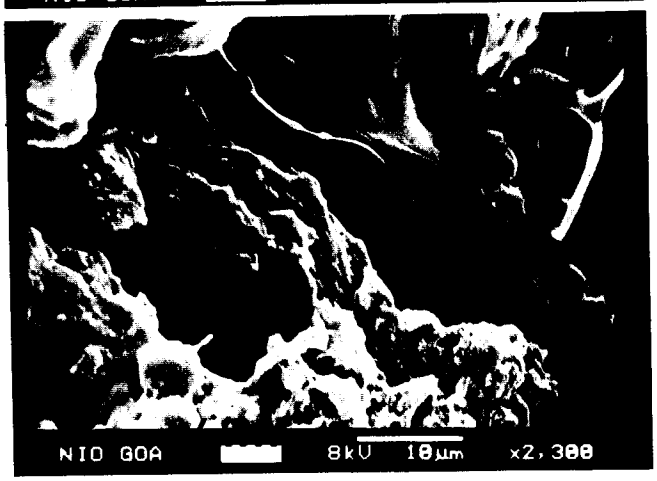


Fig. 4.13

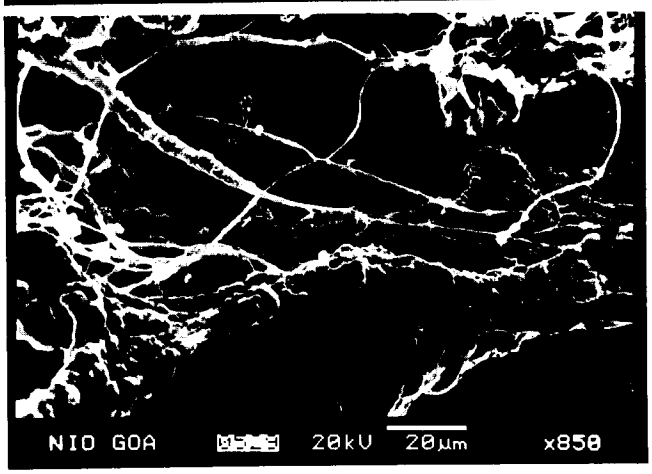


Fig. 4.14

Figs. 4.12 – 4.14. Fungal hyphae traveling between polyp (arrow) and carbonate skeleton (arrow head)

Fig. 4.15. Fungal hyphae seen around a polyp in a transverse section of pink-line syndrome (bar = 50 μm)

4.4. Discussion: -

Studies demonstrate that fungi are a regular component of corals (Kendrick et al. 1982). The present study was carried out over a period of two years and fungi were seen to be constantly associated with healthy-looking, partially dead, bleached and PLS-affected corals. They were found pervading to substantial depths within corals. Data presented show their distribution in corals up to a depth of 3 cm but they were observed occasionally at depths of nearly 7–8 cm and sometimes even deeper. No regular pattern of fungal distribution within corals was observed similar to the black banding seen in *Porites lutea* from Indonesian reefs (Bak & Laane 1987). Black bands in sections of *P. lutea* and *P. lobata* skeletons in Mayotte Island in the Mozambique Channel and Moorea Island in French Polynesia were attributed to the secretion of organic dark pigments by fungi and their conidia (Priess et al. 2000). In our study, we observed a highly patchy distribution of fungal mycelia within the corals. This resulted in a high variability between replicates used for calculating the total biomass, culturable numbers and frequencies as observed by immunofluorescence. Fungal biomass constituted up to 0.05 % of the weight of dead corals. This value is even higher than that reported by Raghukumar et al. (1995) for submerged detritus of leaves of the mangrove *Rhizophora apiculata* Blume (0.015 % of the detrital weight) in Zuari estuary in Goa. Fungal biomass of 3 – 5 mg per cm^3 has been reported in necrotic patches of corals from the Andaman Islands in the Bay of Bengal on the east coast of India (Raghukumar & Raghukumar 1991). Fungi, therefore, are apparently a component of coral skeleton.

Little is known about the diversity of fungi that inhabit corals. Certain 'obligate marine fungi' have been described from dead coral slabs (Kohlmeyer & Kohlmeyer 1987) and the term 'facultative marine fungi' has been coined to accommodate the possibility of terrestrial species of fungi having adapted to the marine environment. Raghukumar & Raghukumar (1998, 1999) hypothesised that species of terrestrial fungi are indeed active in the sea. Kendrick et al. (1982) have isolated species of *Aspergillus*, *Cladosporium* and *Acremonium* in culture from healthy *Porites* colonies in the Caribbean and the South Pacific. Fungi have been isolated from bleached corals of *Millipora complanta* (Te Strake et al. 1988).

In the present study, we have consistently cultured terrestrial fungi from corals under different health conditions. In addition, we have established the presence of the common terrestrial fungus, *Curvularia lunata* (#98 N-28) within healthy and PLS-affected corals by using polyclonal antibodies. This fungus was labelled as a dark non-sporulating (DNS) form in the initial isolations. Several other DNS forms were isolated in this study, although their identity is not known. However, it is possible that these too were terrestrial fungi, possibly belonging to *Curvularia lunata*. This study reiterates the hypothesis of Raghukumar and Raghukumar (1998, 1999) that terrestrial fungi are components of certain habitats in the sea at alkaline pH. Hyaline non-sporulating (HNS) forms were also frequent in the isolations. Immunofluorescence technique revealed the frequent presence of one of these isolates ((#98 N-18) within corals. Presence of such non-sporulating fungi has also been reported by Kendrick et al. (1982) in healthy corals. The terrestrial fungus *Aspergillus sydowii*, causes rapid erosion and death of sea

fans corals in the Caribbean (Smith et al. 1996). More recently, Shinn et al. (2000) reported the presence of terrestrial fungi, especially spores in the dust particles that got blown from Africa to Caribbean, from where they might settle on the coral and trigger mass mortality in coral reef.

In view of the above, the behaviour and role of fungi within healthy and diseased corals merits detailed investigations. In this study, fungi were found within the calcium carbonate skeleton and around polyps. SEM studies showed entry of fungi from the coral carbonate skeleton to the polyps, presence of amorphous 'repair carbonate' around the site of fungal penetration and precipitation of such 'repair carbonate' around the fungal hyphae. Le Campion et al. (1995a) explained this phenomenon of fungal growth and the defence mechanisms of the polyp. During their process of penetration of coral skeleton, their role as bio-eroding agents has been confirmed (Perkins & Halsey 1971, Zeff & Perkins 1979, Le Campion et al. 1995a, 1995b). Fungi in molluscan shells were capable of leaching calcium 3 times more than in control shells without fungi (Raghukumar et al. 1988). The high abundance and biomass of fungi observed by us in corals indicate their potential importance as bio-eroders of calcium carbonate skeletons in the sea. While bio-eroding activities of fungi in dead, or diseased portions of corals is understandable, the possibility of such an activity in normal, healthy corals appears paradoxical.

Priess et al. (2000) observed formation and germination of a conidium inside the coral skeletons of *Porites lutea* but this was noticed rarely in this study. This observation, plus the fact that non-sporulating forms were common in corals suggests that these fungi might survive and propagate by

means other than spore formation, such as hyphal fragments (Raghukumar and Raghukumar 1999).

The possible pathogenic role of fungi associated with polyps in the pink-line zone of corals could not be unequivocally demonstrated in the present study although dense fungal hyphae were found around polyps. Fungi as pathogenic organisms are described for *Aspegillus sydowii* in sea fans, causing mass mortalities (Smith et al. 1996). Association of an unidentified lower fungus with black-band disease in star corals (Ramos-Flores 1983) and necrotic patches in massive corals (Raghukumar & Raghukumar 1991) have been reported, without proof of their pathogenicity. Further studies are required to examine the role of fungi in coral pathogenicity. One possibility is that normal residential fungal flora, and not necessarily extraneous invaders may become opportunistic pathogens under climate variability and anthropogenic stress, as reported for mangrove disease, induced by cyanobacterial symbionts (Rützler 1988).

In conclusion, this study substantiates earlier studies on the presence of fungi in corals and demonstrates that fungi constitute residential flora within corals. The constant presence, pervasive distribution of fungi and a high biomass within the polyp zone confirm this. Fungi have been reported to be associated with micrite tubules of the Devonian era (Kobluk & Risk 1974) and modern carbonate sediments (Perkins & Halsey 1971, Perkins & Tsentas 1976). Association of fungi with present-day corals need not be the effect of modern anthropogenic effects, but the effect of long-term evolutionary association.

5. Isolation, identification and purification of cyanobacteria associated with PLS-affected coral colonies:

5.1. Introduction: -

Ever since the first cyanobacterial disease that causes the black- band disease in corals was reported (Antonius, 1973), many workers were stimulated to discover diseases of corals in different reefs all over the world (Green and Bruckner 2000). In the case of the pink line syndrome, cyanobacteria were seen to be associated with corals adjoining the affected tissue (Ravindran et al. 1999, 2001). However, the various interactions between the cyanobacteria and the coral tissue in PLS are not known. The aim of the present study was to isolate and identify the cyanobacterium associated with the pink line syndrome in order to verify its capability to cause the pink line syndrome in the coral *P. lutea*.

5.2. Methods: -

PLS-affected specimens were collected and sealed in self-sealing polyethylene bags individually under water in the field. They were transported to the field laboratory within an hour, submerged in a bucket of seawater to minimise any temperature rise that may cause stress on the collected colonies. In the laboratory, small portions, approximately a cm^2 of the specimens with pink line were trimmed and washed twice in sterile seawater. These pieces were placed in a tube containing sterile ASN III medium (McLachlan 1973) (see Annexure) and shipped to the main laboratory. The tubes were kept at 12 hours photoperiod using artificial illumination while transporting to the laboratory. These samples were transferred to 100 ml conical flask containing ASN III medium and incubated at 25° C. The culture

flasks were kept close to a window to enable natural light with an average photoperiod of about 10 hrs available to the samples. These flasks were not put under direct sunlight to avoid heating of the medium. They were left undisturbed till the cyanobacterial mat was formed, which took about three weeks to cover the surface of the medium in the flask. This primary culture was subcultured onto ASN III medium with 1.5 % agar. Growing tips of the cyanobacterium was excised and transferred to fresh ASN III broth. The process of transfer to a broth and agar medium was repeated several times, till bacteria-free cultures were obtained. The absence of bacteria was confirmed by phase contrast microscopy and transferring the culture to a nutrient broth and checking for turbidity and bacterial growth. The resulting axenic culture of the cyanobacterium was transferred to ASN III with 1.5% agar slants and sent to National Facility for Marine Cyanobacteria (NFMC), Tiruchirapalli, India for identification.

5.3. Results: -

The cyanobacterium isolated from the pink line syndrome was identified to be *Phormidium valderianum* ((Delp.) Gomont (Fig.5.1). The thallus of the *P. valderianum* was lubricious, formed coils with a bunch of thallus in culture. The thallus was lamellated with dull, green outer surface. The inner surface was colourless. The trichomes were flexuous, densely entangled. They were not constricted at the cross-walls. The ends were not attenuated. The cells were broader than its length with 2.7 μm wide and 4 μm long cells. Axenic cultures were maintained in ASN III medium for further studies.

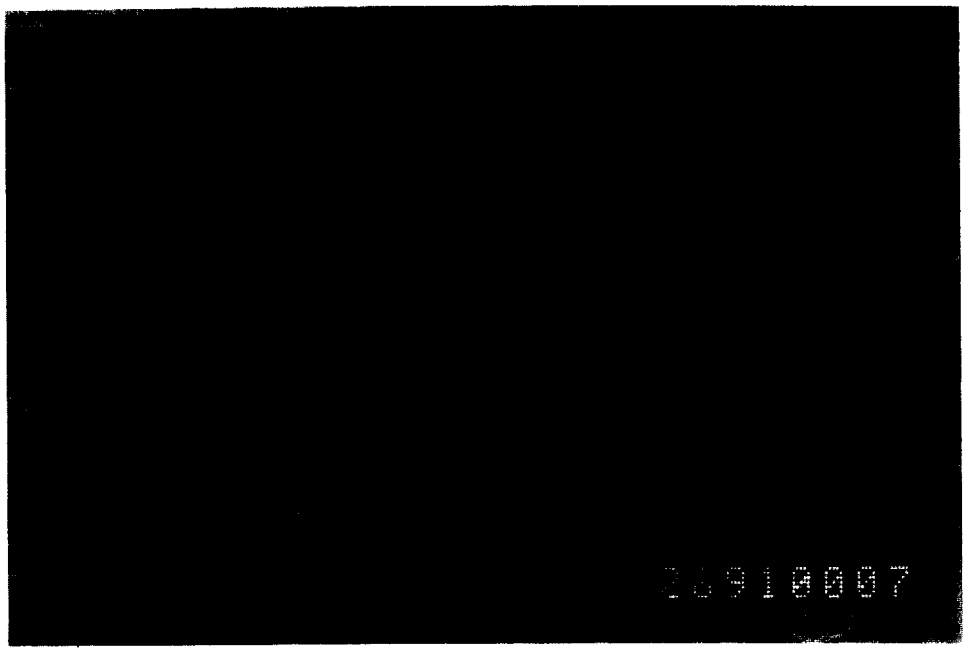


Fig. 5.1. *Phormidium valderianum* as observed under bright field microscopy (bar = 10 μm)

5.4. Discussion: -

The present study records the association of the cyanobacterium *Phormidium valderianum* with the scleractinian coral *Porites lutea* in the Lakshadweep (Ravindran and Raghukumar 2002). This is the first report of the presence of this cyanobacterium in diseased corals anywhere in the world. *Phormidium valderianum* is a common blue-green alga that is distributed all along the Indian coast, being found in open sea, stagnant seawater, backwaters and salt pan (Subramanian et al. 1989; Thajuddin and Subramanian 1992, 1994). Random microscopic observations of the dead corals and other substrates in the lagoon showed that this cyanobacterium is found to be most prevalent in the Kavaratti lagoon. It was found in mucus balls of corals as almost a mono-algal occurrence. In PLS affected colonies, the dead patches were colonised exclusively by *P. valderianum*, thus making it easy to bring it into culture. It is not clear at the moment whether the cyanobacterium colonises the dead patches of the coral as a secondary invader or is a parasite, responsible for PLS. However, unlike the black band disease, where the cyanobacterial colony overgrows the tissue and kills the polyps, *P. valderianum* in PLS affected colonies was never found growing aggressively on the polyp tissue (Ravindran and Raghukumar 2002) and this species is not reported to cause any disease so far.

6. Histology of Healthy and PLS-affected corals:

6.1. Introduction: -

The sensitivity of the scleractinian coral, *Porites* sp., to environmental changes has been considered as a reference for the effects of global atmospheric changes on coral reefs (Edmunds, 1989). Alutoin et al. (2001) have used this coral as a reference species to study the effect of stresses in the environment. *Porites lutea* is one of the major species of scleractinian corals in the Lakshadweep reefs. Any factor that alters the live coverage of this species can potentially result in a phase shift that may convert the coral bed into an algal bed, since several algal species colonise dead coral skeletons (Wellington and Victor 1985; Glynn 1990). Environmental stresses and diseases will be reflected in changes at the cellular level. Therefore, the aim of this study was to observe cellular changes associated with the PLS-affected corals with reference to apparently healthy corals.

6.2. Methods: -

6.2.1. Collection of samples: -

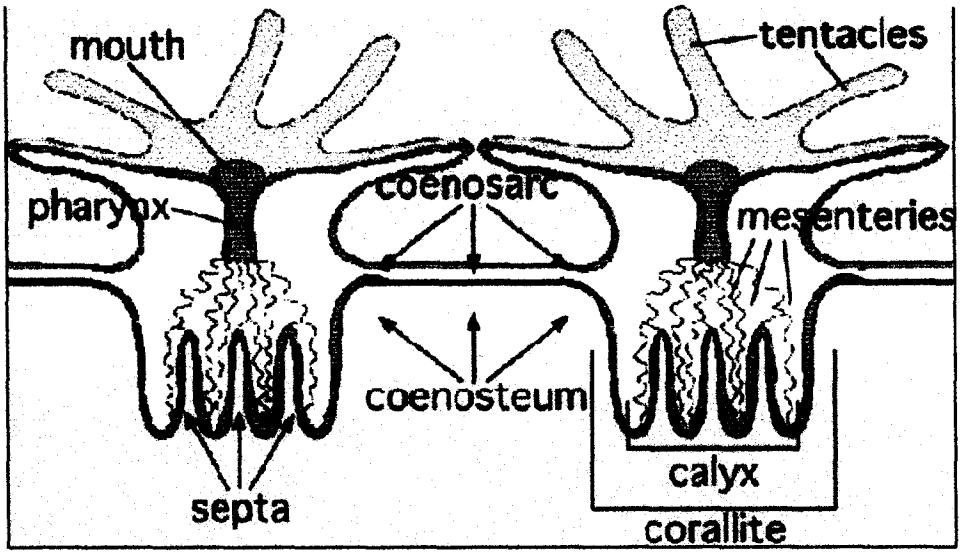
Coral specimens of *Porites lutea* affected by the PLS and healthy colonies were collected from the lagoon in Kavaratti island and were transported in a bucket of seawater to the field laboratory. These specimens were fixed and preserved following the method by Ravindran et al. (2001). Specimens were first fixed in 3% formaldehyde for 24 hours, following which they were transferred to 10% formaldehyde for 48 hours. After the fixation, the samples were washed with fresh tap water several times followed by several washes with 70% ethanol. They were stored in 70% ethanol and transported to the main laboratory.

6.2.2. Histology: -

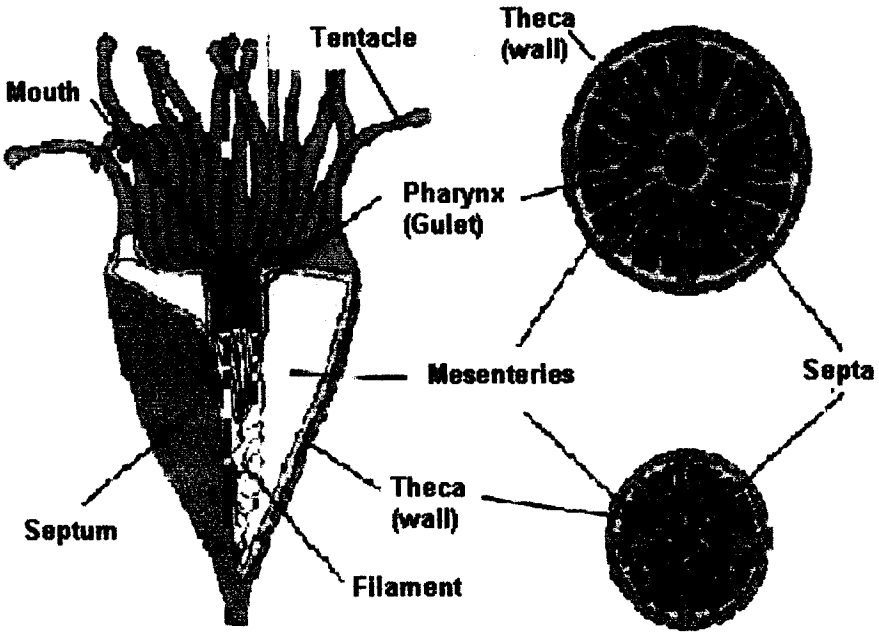
Healthy and PLS-affected specimens preserved in 70% ethanol were cut into small pieces of 0.5-1cm^2 sizes. These were placed in a glass vial and decalcified in a decalcification solution containing 1:1 mixture of 50% formic acid and 20% citric acid (Ravindran et al. 2001), till the entire tissue was released from the skeleton within 3-4 hours. The decalcified specimens were washed several times with distilled water to remove salts and was dehydrated in 30%, 50%, 70%, 90%, 95% and 100% ethanol for 10 minutes each and cleared with 100% acetone. The decalcified tissues were embedded in paraffin wax blocks, following standard histological procedures. The blocks were trimmed and mounted on a specimen holder of the microtome. Thin sections of the size 6 µm were cut using a rotary microtome (Weswox, India, Model 1090A) and double-stained with heamatoxylene (Appendix) and eosin (Appendix). The sections were observed under light microscope (Olympus BX60, Japan) and the cellular details were recorded by photomicrography.

6.3. Results: -

The coral colonies comprising individual polyps are interconnected through a narrow tissue called coenosarc (Fig. 6.1). The polyp that is tubular (Fig. 6.2) in nature has a wall comprising three dermal layers namely, the ectoderm or calicobastic cells that secretes calcium carbonate (the outer layer), the mesogloea (the middle layer) and the gastroderm (the inner layer) (Fig. 6.3). Histological examinations of the PLS affected tissue show that the tissues were degenerated and destroyed. The basic architecture of the three dermal layers was affected (Fig. 6.4, 6.5, 6.6). Most of the calicoblastic epithelium in the PLS-affected tissue was degenerated (Fig. 6.7). The



6.1. Schematic diagram of the coral anatomy



6.2. Schematic diagram of a coral polyp

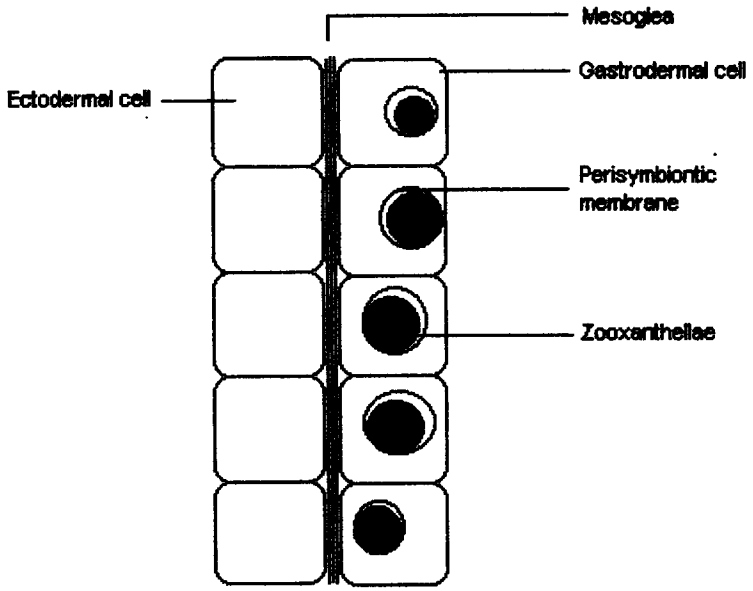


Fig. 6.3. Schematic diagram of the dermal layers of the coral polyp

Fig. 6.4. Dermal layers as observed in the sections of healthy polyp (bar = 25 μm); EC = Ectoderm, GS = Gastroderm

Fig. 6.5. Destroyed tissue of the PLS-affected polyp (bar = 100 μm) EC = Ectoderm, GS = Gastroderm

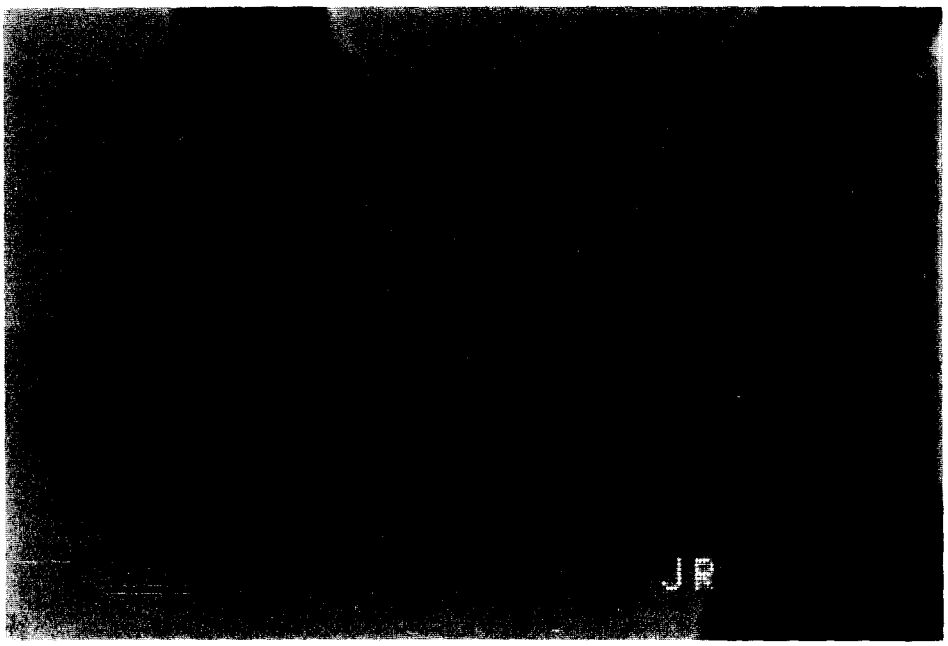


Fig. 6.6. Disturbed dermal layers of a PLS-affected polyp (bar = 10 μm) EC = Ectoderm, GS = Gastroderm

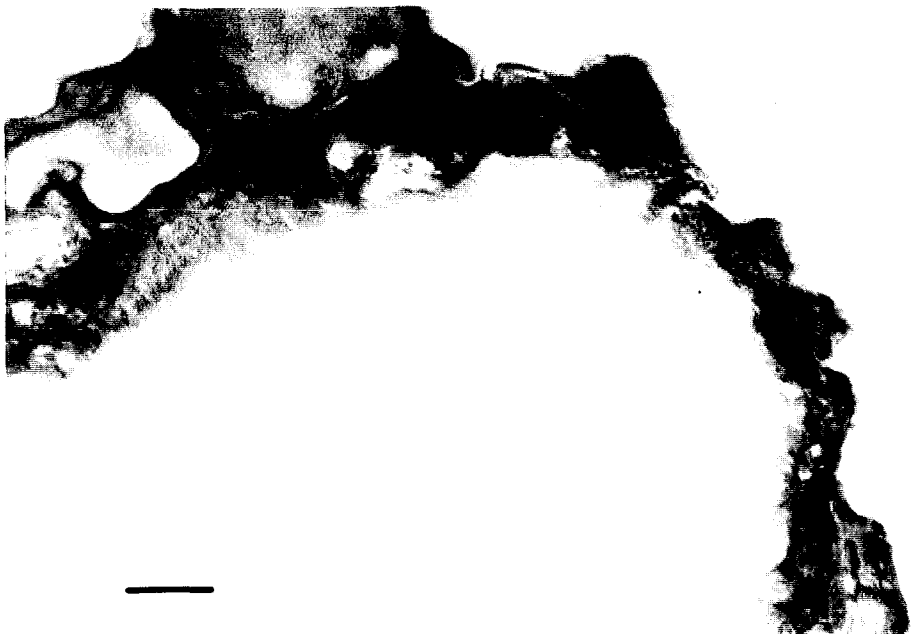


Fig. 6.7. Disturbed calicoblastic epithelium (CL) in the PLS-affected polyp. Bar = 10 μm, GS = Gastroderm

Fig. 6.8. Granular pockets of the calicoblastic epithelium in the PLS-affected polyp (arrow). Bar = 10 μm

gastrodermal layer bordering the calicoblastic layer showed extensive degeneration and stained dark with haematoxylin (Fig. 6.6, 6.7). The ectodermal and calicoblastic layers did not have the normal cellular organisation and appeared granular (Fig. 6.6. 6.7). During the initial stages of destruction, the granules appeared as a layer and the granules were eosin positive (Fig. 6.6, 6.7). These granules were separated into small clusters of the size 7.6 µm (Fig. 6.8). These granular packets get detached from the ectodermal layer and finally the ectodermal layer disappears. The most prominent feature was the presence of large vacuoles in most of the gastrodermal cells having zooxanthellae (Fig. 6.9). A vacuole appeared initially around the zooxanthellae in most of the gastrodermal cells (Fig. 6.10). It is not clear whether the vacuole is present in the cytoplasm or between zooxanthellae and perisymbiontic membrane. The vacuole expanded and made the entire cell appear empty with only zooxanthellae in it (Fig. 6.10). Another prominent feature of the gastrodermal cells was that they were swollen and their cytoplasm did not take the stain (Fig. 6.11). Some of the swollen cells in the gastrodermal layer contained brown non-staining patches (Fig. 6.11). Both ectodermal and gastrodermal cells showed swelling and necrosis (Fig. 6.12). These swollen cells are yellowish to brownish green in colour and non-staining (Fig. 6.12). During degeneration of the gastrodermal layers, the zooxanthellae were released into the coelenteric cavity through various mechanisms. This included gastrodermal cell detachment (Fig. 6.13), as well as zooxanthellae expulsion from the lysed gastrodermal cells (Fig. 6.14) thereby releasing the zooxanthellae into the coelenteric cavity. The gastrodermal cells that detach were mostly those that bordered the

Fig. 6.9

Fig. 6.10

Figs. 6.9 & 6.10. Large vacuoles around the zooxanthellae (arrow). Bar = 10 μm

Fig. 6.11. Gastrodermal cells (GS) of the PLS-affected tissue showing swollen, non-staining and brown non-staining patches. Bar = 10 μm, EC = Ectoderm

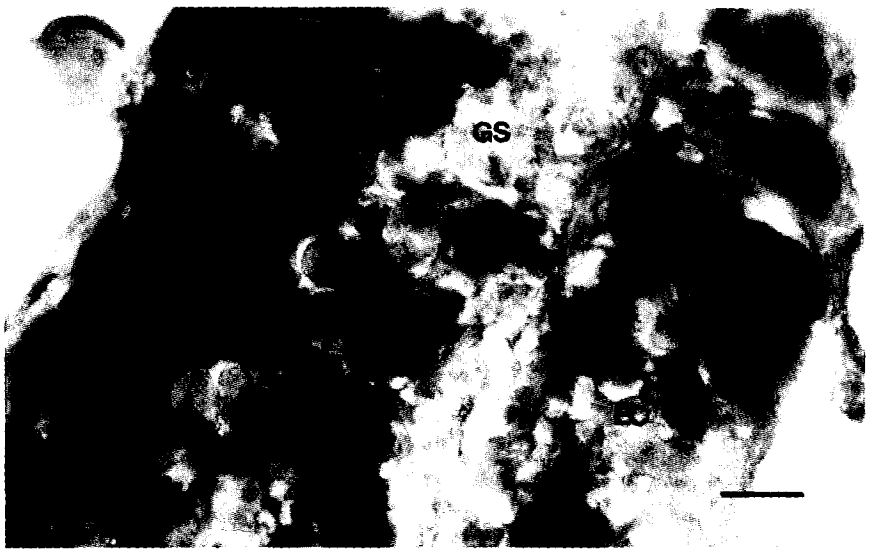


Fig. 6.12. Cells in the ectoderm and gastroderm showing necrosis and brown non-staining patches. Bar = 10 μm, EC = Ectoderm, GS = Gastroderm

Fig. 6.13

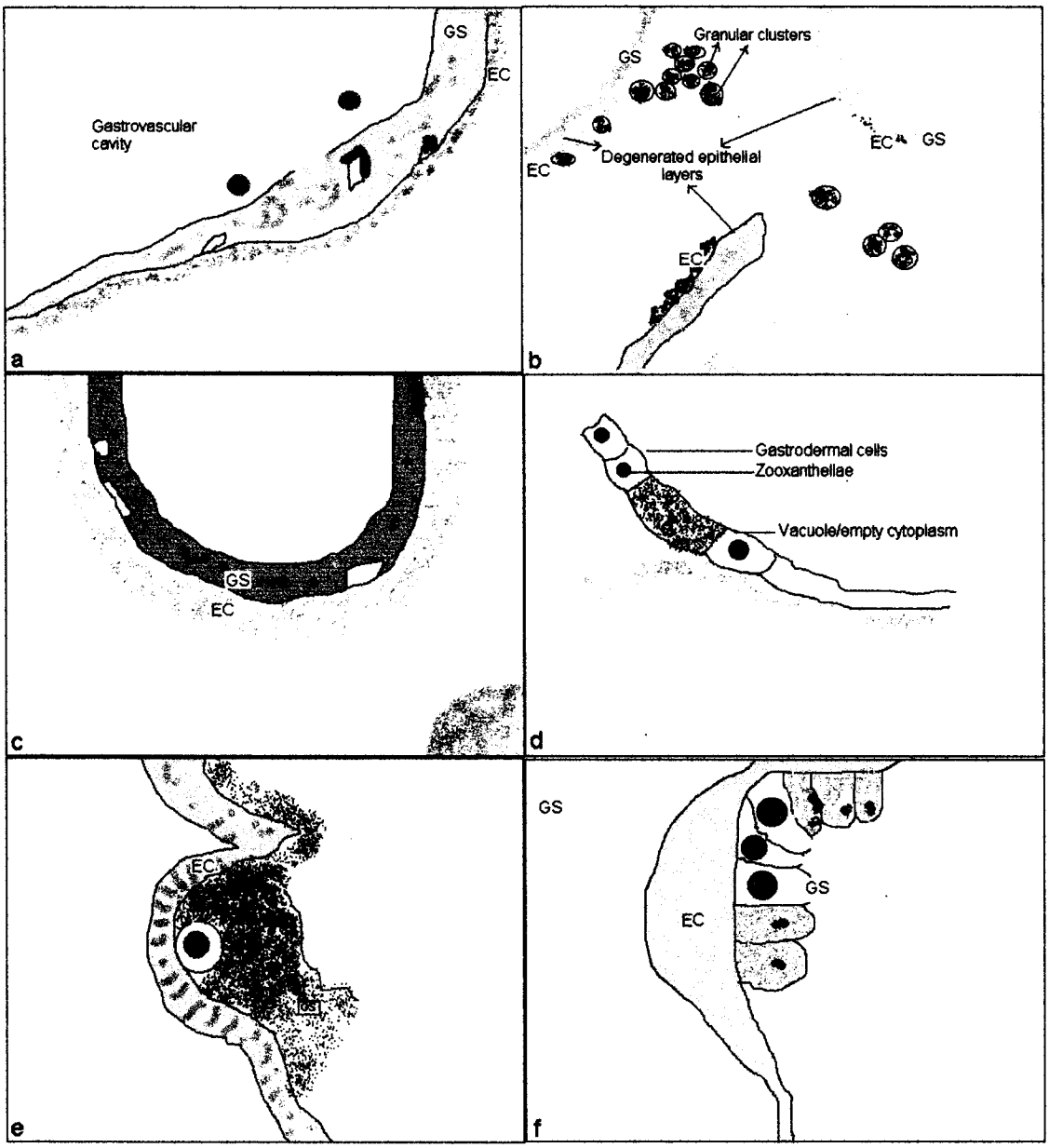
Fig. 6.14

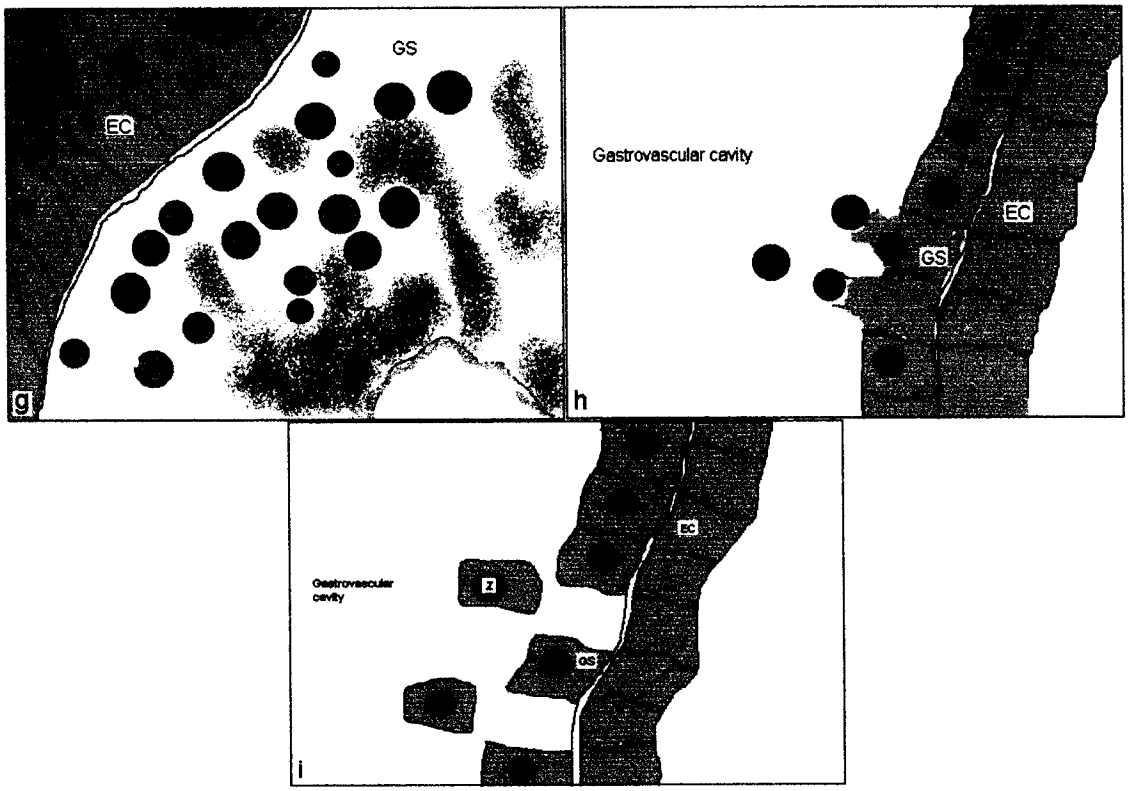
Figs. 6.13 & 6.14. Detachment of gastrodermal cells (GS) and expulsion of zooxanthellae (Z) PLS-affected tissue. Bar = 10 μm

Fig. 6.15. Shrinkage of the cytoplasm (arrow) and empty ectodermal (EC) cells. Bar = 10 μm

Fig. 6.16. Cyanobacteria (arrow) in the debris of PLS-affected polyp (bar = 100 μm)

Fig. 6.17. Schematic representation of events leading to the pink-line syndrome in *Porites lutea*





Note: GS= Gastroderm, EC= Ectoderm, Z= Zooxanthellae

Fig. 6.17a. Zooxanthellae release from the degenerated gastroderm

Fig. 6.17b. Granular clusters in the degenerated ectoderm

Fig. 6.17c. Degenerated calicoblastic ectoderm & gastroderm

Fig. 6.17d. Empty cytoplasm/large vacuole in the gastrodermal cells

Fig. 6.17e.

& 6.17f. Swollen gastrodermal cells

Fig. 6.17g. Browning reactions in the ectoderm and gastroderm

Fig. 6.17h. Zooxanthellae release from the ruptured gastrodermal cells

Fig. 6.17i. Zooxanthellae release to the gastrovascular cavity by the host cell detachment

ectodermal epithelium. The ectodermal layers showed shrinkage of cells with darkly stained nuclear material and because of the shrinkage, this layer appeared empty (Fig. 6.15). Besides all these abnormalities, cyanobacterial filaments were also noticed in association with the debris of the coral tissue in the PLS affected tissue (Fig. 6.16)

6.4. Discussion: -

The PLS-affected tissue of *Porites lutea* showed the following histological abnormalities (Fig 6.17).

1. The calicodermal cells were destroyed and replaced by granular layer.
2. Some of the ectodermal cells were shrunk and others were swollen and showed necrosis
3. The gastrodermal cells, in addition to the swelling and necrosis, expelled their zooxanthellae.
4. Gastrodermal cells and ectodermal cells were highly vacuolated.
5. Invasion of cyanobacteria

The fine balance between cell proliferation and cell death is regulated through a process termed programmed cell death or apoptosis (Thomson 1995). A feature of apoptosis involves cell shrinkage, nuclear and cytoplasmic condensation, membrane blebbing, chromatin compaction and fragmentation of chromosomal DNA (Ryerson and Heath 1996). The appearance of the calicoblastic layer as granular pockets that stains acidic (eosin positive) suggests that it consists of cellular debris. These granules are cytoplasmic fragments, bound by a membrane. This is characteristic of apoptic bodies and the calicoblastic layer itself may be undergoing apoptosis. The membrane bound nature of the granules in the early

stages, loss of the membrane boundary in the later stages and the acidophilic staining of the granules strongly suggests that these granules are apoptic bodies of the calicoblastic layers. The apparent cause for the apoptosis of the calicoblastic cells seems to be acidification of the extracellular space by the presence of the cyanobacterium *Phormidium valderianum*.

Many aquatic photosynthetic microorganisms including cyanobacteria possess an inorganic carbon concentrating mechanism (CCM) that raises the CO_2 concentration at the intracellular carboxylation sites, thus compensating for the relatively low affinity of the caboxylating enzymes for its substrates. In many cyanobacteria like *Synechococcus* sp., *Nannochloropsis* sp and in the diatom *Thalassiosira weissflogii*, the CO_2 efflux were reported during the photosynthesis (Tchernov et al. 1997) and it had been generalised for all the cyanobacterial cells by Badger et al. (2002). The acidic environment caused by the CO_2 efflux from the *P. valderianum* may dissolve the coral skeleton, causing higher concentration of Ca_i^{2+} (inorganic calcium ion). The influx of this Ca_i^{2+} is likely to cause elevated cytosolic Ca_i^{2+} and may stimulate the K^+ efflux. This, in turn, could result in cell shrinkage that probably requires ATP, whose depletion through these processes may result in apoptosis (Trump 1996).

Calcium stains blue with haematoxylin (Luna and Harris 1992) and therefore the dark stained gastrodermal cells and the gaps between the gastrodermal cells might be due to the accumulation of calcium. This shows the abnormal accumulation of the calcium in the intracellular spaces and intercellular junctions of the gastrodermal cells. Increase in the

Ca_i^{2+}, as observed in the gastrodermal cells causes other deleterious effects including activation of proteases, endonucleases and phospholipases that causes DNA breaking (Trump 1996). Vacuolation observed in the gastrodermal and ectodermal cells may reflect increased pinocytic activity, as reported by Papathanassiou (1985) in the brown shrimp *Crangon crangon* (L) that were exposed to the elevated cadmium ions. Appearance of large vacuoles in these cells may be caused by the damage to the osmotic balancing mechanism as reported in the crab *Carcinus maenus* that was exposed to the copper toxicity (Hebel et al. 1999). The osmotic imbalance and necrosis of the gastrodermal cells may cause detachment of the entire layer (Hebel et al. 1999). This could have been the cause of the fragile coral skeleton observed around the PLS affected coral polyps. In addition, nutrient translocation from zooxanthellae to the host may be poor or impaired by the higher zooxanthellae mitotic index, as the nutrients will be utilised by the dividing zooxanthellae.

The Ca^{2+} efflux from the calicoblast cells to external calcifying fluid (ECF) is an energy dependant process (Gattuso et al. 1999). When the nutrient translocation from zooxanthellae to the corals is affected due to higher zooxanthellae proliferation, the calcification is reduced. Elevation of intracellular free calcium will cause cell damage (Trump 1996). The impairment of calcium efflux in the PLS-affected tissues will raise the intracellular free calcium as observed in the gastrodermal cells that stain with haematoxylin and that could lead to cell death. It is hypothesised that the dissolution of skeleton may be proportional to the number of zooxanthellae in corals, as observed in the boring sponge, *Anthosigmella*

varians (Hill 1996). If this mechanism exists, any break down of host control over the maintenance of zooxanthellae number may turn the mutualism into parasitic mode.

7. Pigments in healthy and PLS-affected colonies and the status of zooxanthellae:

7.1. Introduction: -

Diseases of corals cause devastating effects on coral reefs. Since the first report of black band disease (BBD) in corals from the Red Sea (Antonius, 1973), coral diseases have been recognised based on the change of color on the affected colony either in the form of a line or a dead patch. The diseases reported till now include the black band disease (Antonius, 1981a), the white band disease (Gladfelter 1982), the white plague (Richardson, 1998), the yellow band disease (Hayes and Bush, 1990) and the red band disease (Rützler and Santavy, 1983). These have been named after the colour of the line or patch on the diseased colonies. These diseases are either caused by microbial pathogens or unknown abiotic agents as in the shut down reaction (Antonius, 1981b). In addition to the destruction of corals through direct human activity, diseases in scleractinian corals becomes a matter of concern, since destruction by diseases is one of the major contributors to reef degradation (Richardson, 1998).

Lack of study on the physiology of the corals in diseased state was brought to the limelight during the 9th International Coral Reef Symposium in Bali (Smith, 2000). Changing environment causes the corals to succumb to physiological disorders. Other than the well-known physiological disorders like bleaching event, other physiological disorders have neither been described nor reported so far in corals. During a survey in Kavaratti and Kadamat islands in the Lakshadweep, it was found that the scleractinian coral *Porites lutea* showed extensive dead patches in sizes ranging from a few millimetres

to a centimetre with a characteristic pink line surrounding the dead patch. We have reported the appearance of a pink line in the interface of the dead and the healthy looking portions of *Porites lutea* in the Lakshadweep (Ravindran et al. 1999).

Biochemical and physiological changes that prevail during the formation of the pink-line in the PLS-affected colonies of *Porites lutea* were investigated. The sensitivity of the *Porites* sp. to environmental changes has been considered as a reference for the global atmospheric changes (Edmunds and Davies 1989). Since *Porites lutea* is conspicuous and is one of the major species in the Lakshadweep reefs, investigations on the responses of this species to the changing environment could provide valuable information. To understand the status of the symbionts in the PLS-affected polyps, zooxanthellae growth patterns and the concentration of photosynthetic pigments such as chlorophyll a, chlorophyll c were estimated. The coral *Pocillopora damicornis* appears pink because of the presence of a pink colour pigment pocilloporin in the coral polyps (Takabayashi and Hoegh-Guldberg 1995). In order to find the possible production of this pigment in response to the interaction of the polyps with the *P. valderianum*, the presence of pocilloporin was investigated in the polyps of the PLS-affected specimens and the healthy specimens.

7.2. Methods: -

7.2.1. Zooxanthellae status: -

7.2.1.1. Mitotic index: Mitotic Index of the zooxanthellae in healthy and PLS-affected specimens from ten different colonies was determined as described by McAuley (1994). Preserved samples of PLS-affected and control specimens were sub-sampled and decalcified in a decalcifying solution

containing 1:1 ratio of 20% citric acid 50% formic acid (Ravindran et al. 2001). The decalcified tissues were homogenised in a pestle mortar with 1 ml of filter sterilised seawater. The tissue was homogenised and the homogenates were centrifuged to pellet the zooxanthellae leaving the animal tissue in suspension. The pellets were resuspended in fresh filtered seawater and filtered through a 60 µm mesh plankton net to remove the animal debris. The number and the mitotic index (MI) were counted using a Neuboyer Haemocytometer (Wilkerson, 1983). Mitotic index is the % of zooxanthellae cells in doublets.

Specific growth rate (µ) and doubling time (T) was calculated from the mitotic index (MI) as described by Wilkerson et al (1983). The duration of the cell division (t_d) is taken as 11 h as calculated by Wilkerson et al (1983) for asynchronous divisions.

$$\mu = 1/t_d \ln (1+ MI) \text{ divisions day}^{-1}$$

$$T = \ln 2/\mu \text{ days}$$

Cell size of the zooxanthellae was measured under a microscope, using a calibrated ocular micrometer. Doubling time (T) of the zooxanthellae was calculated from the MI (Wilkerson et al. 1983).

Zooxanthellae numbers in the PLS-affected specimens and healthy looking colonies were counted microscopically, using a calibrated ocular grid. The number of zooxanthellae in 50 random quadrants (eyepiece grid) were counted in sections having thirty polyps and the numbers were then normalised for cm^{-2} area.

7.2.1.2. Pigments: The PLS-affected and healthy coral colonies collected from the field were frozen and transported in ice to the main lab. The

methodology as proposed by Myers et al. (1999) was followed. The tissues from the pink line region were scraped using a clean scalpel and placed in preweighed tubes, containing GF/C filter paper pieces to break the cells effectively during homogenisation. The tubes were stored at -20° C till further processing. The tissues were crushed in acetone using a glass rod inside the tube and were centrifuged at 2000 rpm for 10 minutes for the suspended particles to settle. The extracts were scanned in a scanning spectrophotometer (Perkin-Elmer, Lambda 40, USA) in visible range from 400-700 nm and the chl *a*, and chl *c* and carotinoid concentrations were calculated according to the equations of Parsons et al. (1984).

Pocilloporin estimation was carried out according to Takabayashi and Hoegh-Guldberg (1995). About 10 g wet weight of the coral pieces were taken and they were put in 10 ml of cold 0.06 M phosphate buffer (Annexure) for 24 h in the refrigerator. The extract was scanned from 200 nm to 750 nm in the spectrophotometer (Perkin Elmer, Lambda 40, USA). Concentration of the pigment is calculated using the peak value at 560 nm and the extinction coefficient of the pocilloporin mentioned by Dove et al. (1995). The extinction coefficient of the pocilloporin is 34059 + 1635 cm^{-1} m^{-1}. Pigment contents were standardised to surface areas.

7.3. Results: -

The reduced no of zooxanthellae in PLS-affected polyps in-spite of the reduced doubling time indicates that the zooxanthellae are expelled at an equal rate and thus reducing the gross total number of zooxanthellae.

Table 7.1. Zooxanthellae status

	Healthy	PLS
Specific growth rate (μ)	0.13±0.06	0.15±0.08
Doubling time in days (T)	2.55±.078	2.06±0.99
Number cm^{-2} in the colony	$2.7x10^6±1.4x10^6$	$0.7x10^6±0.6x10^6$
Number cm^{-2} in sections	17780±9256	8320±5754
Size in whole tissue preparation (μm)	7.45±0.76	8.75±1.4
Size in sections (μm)	6.2±1.08	7.6±1.2
MI	3.93±2.9	5.75±4.2
Chl *a* (μg cm^{-2})	13.8	54.8
Chl *c* (μg cm^{-2})	10.2	7.2
Carotenoids (μg cm^{-2})	0.009	0.011

Table 7.2. Single factor analysis of variance (ANOVA) between healthy and PLS-affected tissue for various parameters

Parameters	Significance (P)	*df*	F value	F critical
Specific growth rate(μ)	0.298	1, 58	1.1^{NS}	4.01
Doubling time (T)	0.04 *	1, 58	4.6	4
Zooxanthellae number cm^{-2} of the colony	2.2×10^{-9} ***	1, 58	50	12
Zooxanthellae number in cm^{-2} of sections	2.2×10^{-8} ***	1, 98	37.1	11.5
Mitotic Index (MI)I	0.06	1, 58	3.8^{NS}	4
Zooxanthellae size in whole tissue preparation	1.3×10^{-7} ***	1, 98	32.3	11.5
Zooxanthellae size in sections	4.9×10^{-8} ***	1, 98	34.9	11.5
Chl *a*	6×10^{-6} ***	1, 18	39.9	15.4
Chl *c*	0.32	1, 18	1.04^{NS}	4.4
Caroteinoids	0.72	1, 18	0.1^{NS}	8.3

Note: *df*= degree of freedom, Significant at * = 0.05, ** = 0.01, * = 0.001**
NS = nonsignificant, F value > F-critical denotes statistical significance

The results (see tables 7.1 & 7.2) of the zooxanthellae status shows significant variation in doubling time (T), zooxanthellae density in sections and whole colony preparation, zooxanthellae size in sections and in whole tissue preparation. No Pocilloporin peak was detected at 560 nm in the extracts of

PLS-affected polyps and healthy corals. Chl *a* varied significantly in the PLS-affected colony from the healthy one. The mitotic index (MI) and specific growth (μ) did not vary significantly. In pigments, the chl *c* and carotenoids did not vary significantly.

7.4. Discussion:

The doubling time (T) varied significantly but this was not reflected in the mitotic index (Table 7.2). The results further show that the difference in the μ (specific growth rate) is not significant. The μ is used in the calculation of the T using natural logarithms. So, a slight increase in MI will magnify the T value. There could have been high MI in the PLS-affected polyps than what was observed but this was probably not detected because of the heavy loss of the zooxanthellae from the tissue through expulsion as observed in the chapter 3 (Fig. 3. 7).

Zooxanthellae division in the coral is non-phased (asynchronous). The non-phased growth of zooxanthellae is due to the host control on the zooxanthellae division (Wilkerson et al. 1998). The non-phased growth of zooxanthellae is controlled by the host to maximize the supply of photosynthate from zooxanthellae to their host (Belda-Baillie et al. 1993).

The zooxanthellae in the PLS-affected polyps are larger in size than the healthy ones but shows comparatively higher MI (Table 7.1). The zooxanthellae in the host cell are under nutrient limited (Wilkerson 1983; Hoegh-Guldberg 1996) and carbon limited condition (Weis 1993;Goiran 1996). It has been found in the giant clam exposed to enriched nutrients, that the zooxanthellae population was smaller in size and this was attributed to the faster division of the zooxanthellae (Hoegh-Guldberg 1996; Belda-Baillie et al.

1998). Therefore, when the host control on zooxanthellae division is removed by supplying external nutrient and carbon, the zooxanthellae division is faster and the resulting zooxanthellae will be smaller in size. Smith (1986) showed that when only carbon limitation is overcome, the zooxanthellae show only increase in size because P & N are required for the cell division.

There are many reasons for the induced MI in the PLS-affected polyps. Zooxanthellae are carbon limited (Weis 1993; Goiran 1996) and nutrient limited (Wilkerson 1983) in host cells. Excess availability of nutrients and carbon source to the zooxanthellae will overcome the growth limitation. In PLS-affected polyps, the cyanobacterium that is present adjacent to the pink line may be able to supply excess inorganic carbon through carbon concentrating mechanism. Many aquatic photosynthetic microorganisms including cyanobacteria possess inorganic carbon concentrating mechanism (CCM). The HCO_3^- is taken from the ambient water to the cytoplasm. In the cytoplasm, the HCO_3^- is converted into CO_2 by the enzyme carbonic anhydrase (CA) that is in close proximity to the carboxylation site (Tchernov et al.1997). In many cyanobacteria such as *Synechococcus* sp., *Nannochloropsis* sp. and in the diatom *Thlassiosira weisflogic* the CO_2 efflux was reported during photosynthesis (Tchernov et al. 1997). In general, the CO_2 efflux is found in cyanobacterial cells during photosynthesis (Badger et al. 2002). It can be hypothesized at this point that CO_2 efflux from *P. valderianum* in proximity to the PLS-affected polyp, might cause CO_2 influx into the coral tissue. In case of this CO_2 influx into the coral tissue, the carbon-limited zooxanthellae as shown by Smith (1986), will show increased size as was also noticed in the present study.

Bleaching in corals is due to the loss of photosynthetic pigments (Sharp, 1995; Fitt and Warner, 1995) or loss of zooxanthellae (Fitt and Warner, 1995; Lesser et al. 1990). Pigment concentrations are altered in bleached samples mainly because of the reduction in porphyrins, chlorophyll *a* (chl *a*) and chlorophyll *c* (chl *c*) (Ambarsari et al. (1997) or by the sole increase of chl *a* (Jones 1997) or decrease of chl *c* (Lee-shing Fang et al. 1995). A significant reduction in chl *a* concentration was observed in the bleached coral tissues (Le-Tissier and Brown, 1996; Jones and Yellowlees 1997). The effect of solar radiation in the reduction of chl *c* (Lesser et al. 1990; Le Tissier and Brown, 1996) and the protective role of the xanthophyll cycle in corals to prevent radiation related damage (Brown et al. 2000) have been described. Contrary to the bleached specimens, the observations in the PLS-affected tissues showed increased chl *a* concentrations. Whereas, the carotenoids and chl *c* is not altered significantly in the PLS-affected tissues. The apparent increased chl *a* concentrations in the PLS-affected polyps might be due to the enhanced doubling of the zooxanthellae. However, the density of zooxanthellae was not high due to the preferred expulsion of the doubling zooxanthellae from the host tissue as was noticed by Suharsono and Brown (1992). The other reason for the increased chl *a* concentrations might be the increased size of the zooxanthellae in the PLS-affected tissue.

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 pCO_2

around the polyps as described in the various photosynthetic organisms like cyanobacteria to diatom (Tchernov 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 pCO_2 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) Boedijin, 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 pCO_2 around the polyps: -

To generate CO_2, 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, CO_2 generated in the flask was passed on the healthy colonies through the base of a pipette tip (Fig. 8.2). Three such CO_2 generating set-ups were made to pass CO_2 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^{-5}M of DCMU. The inoculum was placed in the inoculum housing in the experimental coral sample.

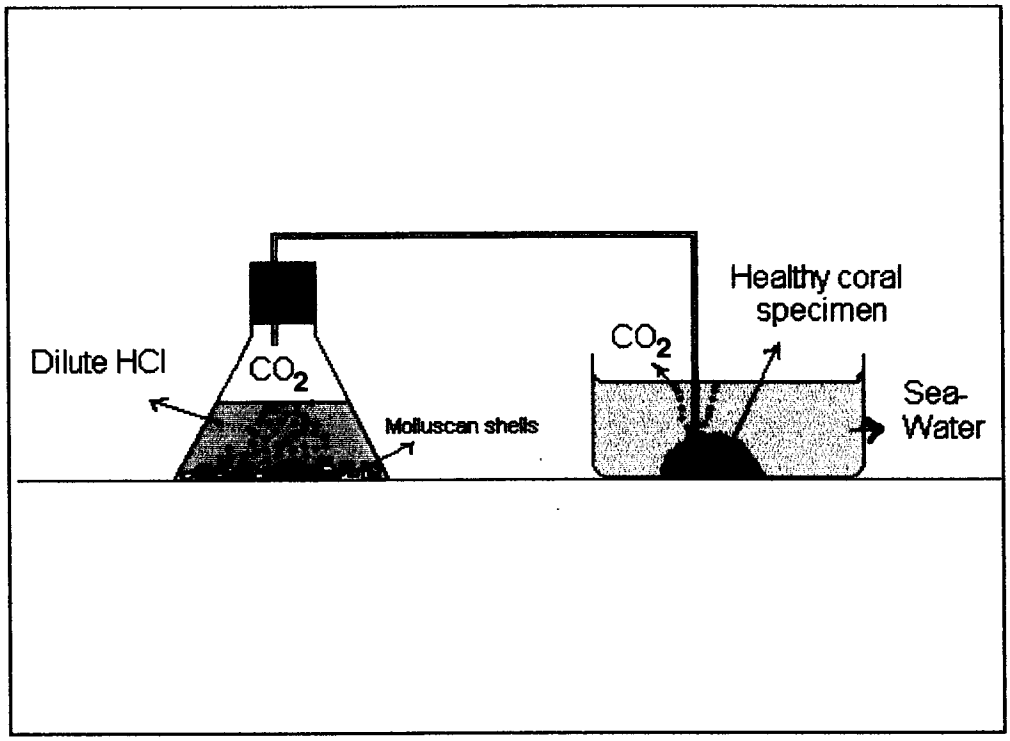


Fig. 8.2. Schematic diagram of the CO_2 generation and experimental design

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

Fig. 8.3 (bar = 3 cm)

Fig. 8.4 (bar = 2 cm)

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 pCO_2 around the coral polyps by passing CO_2 yielded some interesting result. After two weeks, the polyps adjacent to the CO_2 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

Experiments	Results
Inoculation with fungi	-
Inoculation with the cyanobacterium *P. valderianum*	+
Elevation of pCO_2 around polyps	+
Elevation of heterotrophic activity	-
Effect of non-photoactive cyanobacteria	-
Effect of acidic pH (pH 5.0) on coral polyps	-

8.4. Discussion: -

The result proves that there is a correlation between the pCO_2 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 pCO_2 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 *Synecococcus* 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 CO_2, and this, in turn, will dissolve the skeleton of the coral as observed in the PLS affected corals. The elevation of the pCO_2 due to the CCM of the cyanobacteria will enhance the availability of the inorganic carbon (C_i) 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 CO_2. In this condition, the lower pH around the polyps causes the skeletal dissolution that may probably enhance the availability of the C_i 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 CO_2 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 mutualisitc 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 zooxanrthellae. 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.

9. Metabolic state of Healthy and PLS-affected tissues: -

9.1. Introduction: -

The ratio of RNA/DNA has been used as a biochemical growth-rate indicator. This provides an estimate of growth rates and metabolic status in a wide variety of marine organisms, such as larval fish (Bulow 1987; Buckley et al. 1999; Kawakami et al. 1999; McNamara et al. 1999), phytoplankton (Dorth et al. 1983), copepods (Nakata et al. 1994, Wagner et al. 1998, Biegala et al. 1999), and marine invertebrates (Wright & Hetzel 1985, Frantizis et al. 1992, Pierce et al. 1999, Wo et al. 1999). RNA concentration in tissue or samples often reflects the ratio of protein synthesis. A relationship with organismal growth was established early on (Sutcliffe 1965, 1970). The RNA/DNA ratio provides an index of protein synthetis capacity per cell, since the amount of DNA per cell is assumed not to vary with growth rate, food density, and temperature and may be affected by gametogenesis and developmental stage.

Coral respond to the stress events by changes in growth rates, loss of zooxanthellae, aberrant fecundity and reduced planula larval survival, and changes in metabolism (Brown and Howard 1985). All of these responses involve changes in energy allocation. Brown and Howard (1985) concluded that by an extrapolation of the approach of Bayne and Widdows (1978) for other marine invertebrates, energy budgets that may provide early indications of environmental stress in corals might be understood. DNA and RNA ratio can be used as indicator of starvation and growth (Bulow 1987; Clemmensen 1987). The quantity and ratio of nucleic acids are reliable and sensitive indicators of pollution induced (Wang and Sticle 1988) and nutritional stress

(Wang and Sticle 1986). Various indications of the cellular status using the RNA/DNA ratio have been summarized by Lemmens (1995). In addition to the RNA/DNA ratio, the protein profiles will give an overview of the protein expression in the different state of the cell.

9.2. Methods: -

9.2.1. RNA/DNA ratio: -

The method described by Lemmens (1995) was followed in order to analyze the RNA/DNA ratio in the PLS affected and healthy coral specimens,. The coral samples with PLS and healthy looking specimens were collected and transported in the bucket of seawater to the field laboratory. The samples were frozen immediately and transported to the main laboratory for further analysis. The samples were cut into small pieces. Areas of the small pieces were measured by plotting the piece on a graph paper. The measured pieces were scrapped till the entire tissue is removed from the skeleton. The total removal of tissue was ensured by the exposure of white skeleton. The scrapped tissue was homogenized with the ice cold Tris-buffer (Annexure) in a glass pestle-mortar kept in the ice bath. 800 µl of the homogenate was transferred to an microfuge tube. To the homogenate, 100 µl of proteinase K (2 mg Proteinase K ml^{-1}) and 100 µl of SDS solution (20% in Tris buffer) was added. This mixture was left at room temperature for fifteen minutes while stirring alternatively. The mixture was centrifuged after incubation for fifteen minutes at 10,000 g in a cooling centrifuge (SORVALL SUPER T21, Sorvall products, L.P, USA) at 4° C for fifteen minutes. A sub-sample of 300 µl of the supernatant was transferred into an microfuge tube in duplicate. In each tube, 300 µl of 80% phenol (Tris-saturated phenol procured from Bangalore Genei

Pvt. Ltd) and 300 µl chloroform/Isoamyl alcohol (24:1; v/v) were added. The mixture was vortexed for 10 minutes at room temperature. The mixture was centrifuged for ten minutes at 10,000 g. The phenol-chloroform/Isoamyl alcohol phase (lower phase) was removed with a micropipette. The extraction procedure using the phenol-chloroform-isoamyl alcohol mixture was repeated thrice. To the final supernatant solution that contains nucleic acids, 300 µl of chloroform/isoamyl alcohol was added, vortexed for a minute and centrifuged for five minutes in 10,000g. Chloroform/isoamyl alcohol phase (lower phase) was removed. The chloroform-isoamyl alcohol wash was repeated thrice and the supernatant was transferred to a new microfuge tube. To the 100 µl of the supernatant, 3 ml of ice cold bis-benzimide (0.1 mg l^{-1}) was added to estimate the DNA concentration and Ethidium Bromide (10mg l^{-1}) was added to estimate the total nucleic acid concentration (DNA + RNA). The nucleic acid content was determined fluorometrically. Fluorescence was measured for Bis-Benzimide at 365 nm excitation with emission at 590 nm and for Ethidium Bromide at 352 nm excitation with the emission at 448 nm. This value was validated with the standard DNA solution. The entire sequence of the procedure is summarized below.

Collection of sample &

Freezing the sample

↓

Homogenise in ice cold Tris-buffer

Using a glass pestle and mortar

↓

Transfer 800 µl sub-sample to microfuge tube

Add 100 µl Proteinase K solution (2mg Proteinase K ml^{-1})

and 100 µl of SDS solution (20% in Tris buffer). Incubate for

15 minutes at room temperature while alternating stirring on

Vortex mixture

↓

Centrifuge for 15 minutes (~10,000 g)

↓

Transfer a 300 µl subsample of supernatant into

An Microfuge tube (duplicate here)

↓

A 1 Add 300 µl of 80% phenol and 300 µl chloroform/isoamyl

alcohol (24:1; v/v). Mix for 10 min on a vortex mixure at room temp

↓

A2 Centrifuge for 10 minutes at ~10,000 g

↓

A3 Remove phenol-chloroform/isoamyl phase (lower phase)

with a micropipette

↓

Repeat steps A1-A2-A3 with supernatant containing nucleic acid

↓

Add 300 µl chloroform/isoamyl alcohol to

B1 Supernatant, containing nucleic acids mix for

1 min on a vortex at room temp

↓

B2 Centrifuge for 5 minutes at ~10,000 g

↓

Remove chloroform/isoamyl phase

(lower phase) repeat steps B1-B2 with

supernatant, containing nucleic acids

↓

Transfer supernatant to new microfuge tube.

This will be used for fluorometric determinations

and should be kept below 4° C for a short storage period

and below –20° C for a longer storage period

↓

Use 100 µl supernatant (approximately 1 µg DNA

and RNA) on 3 ml of ice cold Bis-Benzimide (BBZ)

or Ethidium Bromide (EB) solution in 3 ml cuvettes

EB solution: 10mg l^{-1} Tris buffer

BB2 solution: 0.1mg BBZ l^{-1} Tris buffer

↓

Determine nucleic acid content fluorometrically

BBZ excitation at 365 nm; emission at 590 nm

EB excitation at 352 nm; emission at 448 nm

9.2.2. Protein profile: -

To get the protein profile of the PLS affected and healthy tissue, Laemmli's (1970) method was followed. Ingredients of all the reagents used in

SDS-PAGE are given in the annexure. The PLS affected coral tissues and the healthy specimens were collected from the lagoon and transported to the field laboratory in a bucket of seawater. The specimens were frozen in the liquid nitrogen and transported to the main laboratory in the liquid nitrogen. Small pieces of the size 2 cm^2 were scrapped from the frozen tissue and were put in the microfuge tube. The tissues in the microfuge tubes were lysed by freeze thawing by alternatively dipping in the liquid nitrogen and room temperature. The lysed cells were heated at 95° C for 4 minutes in 950 µl of the sample buffer, along with 50 µl of ß-mercaptoethanol. After the heating, the samples were frozen.

The polyacrylamide gel was prepared according to the Bio-Rad Mini-Protean® 3 Cell Instruction manual (Bio-Rad, California, USA). Resolving gel monomer (15%) was degassed by 20 minutes. To the degassed mixture, 50 µl 10% ammonium persulphate (APS) and 5 µl N,N,N',N'-tetramethylethelenediamine (TEMED) were added and after a brief mixing, the solution was poured in the BIO-RAD Mini-Protean 3 system gel caster. Stacking gel monomer (6%) was degassed for 20 min. To the degassed mixture, 50 µl APS and 10 µl TEMED were added and after a brief mixing, the solution was overlaid on the resolving gel without disturbing the resolving gel. This setup was left undisturbed for an hour for the polymerization of the gel.

The electrode buffer was filled in the inner and outer chamber of the unit. The frozen concentrated protein samples were brought to the room temperature prior to loading the gel. In the stacking gel, one well was loaded with 12 µl of low molecular weight marker, starting from 3 to 43 kDa (Bangalore Genei, Pvt. Ltd) and remaining wells were loaded with protein

extracts from PLS and healthy samples. The sample of each well was having 2 µg of total protein. The gel was run at 60 volts for 45 minutes. The gel was removed from the unit and stained with Coomassie Blue for 30 minutes. The stained gel was destained in the destaining solution overnight to remove the excess stain.

9.2.3. Protein estimation: -

The method followed to estimate the protein content of the PLS affected and healthy specimens was as described by Lowry et al. (1951). The PLS and healthy specimens were collected from the lagoon and transported to the field laboratory in a bucket of seawater. The specimens were frozen immediately and transported to the main laboratory in the frozen condition. The area of the samples was measured using graph paper and the tissue from the specimen with measured area was scrapped and put in a clean acid washed glass test tube. To the samples in the test tube, 5 ml of the alkaline copper tartrate reagent (annexure) was added and mixed and allowed to stand for 10 minutes. To these tubes, 0.5 ml Folin reagent, diluted with water in 1:3 ratio was added and allowed to stand for 20 minutes. The optical density was measured in a spectrophotometer (SHIMADZU-UV1201, Japan) at 750 nm. Bovine Serum Albumin (BSA) was used as a standard. The results were analyzed by single factor ANOVA for statistical significance using Excel spread sheet program in MS office 2000.

9.3. Results: -

The RNA/DNA ratio showed no significant difference between the healthy and PLS specimens ($P = >0.05$, $n=10$). The protein profile between the PLS affected and healthy specimens showed slight variations. The healthy

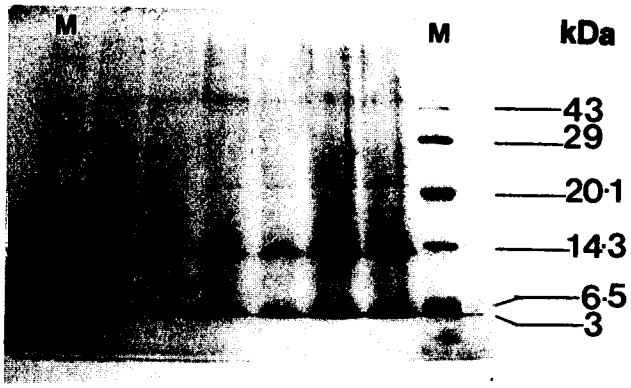


Fig. 9.1. SDS-PAGE of the protein extracted from healthy polyps

Fig. 9.2. SDS-PAGE of the protein extracted from PLS-affected polyps. Notice extra-band at 29 kDa.

specimens showed few protein bands compared to the PLS-affected specimens (Figs. 9.1, 9.2). The protein bands in the healthy specimens are approximately in the range of 3, 14, 20 and 43 kDa whereas the PLS-affected colonies expressed the proteins that were approximately 3, 14, 20, 29 and 43 kDa. In summary, the 29 kDa band is absent in the healthy specimens. The 14 kDa band in PLS-affected specimen was darker in comparison to the healthy specimens. The protein content between the PLS-affected and healthy specimens was not statistically significant ($P = >0.05$, $n=10$) as observed by single factor ANOVA.

9.4. Discussion: -

The PLS-affected colonies expressed a kind of protein that is close to 29 kDa in addition to the high expression of 14 kDa band. This gives some indications that the cells in the PLS affected colonies responded to the diseased conditions. The unaltered state of RNA/DNA ratio, insignificant protein variation in PLS and healthy samples and difference in the protein expression between the PLS and healthy specimens indicate that the cells undergo degenerative processes without synthesising any protein. The protein content of the PLS affected specimens are the same as in healthy one but 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. The *Acropora palmata* coral symbiosis gets only 30% of their nitrogen requirement from the environment and rest through host feeding (Paramet 1989) and the 90% of the nitrogen available for the zooxanthellae in the coral *Stylophora pistillata* is recycled (Rahav et al. 1989). The corals in the Lakshadweep are under oligotrophic conditions that may not get surplus

nitrogen to synthesis new protein in response to external threat to the cell, thereby depending upon the recycled nitrogen source as described by Vierstra (1993) in plants. Vierstra (1993) proposed that an increase in the protease in the plant cell might be related to recovery of nitrogen from non-essential proteins. It is possible that the expression of 29 kDa in PLS specimen may be a protease that is produced to cleave the non-essential protein in the affected specimen to synthesise a new protein that could probably protect the cell. It is not known why the high expression of the ~14 kDa protein was found in the PLS-affected specimens. Bangyeekhun et al. (2001) worked on a gene that encodes the subtilisin, serine protease. They conceptualized that the pre-protein peptides are 29 kDa and finally the mature enzyme is synthesised after cleaving many peptides yielding a 25 kDa enzyme, the subtilisin. The 29 kDa protein might also be a small heat shock protein (Hsp) that are produced in cells in response to stress (Paul van den Ussel et al. 1999).

10. Summary and Conclusion:

Coral reefs are renowned for their spectacular diversity and have significant aesthetic and commercial values, particularly in relation to tourism and fisheries. Coral reefs are being degraded on a global scale by a wide range of impacts, including mass bleaching events, diseases, pollution and several human activities. Diseases can be divided into biotic and abiotic diseases. The parasitic relationship between a host and its symbionts eventually leads to the biotic disease of host. The biotic disease can be recognised when the presence of a pathogen results in a distinct deviation from normality as displayed by functional or structural deficit.

During the surveys in the Lakshadweep islands, extensive partial mortality was observed in the dominant scleractinian coral species *Porites lutea*. Most of these corals showing partial mortality were clearly distinguished by a pink line around the dead patch which was colonized by the cyanobacterium *Phormidium valderianum* and the width of the line ranged from a few mm to a cm. The pink coloration is of the tissue rather than that of the skeleton or from any associated organisms. The disease incidence is higher in the southern lagoon than the northern lagoon. This is attributed to the slower current velocity that is prevailing in the southern lagoon that facilitates relatively calm water for the settlement of any pathogen on the coral colonies.

No dense bacterial populations were found in association with the PLS-affected polyps. A hyaline non-sporulating fungus and the dark melanized *Curvularia lunata* were frequently isolated from the PLS-affected tissue. Their associations with the PLS-affected polyps were confirmed by the

immunofluorescence detection method. Verification by Koch's postulate showed that these fungi were not the disease-causing agents. On the other hand, the cyanobacterium *Phormidium valderianum* which was isolated from the pink line successfully induced the disease syndrome (PLS) in *P.lutea*. The abiotic factors, which are detrimental to the host when the cyanobacterium interacts with the polyps, are the CO_2 produced by the cyanobacterium and the resulting acidic microenvironment.

The histological studies showed that the PLS-affected polyps underwent extensive tissue mortality with a variety of cellular events that lead to the death of the polyps. Some of the features of the cellular events are destruction of ectodermal and calicoblastic cells by a phenomenon similar to apoptosis, leaving behind cellular debris in these granular layers. The gastrodermal cells were ruptured, vacuolated and mostly free from their zooxanthellae. The expelled zooxanthellae were found on the surface of the coral colony and in the coelenteron. Most of the gastrodermal cells were found detached from the wall. Calcium accumulation was also found to be common in the gastrodermal cells.

Anomaly of the zooxanthellae population in the PLS-affected polyps was triggered by the cyanobacterial carbon concentration mechanism (CCM) found in *P. valderianum*. The cyanobacterial production of the CO_2 through its CCM, supplies extra CO_2 for the carbon-limited zooxanthellae. Thus, they overcome the host control on their division. Satisfying the inorganic demand of the zooxanthellae minimises its photosynthate translocation to the host. This causes stress that finally leads to death of the host cells.

There was very little change in the protein content and in the RNA/DNA ratio of between the PLS-affected and the healthy polyps. The protein profile showed that there was a new production of 29 kDA protein in the PLS-affected polyps. This might be due to the recycling of the non-essential proteins available in the cytoplasm to produce essential proteins as the PLS-affected polyps were depleted in energy by the low zooxanthellae density and an increased growth rate of the zooxanthellae. Alternatively, it needs to be confirmed whether this new protein is a heat shock protein synthesized in response to the stress.

10.1. Conclusion:

It is suggested in the present study that a foreign organism may induce disease symptoms not necessarily through parasitic interactions, but by the generation of abiotic factors such as acidic microenvironment in the vicinity of the host. Similar symptoms can be generated through the induction of the particular abiotic factors such as pCO_2 even in the absence of a foreign organism. In a disease where a pathogen is involved, the response can be immunological. On the other hand, disease symptoms that are produced by abiotic factors generated by a foreign organism, such a response may not be elicited from the host. As a result physiological disorders are produced in the host. The present study describes a novel approach to research in coral pathology. The sequence of events that is proposed to be the cause for the formation of the pink line in the coral is as follows.

1. The cyanobacterium *P. valderianum* settles on the coral colony.

2. The cyanobacterial carbon concentration mechanism (CCM) produces CO_2 (see Chapter 7), surplus amounts of which leaks out from their cell.
3. This becomes the source of excess C_i required for the carbon limited zooxanthellae in the host cell.
4. Zooxanthellae grow and overcome the host control.
5. Host looses its share of organic carbon from the zooxanthellae as this is used by the dividing zooxanthellae.
6. Calcification and growth of the host polyps is retarded.
7. The acidic environment created by the release of CO_2 may cause mortality of coral polyps and skeletal erosion.
8. The synergistic effects of CO_2 influx and acidic environment around the polyps might form the pink coloration of the host.

ANNEXURE

1. **Phosphate buffered saline (PBS)**
   8.0 g NaCl
   0.2 g KCl
   0.2 g KH_2PO_4
   1.15 g Na_2HPO_4
   Distilled water 1000 ml

2. **ASN III medium**

NaCl	25 g
$MgCl_2.6H_2O$	02 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.2H_2O$	0.5 g
Citric acid	0.003 g
EDTA (Di sodium salt)	0.0005 g
Ferric Ammonium citrate	0.0003 g
Na_2CO_3	0.02 g
Trace metal mix	1 ml (see below)

Filter sterilise the following and add separately

$NaNO_3$	0.75 g
$K_2HPO_4.3H_2O$	0.02 g
Distilled water	1000 ml
pH	7.5

Trace metals:

H_3PO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO_4.7H_2O$	0.222 g
$Na_2MnO_4.2H_2O$	0.390 g
$CuSO_4.5H_2O$	0.079 g
$COCl_2.6H_2O$	0.049 g
Distilled water	1000 ml

3. **Tris buffer**

Tris	0.05 M
NaCl	0.1 M
0.01 M Disodium EDTA	0.01 M
pH	9

4. **15% resolving gel monomer**

Distilled water	2.4 ml
30% Acrylamide/Bis-Acrylamide	5 ml
Resolving buffer	2.5 ml
10% SDS	0.1 ml

5. **6% stacking gel monomer**

Distilled water	5.4 ml
30% Acrylamide/Bis-Acrylamide	2 ml
stacking buffer	2.5 ml
10% SDS	0.1ml

6. **Sample buffer**

Deionised water	5.55 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
Glycerol	3.0 ml
0.5% (w/v) Bromophenol blue	0.2 ml
Total Volume	10.0 ml

7. **Coomasie blue stain**

Coomasie blue R-250	0.5 g
Methanol	40 ml
Acetic acid	10 ml
Distilled water	50 ml

8. **Destaining solution**

Methanol	500 ml
Acetic acid	100 ml
Distilled water	1000 ml

9. **Electrode buffer**

Tris	1.2 g
Glycine	5.7 g
SDS (10%)	4 ml
Distilled water	200 ml

Adjust the pH to 8.3 make the final volume to 400 ml using distilled water.

10. **Stacking buffer**

Tris	6.1 g
Distilled water	100 ml
pH	6.8

11. **Resolving buffer**

Tris	18.2 g
Distilled water	100 ml
pH	8.8

12. **Alkaline copper tartrate**

Solution A
2% solution of Na_2CO_3 in 0.1 N NaOH
Solution B
0.5% solution of $CuSO_4.5H_2O$ in 1% solution of potassium sodium tartrate

Mix solutions A and B in the ratio of 1: 50 (v/v)

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1. Ravindran J, Raghukumar C, Raghukumar S (1999) Disease and stress induced mortality of corals in Indian reefs and observations on bleaching of corals in Andamans. Current Science 76: 233-237
2. Ravindran J, Raghukumar C, Raghukumar R (2001) Fungi in *Porites lutea*: association with healthy and diseased corals. Diseases of Aquatic Oganisms 47: 219-228
3. Ravindran J, Raghukumar C (2002) Pink line syndrome (PLS) in the scleractinian coral *Porites lutea*. Coral Reefs 21: 252

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1. Mukundan, T. and Kishore, K., *Curr. Sci.*, 1991, **60**, 355–362.
2. Constantine, G., in *Biology of Bats* (ed. Wimsatt, W. A.), Academic Press, New York, 1970, vol. 1, pp. 319–322.

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Table 1. Fungi isolated from the polyp zone of *Porites lutea* collected from the lagoon in Kavaratti, Lakshadweep Island. Dead patch: coral with necrotic patch; ND: no data; PLS: coral showing pink-line syndrome

Fungal species	Frequency of isolation (%)					
	Healthy		PLS		Dead patch	
	1998	1999	1998	1999	1998	1999
Dark, non-sporulating forms	22	0	0	25	60	ND
Orange, non-sporulating forms	6	0	0	0	0	ND
Hyaline, non-sporulating forms	0	36	25	0	7	ND
Acremonium sp.	0	0	0	0	13	ND
Fusarium sp.	11	0	0	0	0	ND
Aspergillus sp.	17	7	25	15	7	ND
Cladosporium sp.	0	7	8	5	0	ND
Mycelial yeasts	0	21	0	0	0	ND
Labyrinthula sp.	0	0	0	5	0	ND
Unidentified	0	0	0	5	0	ND
Chaetomium sp.	0	0	0	10	0	ND
Aureobasidium sp.	0	0	0	5	0	ND
Total	56	71	58	70	87	ND

from the coral skeleton and spread around the polyps (Figs 15, 16 & 17). The fungal hyphae were covered by remineralised carbonate precipitate (Figs 10, 13 & 17) and most often fresh accretion of amorphous repair carbonate around fungal hyphae was noticed (Figs 12 & 13). Whole polyps removed after careful decalcification of the surface layer of coral in the pink-line zone also revealed the presence of fungal hyphae around the periphery of polyps (Fig. 18). Sections of polyps from healthy and PLS-affected corals stained with basic fuchsin showed abundant fungal hyphae around the polyps (Fig. 19).

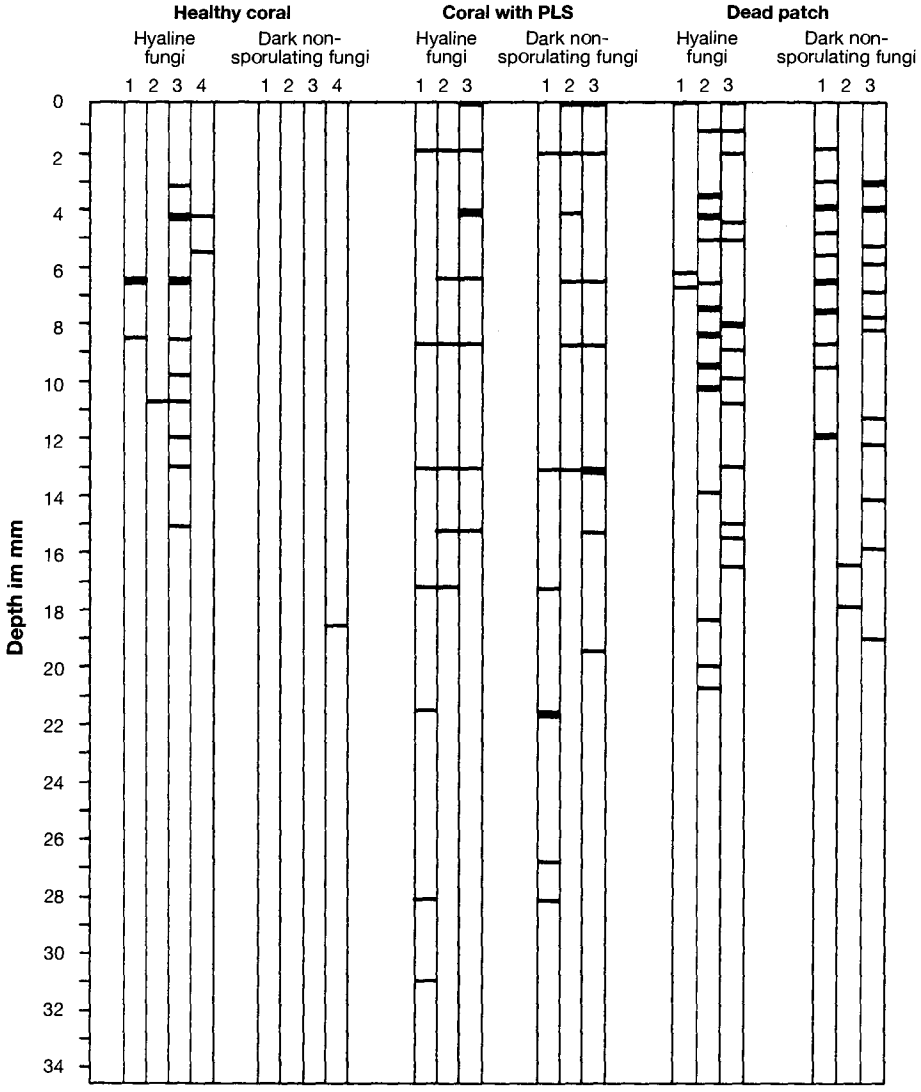

Fig. 6. Vertical sections of coral. Vertical distribution of fungi within healthy, pink-line syndrom-affected and partially dead corals. The horizontal bars represent fungal presence detected by Calcofluor staining (as described in 'Materials and methods') at a particular depth in coral. The dark bars represent detection of fungi in more than 2 transects in a vertical section of the coral. Numbers 1 to 4 represent replicate coral colonies examined

DISCUSSION

The scleractinian coral *Porites lutea* in 4 different stages of health were examined in this study. The first of these is corals affected by the pink line, a syndrome being reported for the first time (PLS). A similar syndrome was reported to occur in massive corals from Sri Lanka in 1998 (P. Weerakkody pers. comm.). It is possible that lowered host resistance due to stress-related factors induced the PLS. We are investigating the biochemical nature of the pink band observed in these corals. The pink band between the dead and healthy coral tissues may play a role in the coral's immunological and chemical defence systems similar to the pink band observed in the tissues of

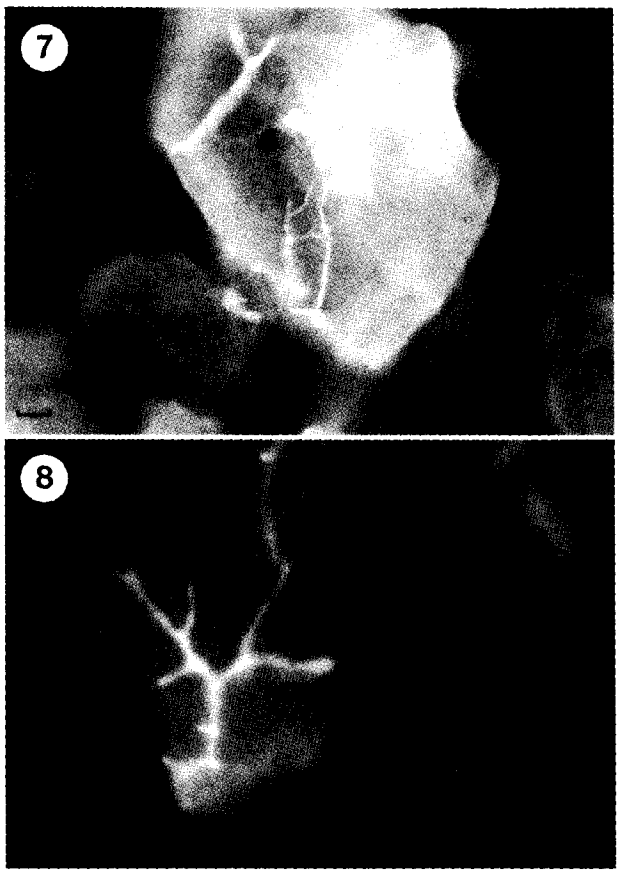

Fig. 7. Immunofluorescence detection of the hyaline, non-sporulating fungus strain 98 N-18 inside coral. Scale bar = 20 µm. Fig. 8. Immunofluorescence detection of the dark, non-sporulating fungus strain 98 N-28, *Curvularia lunata*, inside coral. Scale bar = 10 µm

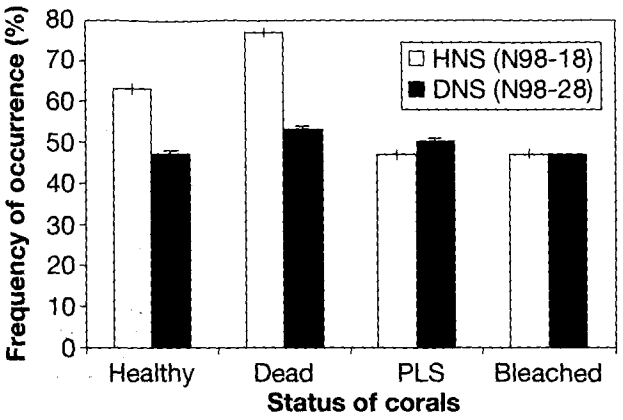

Fig. 9. Percentage frequency occurrence of the hyaline, non-sporulating (HNS) (strain 98 N-18) and dark, non-sporulating (DNS) forms (*Curvularia lunata*, strain 98 N-28) in healthy, dead and PLS-affected corals of *Porites lutea*, detected by immunofluorescence probes

pink colonies of *Pocillopora damicornis* at the contact sites between non-similar colonies (Dove et al. 1995). We also studied 'bleached' specimens of *P. lutea*. A coral-reef bleaching event is 1 of the examples of a response to environmental stress such as elevated temperature, salinity or illumination, excessive sedimentation, turbidity or chemical pollution (Williams & Williams 1990). In addition to the above, we examined healthy-looking corals devoid of any symptoms of damage and disease, as well as necrotised and dead patches on 'partially dead corals' (Meesters et al. 1996).

Our studies show that fungi are a regular component of corals. The present study was carried out over a period of 2 yr and fungi were seen to be constantly associated with healthy-looking, partially dead, bleached and PLS-affected corals. They were found extending to substantial depths within corals. Data presented show their distribution in corals up to a depth of only 3 cm but they were observed occasionally at depths of nearly 7 to 8 cm and sometimes even deeper. No regular pattern of fungal distribution within corals was observed similar to the black banding seen in *Porites lutea* from Indonesian reefs (Bak & Laane 1987). Black bands in sections of *P. lutea* and *P. lobata* skeletons in Mayotte Island in the Mozambique Channel and Moorea Island in French Polynesia were attributed to the secretion of organic dark pigments by fungi and their conidia (Priess et al. 2000). In our study, we observed a highly patchy distribution of fungal mycelia within the corals. This resulted in a high variability between replicates used for calculating the total biomass, culturable numbers and frequencies as observed by immunofluorescence. Fungal biomass constituted up to 0.05% of the weight of dead corals. This value is even higher than that reported by Raghukumar et al. (1995) for submerged detritus of leaves of the mangrove *Rhizophora apiculata* Blume (0.015% of the detrital weight). Fungal biomass of 3 to 5 mg cm^{-3} has been reported in necrotic patches of corals from the Andaman Islands in the Bay of Bengal on the east coast of India (Raghukumar & Raghukumar 1991). Fungi, therefore, are an apparently important component of corals.

Little is known of the diversity of fungi that inhabit corals. Certain 'obligate marine fungi' have been described from dead coral slabs (Kohlmeyer & Volkmann-Kohlmeyer 1987) and the term 'facultative marine fungi' has been coined to accommodate the possibility of terrestrial species of fungi having adapted to the marine environment. Raghukumar & Raghukumar (1998, 1999) hypothesised that terrestrial fungi are indeed active in the sea. Kendrick et al. (1982) have isolated species of *Aspergillus*, *Cladosporium* and *Acremonium* in culture from healthy *Porites* colonies in the Caribbean and the

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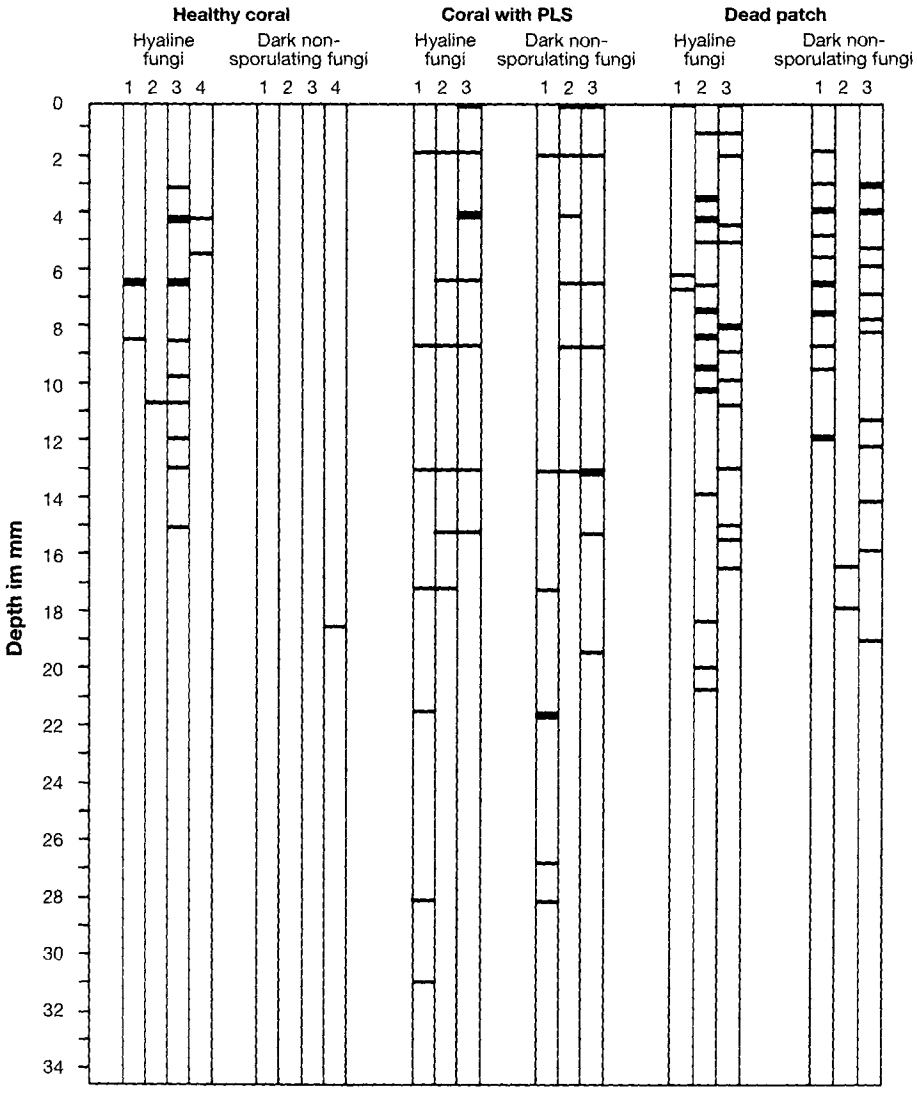

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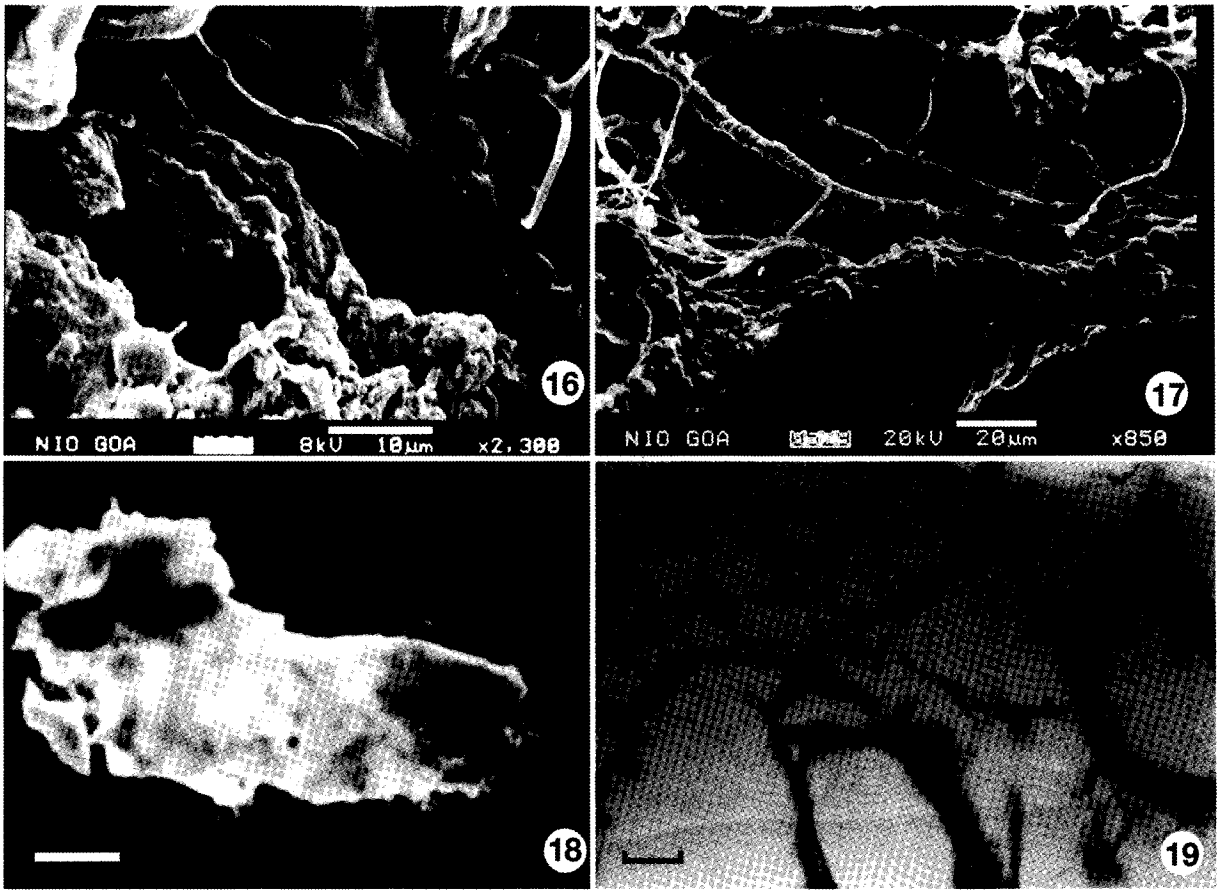
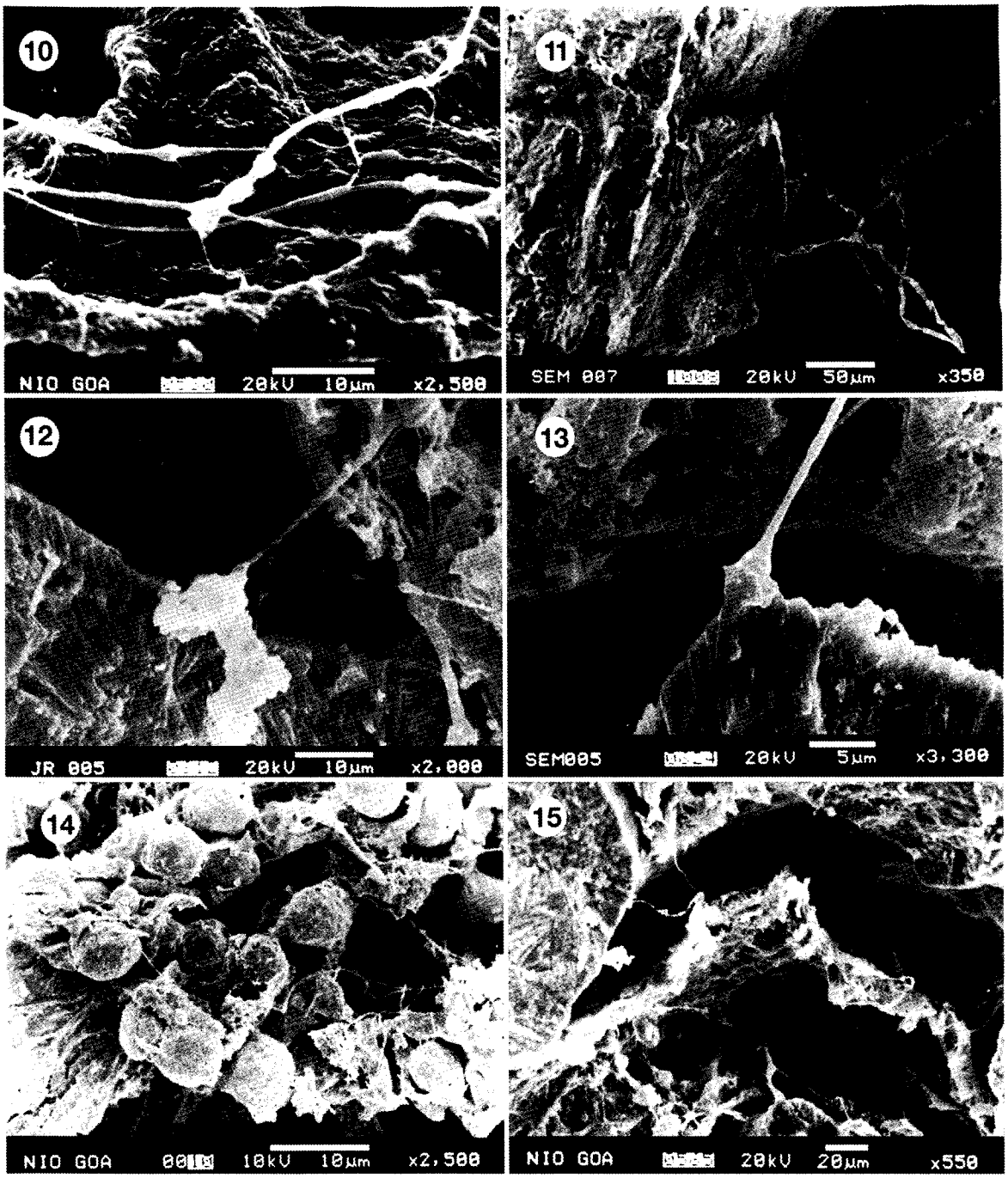


Figs 16 & 17. Fungal hyphae travelling between polyp and carbonate skeleton. Fig. 18. Fungal hyphae around a polyp picked up from the pink line. Scale bar = 370 µm. Fig. 19. Fungal hyphae (arrow) seen around a polyp in a transverse section of pink-line syndrome-affected coral. Scale bar = 20 µm

This study reiterates the hypothesis of Raghukumar & Raghukumar (1998, 1999) that terrestrial fungi are components of certain habitats in the sea. HNS forms were also frequent in our isolations. Immunofluorescence technique revealed the frequent presence of 1 of these isolates (# 98 N-18) within corals. The presence of such non-sporulating fungi has also been reported by Kendrick et al. (1982) in healthy corals. The terrestrial fungus *Aspergillus sydowii* causes rapid erosion and death of sea fan corals in the Caribbean (Smith et al. 1996). More recently, Shinn et al. (2000) reported the presence of terrestrial fungi, especially spores in the dust particles that are blown from Africa to the Caribbean, where they settle on the coral and trigger mass mortality in coral reef.

In view of the above, the behaviour and role of fungi within healthy and diseased corals merit detailed investigations. In this study, fungi were found within the calcium carbonate skeleton and around polyps. SEM studies showed entry of fungi from the coral carbonate skeleton into the polyps, the presence of amorphous 'repair carbonate' around the site of fungal penetration and precipitation of such 'repair carbonate' around the fungal hyphae. Le Campion-Alsumard et al. (1995a) explained this phenomenon of fungal growth and the polyp's defence mechanisms. During their process of penetration of coral skeleton, their role as bioeroding agents has been confirmed (Perkins & Halsey 1971, Zeff & Perkins 1979, Le Campion-Alsumard et al. 1995a,b). In molluscan shells with fungi, 3 times more calcium was leached than in control shells without fungi (Raghukumar et al. 1988). The high abundance and biomass of fungi that we observed in corals indicate their potential importance as bioeroders of calcium carbonate skeletons in the sea. While bioeroding activities of fungi in dead or diseased portions of corals are understandable, the possibility of such an activity in normal, healthy corals appears paradoxical.

Priess et al. (2000) observed formation and germination of a conidium inside the coral skeletons of *Porites lutea*. However, we rarely noticed this behaviou within corals. This observation, plus the fact that nor sporulating forms were common in corals suggests th these fungi might survive and propagate by mea



Figs 10 & 11. Fungal hyphae with swellings ramifying within coral skeleton as observed under a scanning electron microscope Fig. 12. Deposition of amorphous repair carbonate around the invading fungal hypha. Fig. 13. A fungal hypha showing basal swelling at the site of emergence from the carbonate skeleton. Fig. 14. A group of 'conidia-like' structures. Fig 15. Fungal hyphae travelling between polyp and carbonate skeleton

South Pacific. Fungi have been isolated from bleached corals of *Millipora complanta* (Te Strake et al. 1988). In the present study, we consistently cultured terrestrial fungi from corals under different health conditions. In addition, we have established the presence of the common terrestrial fungus, *Curvularia lunata* (# 98 N-28), within healthy and PLS-affected corals using polyclonal antibodies. This fungus was labelled as a DNS form in our initial isolations. We isolated several other DNS forms in our study, although their identity is not known. However, it is possible that these too were terrestrial fungi, possibly belonging to *Curvularia lunata*.

other than spore formation, such as hyphal fragments (Raghukumar & Raghukumar 1999).

The possible pathogenic role of fungi associated with polyps in the pink-line zone of corals could not be unequivocally shown in the present study although dense fungal hyphae were found around polyps. Fungi as pathogenic organisms are described for *Aspegillus sydowii* in sea fans, causing mass mortalities (Smith et al. 1996). Association of an unidentified lower fungus with black-band disease in star corals (Ramos-Flores 1983) and necrotic patches in massive corals (Raghukumar & Raghukumar 1991) has been reported, without proof of their pathogenicity. Further studies are required to examine the role of fungi in coral pathogenicity. One possibility is that normal residential fungal flora, and not necessarily extraneous invaders, may become opportunistic pathogens under climate variability and anthropogenic stress, as reported for mangrove disease, induced by cyanobacterial symbionts (Rützler 1988).

In conclusion, our study substantiates earlier studies on the presence of fungi in corals and shows that fungi are residential flora within corals. The constant presence and pervasive distribution of fungi, and a high biomass within the polyp zone confirm this. Fungi have been reported to be associated with micrite tubules of the Devonian era (Kobluk & Risk 1974) and modern carbonate sediments (Perkins & Halsey 1971, Perkins & Tsentas 1976). Association of fungi with present-day corals need not be the effect of modern anthropogenic effects, but may be the effect of long-term evolutionary association.

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Pink line syndrome (PLS) in the scleractinian coral Porites lutea

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Fig. 1. The coral *P. lutea* around Kavaratti Island

We describe here an unreported diseased state of *Porites lutea* (Milne-Edwards and Haime) on the Kavaratti reef of the Lakshadweep group of islands (11° N; 71°E). Pink line syndrome (PLS) causes partial mortality of the coral *P. lutea* around Kavaratti Island (Fig. 1), and about 10% of colonies were found to be affected by PLS. The dead patches were colonized by a cyanobacterium identified as *Phormidium valderianum* (Delp.) Gomont. Pink-colored polyps, forming a pink line, border these patches. Similar to the widely described black-band disease (BBD) in corals (Antonius 1981), PLS has a well-defined pink line varying in thickness from a few millimeters to a centimeter, bordering the dead patch in the affected colonies. Unlike BBD, where the black color is from a band mainly consisting of the cyanobacterium *Phormidium corallyticum*, migrating over and killing the polyps underneath (Richardson 1996), the pink line in PLS is due to the pink coloration of the animal tissue bordering the dead patch colonized by the cyanobacterium *P. valderianum*. In addition to the cyanobacterium, fungi such as *Curvularia lunata* (Wakker) Boedijin and a hyaline non-sporulating form were isolated and found to be frequently associated with PLS tissue and the dead patch (Ravindran et al. 2001). Koch's postulate experiments using the cyanobacterium and the fungi proved that the cyanobacterium *P. valderianum* is the causal organism inducing pink coloration in the coral tissue. Histological examination revealed that the cyanobacterium did not penetrate the tissue at any contact point, but the filaments were found in the debris of destroyed tissue. Moreover, tissue degeneration and zooxanthellae expulsion from the PLS tissue occurred.

Large-sized but reduced numbers of zooxanthellae with shorter doubling time in the PLS tissue in comparison to the healthy tissue indicates physiological disruptions in the PLS tissue. Various factors that could elicit response in the coral tissue by the presence of cyanobacterial biofilm adjacent to the polyps were tested. Elevated levels of pCO2, expected to be present around the pink polyps owing to the presence of the cyanobacterium, appear to be inducing pink coloration in the affected tissues.

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Disease and stress-induced mortality of corals in Indian reefs and observations on bleaching of corals in the Andamans

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A study was carried out in the Lakshadweep and Andaman islands and the Gulf of Kutch to assess the health of corals in Indian reefs. Disease, predation and stress were the major factors of coral mortality. Death caused by diseases – the black band disease (BBD), the white band disease (WBD) – necrotic lesions, and bleaching was observed in Kavaratti and Kadamat islands of Lakshadweep. The predatory starfish, *Acanthaster planci*, grazing on coral polyps was also noticed in these reefs. Large-scale silt deposition in the intertidal zone of Paga, Boria, Vadinar and Mangunda reefs in the gulf of Kutch buried the coral colonies and appeared to be the main cause of coral mortality. A severe incidence of coral bleaching was observed during July 1998 in some reefs in the Andamans. While more than 85% of corals near Ross island and Marina Park exhibited partial bleaching, up to 10% were totally bleached.

SEVERAL diseases and stress reactions in scleractinian corals from various parts of the world have been reported[1,2]. Many of these diseases are microbially

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induced. The bacterium, *Sphingomonas* sp. has been proved to be a pathogen in Florida corals[3]. The cyanobacterium *Phormidium corallyticum*[1] causes black band disease (BBD) in *Montastrea* sp[4]. For many other diseases, such as the white band disease (WBD), microbial causative agent has not been established. Raghukumar and Raghukumar[5] reported the occurrence of a fungus in association with diseased corals in Andaman waters. Increasing incidence of coral diseases is a concern for conservation of coral ecosystems, and therefore regular monitoring of coral reefs is recommended[6]. Although information on conservation and management in Indian coral reefs has been presented by Pillai[7], a systematic survey of the present status of corals in Indian reefs is lacking. Therefore, we have initiated monitoring the status of health in Indian coral reefs, particularly in the Lakshadweep islands of the Arabian sea. We are presenting here our preliminary observations on the types of coral mortality occurring in these waters.

Surveys were conducted in: i) Kavaratti atoll and Kadamat atoll in the Lakshadweep group of islands in the Arabian sea; ii) Vadinar, Paga, Boria and Mangunda reefs (Gulf of Kutch) in the western coast of India, and iii) Ross island, North Bay, and Marina park in the vicinity of Port Blair, Andaman islands (Figure 1). Observations were made in the intertidal region of the windward side of the island, the lagoon and the reef slope of the leeward sides of the island, in Kavaratti atoll and Kadamat atoll. Observation and collections of corals in the sub-tidal regions were made by skin and scuba diving. Presence of fungi in corals was detected by examining sections of 2–3 mm thickness under OLYMPUS SZH10 zoom stereo microscope. The same sections were examined for the presence of fungi after staining with 0.01% calcoflour (Sigma) for 5–10 seconds[8] under OLYMPUS BX 60 epifluorescence microscope with blue excitation filter. Bleaching of corals in Andamans was estimated by snorkelling over parallel transects of 25 m length. A total of 15 such transects were covered during one survey.

We observed four distinct kinds of disease symptoms in the Lakshadweep: i) necrotic lesions; ii) fleshy-algal over growth; iii) black band disease, and iv) white band disease.

Necrotic lesions were observed very frequently in *Porites lutea* which is common in the lagoons of Kavarati and Kadamat islands. The disease is characterized by the presence of single or multiple necrotic patches on coral colony. Two types of necrotic patches were observed. In one type, the patches were surrounded by healthy polyps (Figure 2 *a*). This type occurred mostly on the upper side of the corals. In the second type the lesions occurred at the bottom of the coral in contact with the sediment and these lesions were not surrounded by healthy tissues. The necrotic patches

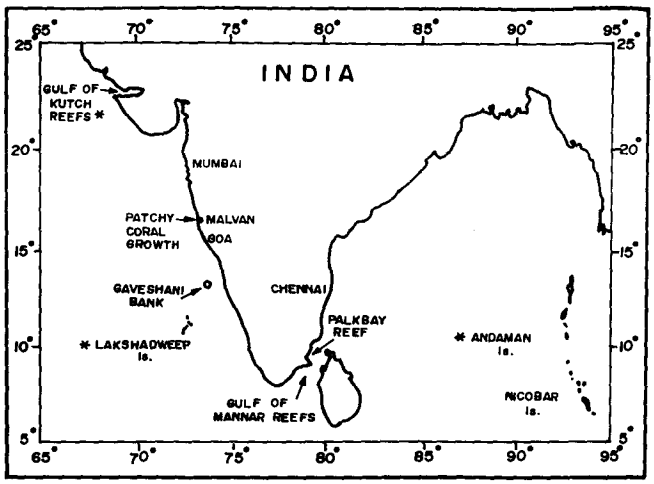

Figure 1. Location of sampling sties.

were devoid of polyps, and were colonized by fleshy algae and cyanobacteria. The margins of the necrotic patches, showing a pink coloration, were surrounded by polyps. Both hyaline and melanized fungi were observed in these specimens.

Necrotic lesions were also extremely prevalent in *Porites* sp. in Marina Park, Andaman (Figure 2 *b*). About 90% of corals in these beds, comprising specimens of 15–30 cm in size, were affected.

Encrusting fleshy red algae were found on a few occasions on *Porites lutea* in Kavaratti lagoon, causing death of the underlying polyps (Figure 2 *c*).

The BBD was noticed occasionally on *Porites* sp. in Kavaratti atoll. This was characterized by a necrotic patch surrounded by a black line or band (Figure 2 *d*). The band width varied from 3 to 6 mm. The black band found on the small vertical lobes of the colony appeared to advance on the colony as the necrotic patch expanded upwards from the bottom. The dead portions of the corals were colonized by algae.

The WBD was found on several massive corals in the reef slope of Kavaratti island. In this case a white band was present around a necrotic patch on the coral colony. The dead portion was devoid of polyps and appeared white. Older patches were colonized by algae.

Coral bleaching was common in Lakshadweep reef, although the incidence was low during our survey from April 96 to Feb. 98. Fully- and partially-bleached colonies were frequency noticed. Bleached colonies under water are easily identifiable by their white skeleton which is devoid of pigments. Colonies showing bleaching were observed to secrete excess mucus. While the fully-bleached colonies appeared totally white, partially-bleached ones showed moderate loss of their original colour and were also observed to secrete excess mucus.

A very high incidence of bleaching was noticed in July 1998 in the three Andaman reefs surveyed

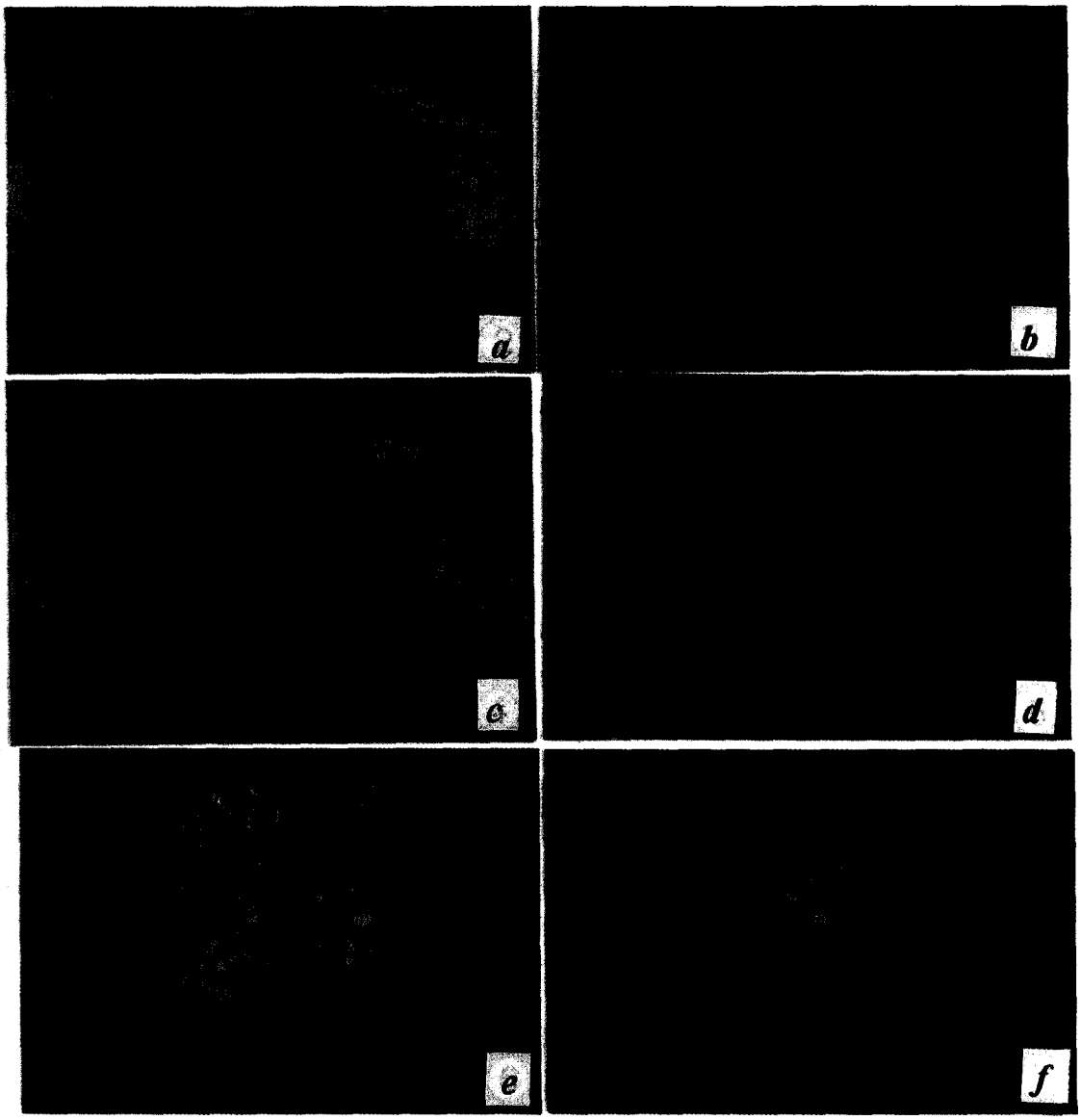

Figure 2 ***a–f.*** ***a***, Necrotic lesions in *Porites* sp. at Kavaratti island, Lakshadweep; ***b***, Bleaching and necrotic lesions in *Porites* sp. at Ross island, Andamans; ***c***, Algal over-growth in *Porites* sp. at Kavaratti island, Lakshadweep; ***d***, Black band disease (BBD) of a scleractinian coral, Kavaratti island, Lakshadweep; ***e***, Partial bleaching of *Acropora* sp. at Ross island, Andamans; ***f***, *Acropora* sp. grazed by the starfish *Acanthaster planci.* at Kadamat island, Lakshadweep (white patches).

Table 1. Bleaching in the Andaman reefs

Station	Bleaching in massive corals (%)	Bleaching in branched corals (%)
Ross island	100	91
North Bay	98	100
Marina Park	83	89

(Figures 2 *b*, *e*; Table 1). More than 90% of the massive corals and more than 75% of the branched ones were affected in the three sites: Ross island, North Bay, and Marina Park. We have isolated a number of fungi from coral colonies with the corals showing: (i) necrotic lesions, and (ii) bleaching. We have isolated a number of fungi from healthy colonies as well.

The crown-of-thorns star fish, *Acanthaster planci*, a well-known predator of coral polyps was observed in Kavaratti lagoon as well as reef slopes of Kadamat island. The star fish was found in considerable numbers in the latter site. Corals grazed by the star fish appeared white and were devoid of polyps (Figure 2 *f*).

Corals with necrotic lesions, fleshy algal over growth, and silt deposition showed the presence of melanized

Table 2. Causes of coral mortality in different locations

Area surveyed	Location	Period of time	Colony	Cause of mortality
Kavaratti	Lagoon	Apr. 96–Nov. 98	*Porites lutea*	Necrotic patch
Kavaratti	Lagoon and intertidal	Nov. 97 and Feb. 98	*P. lutea*	Bleaching
Kavaratti	Lagoon	Nov. 97 and Feb. 98	*Acropora palmata*	Bleaching
Kavaratti	Reef slope	Nov. 97 and Feb. 98	*A. humilis*	Bleaching
Kavaratti	Lagoon	Apr. 97	*P. lutea*	Predation by *Acanthaster planci*
Kavaratti	Lagoon	Apr. 97–Feb. 98	*P. lutea*	Black band disease
Kavaratti	Lagoon	Apr. 97–Feb. 98	*P. lutea*	Coralline algal over-growth
Kavaratti	Reef slope	Feb. 98	*Porites* sp.	White band disease
Kadamat	Reef slope	Feb. 98	*Acropora* sp.	Bleaching
Kadamat	Reef slope	Feb. 98	*Porites* sp.	Bleaching
Kadamat	Reef slope	Feb. 98	*Acropora* sp.	Predation by *Acanthaster planci*
Kadamat	Reef slope	Feb. 98	*Goniastraea* sp.	Bleaching
Kadamat	Reef slope	Feb. 98	*Siderastraea* sp.	Algal over-growth
Kadamat	Reef slope	Feb. 98	*A. humilis*	Algal over-growth
Kadamat	Reef slope	Feb. 98	*Acropora* sp.	Algal over-growth
Kadamat	Reef slope	Feb. 98	*Acropora* sp. (table coral)	Algal over-growth
Vadinar	Reef flat	Aug. 96	*Favia* sp.	Bleaching
Vadinar	Reef flat	Aug. 96	*Pseudosiderastrea* sp.	Bleaching
Vadinar	Reef flat	Aug. 96	*Favia* sp.	Sedimentation
Vadinar	Reef flat	Aug. 96	*Pseudosiderastrea* sp.	Sedimentation
Vadinar	Reef flat	Aug. 96	*P. lutea*	Sedimentation
(Paga reef)	Reef flat	Sep. 97	Dead reef	Sedimentation
(Mangunda reef)	Reef flat	Sep. 97	Dead reef	Sedimentation
Ross island, Marina Park and North Bay (Andaman island)	Fringing reef	July 98	All branching and massive corals	Bleaching
Ross island, Marina Park and North Bay (Andaman island)	Fringing reef	July 98	*Porites* sp.	Necrotic lesion

fungal hyphae. These fungal filaments were observed to be present from surface to subsurface and deeper in the coral section (Figure 3).

Silt deposition appeared to be the major cause of coral mortality in the interdial regions of the Gulf of Kutch. Death of corals appeared to have been caused by settlement of the fine sediment on the coral colonies, smothering the polyps to death. The thickness of the sediment on the colony ranged from a few millimeters to several centimeters.

This is the first report on the occurrence of coral disease and mortality in the Lakshadweep islands and the Gujarat coast. The disease symptoms observed by us closely resembled those reported earlier in literature from coral reefs in many parts of the world[1]. Necrotic lesions, resulting in partial mortality, are common among scleractinian corals[9] and symptoms of partial mortality described earlier by other workers were observed by us during our present survey. Such necrotic patches may be caused by fishes, divers and diseases. Microbial pathogens might also cause such dead patches. We have observed fungal hyphae in healthy, diseased and dead portions of *Porites lutea* showing necrotic patches. Fungal hyphae have also been reported in association with black band disease[10]. Involvement of fungi in causing partial mortality in *Porites lutea* observed by us needs to be confirmed by applying Koch's postulates.

The BBD is a widely reported disease from various parts of the world[1]. However, we noticed this only on a few occasions. The cyanobacterium, *Phormidium corallyticum*, is the known cause of BBD. We too noticed the presence of a *Phormidium* sp. in association with the BBD. The WBD observed by us in our survey is similar to that reported by Gladfelter[11]. The causal agent of WBD is not known.

Figure 3. Endolithic fungal filaments seen in the vertical section of *Porites* sp. at Kavaratti island, Lakshadweep.

Bleaching was a common symptom observed in our survey. Factors that trigger this physiological status have still not been identified. Stress-inducing factors like elevated sea surface temperature (SST) (ref. 12),

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Fungi in *Porites lutea*: association with healthy and diseased corals

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ABSTRACT: Healthy and diseased scleractinian corals have been reported to harbour fungi. However, the species of fungi occurring in them and their prevalence in terms of biomass have not been determined and their role in coral diseases is not clear. We have found fungi to occur regularly in healthy, partially dead, bleached and pink-line syndrome (PLS)-affected scleractinian coral, *Porites lutea*, in the reefs of Lakshadweep Islands in the Arabian Sea. Mostly terrestrial species of fungi were isolated in culture from these corals. Hyaline and dark, non-sporulating fungi were the most dominant forms. Fungal hyphae extended up to 3 cm within the corals. Immunofluorescence detection using polyclonal immunological probes for a dark, initially non-sporulating isolate (isolate # 98-N28) and for a hyaline, non-sporulating fungus (isolate # 98-N18) revealed high frequencies of these in PLS-affected, dead and healthy colonies of *P. lutea*. Total fungal biomass accounted for 0.04 to 0.05% of the weight of corals in bleached corals and was higher than in PLS-affected and healthy colonies. Scanning electron microscopy revealed the presence of fungi within the carbonate skeleton and around polyps. Fungi appear to be a regular component of healthy, partially dead and diseased coral skeleton.

KEY WORDS: Scleractinian coral · *Porites lutea* · Bleaching · Pink-line syndrome · Fungi · Immunofluorescence probe · Lakshadweep Islands · Arabian sea · India

INTRODUCTION

Several publications document the occurrence of fungi in healthy and diseased corals (Ramos-Flores 1983, Le Campion-Alsumard et al. 1995a, Smith et al. 1996). Kendrick et al. (1982) isolated terrestrial species of fungi in culture from a number of species of live corals and Le Campion-Alsumard et al. (1995a) suggested a symbiotic role for fungi in healthy corals. Raghukumar & Raghukumar (1991) found them in necrotic patches of a massive coral. A number of new fungi have been described from dead corals (Kohlmeyer & Volkmann-Kohlmeyer 1987).

Because of widespread mortality of coral reefs, documenting coral diseases has assumed importance in recent years and the spread of several old and new diseases has been reported from various parts of the world (Kushmaro et al. 1996, Goreau et al. 1997, 1998, Richardson 1998). Pathogenic organisms are identified for some of these, whereas for others the causal organisms are not yet known. Fungi are well known as plant and animal pathogens on land, and several fungal diseases have also been reported from the marine environment (Raghukumar 1996, Richardson 1998). A terrestrial species of fungus has also been shown to cause a disease in soft corals (Smith et al. 1996). In view of these reports, it is of interest to examine whether fungi associated with diseased corals are primary pathogens. We investigated the presence of fungi, and their species composition, prevalence and biomass in healthy, partially dead, bleached and pink-line syndrome (PLS)-affected scleractinian coral *Porites lutea*.

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MATERIALS AND METHODS

Study area. Diseases of corals were surveyed in the lagoon and intertidal zones of Kavaratti Island of the Lakshadweep archipelago off the south-west coast of India. The Lakshadweep archipelago comprises 36 islands with 12 atolls and lies between 10–12° N and 71° 40′–74° E in the Arabian Sea (Fig. 1) and forms the northern part of the Laccadive-Chagos Ridge. Samples of *Porites lutea* were collected in the lagoon at a depth of 3 to 5 m and samples of *P. compressa* were collected from the intertidal zone of reef flat in Kavaratti Island. Bleached and PLS-affected corals were collected in October and November 1998 and April 1999.

Frequency of fungi isolated from corals. Small pieces from the surface of healthy-looking colonies of *Porites lutea* and those showing PLS were chiselled and collected in sterile plastic bags under water. The samples were taken in ice and processed within 2 h of collection. The coral samples were broken further into smaller pieces inside the same plastic bags, thereby avoiding exposure of the broken surface to air to avoid contamination by aerial opportunistic fungi. The broken pieces ranging in size from 3 to 5 mm^3 were directly plated on 1:5 diluted corn meal agar medium (HI-MEDIA, Mumbai, India) prepared with half-strength sea water and fortified with streptomycin (0.5 g l^{-1} medium) and penicillin (100 000 U l^{-1} medium) to inhibit bacterial growth. The presence of aerial fungi in the experimental site was tested by exposing 3 to 5 Petri plates containing corn meal agar medium to the air for 10 s during the isolation procedure. Subsequent comparison of these plates with experimental plates facilitated the detection of aerial contamination in isolation plates. Fungal hyphae emerging from coral pieces after 5 to 7 d of incubation in the dark were identified up to generic level. Frequency of fungi isolated from corals is expressed as a percentage.

Estimation of biomass of fungi. Coral samples from the polyp zone (up to 5 mm below surface) were weighed and decalcified in a decalcification solution (1:1 ratio of 20% citric acid 50% formic acid). The resulting material was homogenised in a VirTis homogeniser (VirTis Co., Gardiner, NY, USA) at 5000 rpm for 15 s and filtered over a 0.22 µm pore size Nuclepore polycarbonate filter paper under vacuum. The filter paper was stained with 0.01% Calcofluor, an optical brightener that stains chitin and cellulose (Sigma, St Louis, MO, USA) for 5 to 10 s (Mueller & Sengbusch 1983). After the excess stain was washed with distilled water, the filter was mounted with a drop of non-fluorescent immersion oil and a cover slip was applied. The slides were stored in the dark at 5°C until further examination.

The slides were observed under an epifluorescence microscope with a blue excitation filter (Olympus BX-60, Tokyo, Japan). The images of fungal hyphae were captured with a charge-coupled device camera (Optronics Engineering, Goleta, CA, USA) and digitised using a frame-grabber card (Oculus TCX, Coreco Inc., Quebec, Canada). Since some coral detritus also showed fluorescence, images were manually traced in conjunction with Optimas 6.0 image analysis software (Optimas Corporation, Bothell, WA, USA) calibrated to the particular objective. Total length and average width of the mycelia for the total area were estimated using the software to calculate fungal biovolume. Mycelial fungal biomass was determined by assuming a mass density of 0.2 g cm^{-3} (Newell et al. 1986) and expressed as mg fungal biomass g^{-1} wet weight of

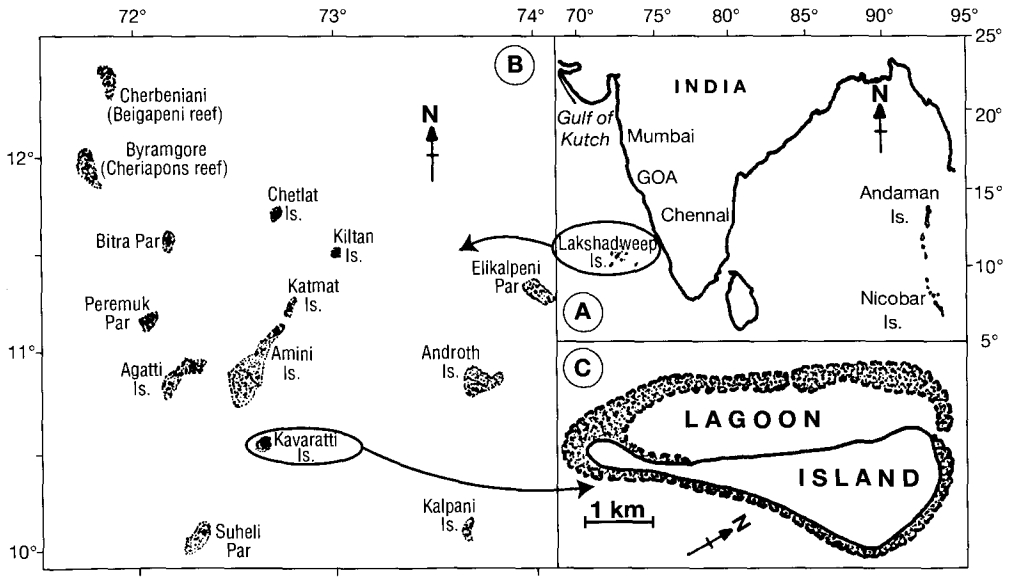


Fig. 1. Site of sampling: (A) Lakshadweep islands situated on the south-west of India in the Arabian Sea; (B) the group of islands of the Lakshadweep archipelago; (C) Kavaratti island with lagoon and a reef enclosing lagoon and island

coral tissue. About 3 to 10 coral pieces were examined as replicates for this study.

Vertical distribution of fungi in corals. PLS-affected coral, the dead portion from partially dead corals and healthy-looking colonies of *Porites lutea* were cut into 1 cm thick sections with an electric rock cutting saw. These sections were washed with a jet of water to remove free particles and stained with 0.01% Calcofluor solution for 2 to 3 min. After excess stain was washed off, the sections were examined under an epifluorescence microscope, using a blue excitation filter for the presence of fungi. Dark, non-sporulating (DNS) fungi did not stain with Calcofluor but could nonetheless be detected directly. The results are qualitatively expressed as presence or absence of fungi at various depths in the coral sections.

Scanning electron microscopy of corals. Healthy-looking pieces of *Porites lutea* and those showing PLS collected from a 1 to 3 m depth in the lagoon were fixed immediately with 3% formalin prepared with sea water for 24 h. They were further fixed with a 10% formalin solution for 48 h. The fixed samples were rinsed thrice with tap water and were preserved in 70% ethanol and stored till use. Fixed samples were later decalcified (see above) and washed twice in distilled water and dehydrated in a graded acetone series. The tissues were dried critically using liquid CO_2 in a critical point freeze drying apparatus (Structure Probe Inc., West Chester, PA, USA). The whole specimens (containing tissue and skeleton) were prepared in a similar manner without the decalcification step for scanning electron microscopic (SEM) studies. Critically dried specimens were sputter coated with gold-palladium and examined under an SEM to observe the fungi (model 5800 LV, JEOL, Akishima, Japan).

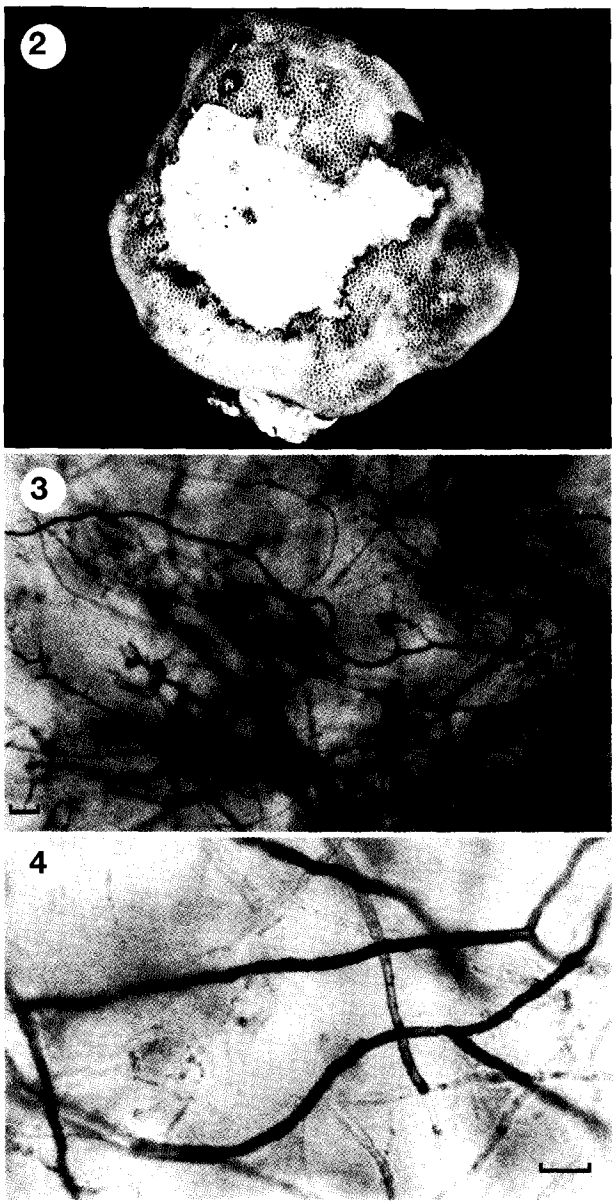
Immunofluorescence detection of selected fungi in *Porites lutea*. Antibodies were raised commercially (Genei India Pvt. Ltd, Bangalore, India) for 2 of the most frequently isolated fungi, a dark mycelial fungus (isolate # 98 N-28, *Curvularia lunata* (Wakker) Boedijn) and a hyaline, non-sporulating (HNS), unidentified isolate (Isolate # 98 N-18) obtained from a partially dead and a PLS-affected coral, respectively. About 2 mg of fungal pellet was crushed in 2 ml 0.15 M NaCl and centrifuged, and 1 ml supernatant was emulsified with 1 ml Freund's adjuvant. This was injected subcutaneously at multiple sites on the back of New Zealand male white rabbits (2 rabbits for each fungus). The first booster injection was given after 6 wk of the primary injection and the second booster was given after a further 4 wk. The antibody titre was monitored by Dot ELISA after 10 d of the booster injection and yielded a titre value of 1:2000 for isolate # 98 N-18 and 1:5000 for isolate # 98 N-28. The antibodies thus obtained were diluted 4 times in phosphate buffered saline (PBS) containing 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4 in 1000 ml distilled water (Mendoza et al. 1995). The coral specimens were crushed into a coarse powder and incubated with 50 µl of the diluted antibody solution in an Eppendorf tube for 1 h at 25°C. The mixture was vortexed after dilution to 1000 µl with PBS solution. The slurry was centrifuged at 4000 rpm for 1 min. After the supernatant was decanted, the coral debris was similarly washed 3 times with 1000 µl of PBS. The samples were incubated in 50 µl of the secondary antibody (Genei India Pvt. Ltd), and the goat anti-rabbit anti-serum was tagged with fluroscein isothiocyanate for 1 h at 25°C. The excess stain was washed with PBS as described. The coral debris was transferred onto a slide and viewed under blue excitation (excitation wave length 400 to 440 nm and barrier filter # 475) for the presence of fungi and photographed. Specificity of the antiserum was confirmed by the absence of immunofluorescence staining by 4 species of *Aspergillus*, *Cladosporium* sp. and mycelial yeast isolated from *P. lutea*. Staining of coral sections with pre-immune sera did not show any immunofluorescence.

Histology. To detect fungi within polyps, coral polyps were removed from formalin fixed coral pieces following decalcification as described above. The polyps were dehydrated in a graded series of ethanol and embedded in wax and sectioned. The sections were stained with basic fuchsin (Bancroft 1995).

RESULTS

Bleached corals were collected 5 mo after the major coral reef bleaching event reported from all over the world including the Andamans in the Bay of Bengal (Ravindran et al. 1999). Line transect observations carried out in October 1998 and April 1999 in the lagoon at Kavaratti Island revealed the presence of bleaching and PLS in *Porites lutea* colonies. In the PLS-affected corals, the coral tissue around the dead patch appeared bright pink in otherwise pale brown morphotypes of *P. lutea* (Fig. 2).

Several fungi from PLS-affected corals as well as from dead, bleached and healthy ones were isolated from the surface of the coral up to 5 mm depth (Table 1). The highest frequency of fungi occurred in dead patches. There were no differences in the frequencies of fungi occurring in healthy and PLS-affected colonies of *Porites lutea*. Most of the identified species of fungi belonged to terrestrial genera. DNS and HNS forms were the most dominant in all the healthy-looking and stressed corals (Table 1). One of the DNS strains, # 98 N-28, isolated from a dead patch during 1998 sporulated in subsequent culture, although poorly, and was identified as *Curvularia lunata* (Wakker) Boedijin. The



Figs 2 to 4. Pink-line syndrome in a *Porites lutea* colony in the lagoon of Kavaratti. Fig. 3. Fungal hyphae and algal filaments from the polyp zone, visible after decalcification of the surface layer. Scale bar = 20 µm. Fig. 4. Septate hyphae of a dark, non-sporulating fungus seen after decalcification of the surface layer. Scale bar = 20 µm

other dominant and frequently isolated fungi from these corals were various species of *Aspergillus*, a species of *Cladosporium* and a mycelial yeast. On decalcification of the top 5 mm layer, black septate fungal mycelia interspersed with thin hyaline fungal mycelia, and cyanobacterial and algal filaments were observed (Figs 3 & 4).

Fungi were isolated from almost all healthy as well as PLS-affected colonies of *Porites compressa* from the intertidal zone. Here too the HNS (about 30% frequency) and DNS fungi (about 60% frequency) were isolated.

Sections of corals stained with Calcofluor revealed the presence of fungi as bright blue septate filaments under the epifluorescence microscope in contrast to bright orange cyanobacteria or red chlorophycean algal filaments. The highest fungal biomass occurred in bleached corals collected in October and November 1998, nearly 5 mo after the major bleaching event, whereas the fungal biomass was lower in corals with dead necrotic patches and very negligible in PLS-affected corals (Fig. 5). Examination of the depth-wise distribution of the hyaline and dematiaceous fungi revealed that they pervaded the PLS-affected corals and necrotised patches of partially dead corals more frequently than the healthy ones (Fig. 6).

An isolate of HNS fungi (# 98 N-18) as well as *Curvularia lunata* (# 98 N-28) were detected in corals by the immunofluorescence probes (Figs 7 & 8). Branched mycelia of both the fungi adhered closely to calcium carbonate particles and often appeared to be present within the carbonate skeleton (Figs 7 & 8). Frequencies of *Curvularia lunata* (isolate # 98 N-28) were similar in healthy, PLS-affected and dead corals (about 50% frequency). The HNS form (isolate # 98 N-18) was found to be more frequent in the healthy (60% frequency) and partially dead corals (80% frequency) than in PLS-affected corals with 50% frequency (Fig. 9).

SEM of the healthy, bleached and PLS-affected corals showed that fungi perforated the coral skeleton (Figs 10, 11 & 12). The hyphae showed distinct swellings at regular intervals (Figs 10 & 11). Fungal hyphae showed basal swellings on emergence from the carbonate skeleton (Figs 12 & 13). Occasionally, we also observed 'conidia-like' structures within the coral skeleton (Fig. 14). Ramifying fungal hyphae emerged

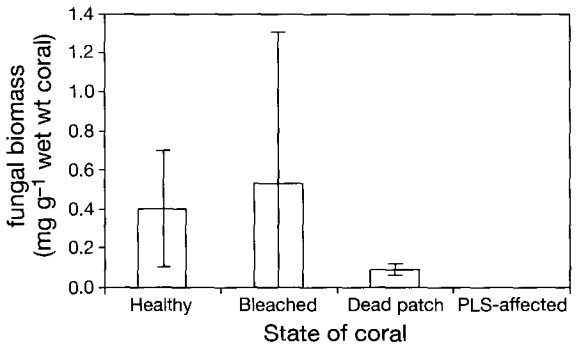


Fig. 5. Fungal biomass in mg g^{-1} coral wet weight. Preweighed crushed corals were stained with the optical brightener Calcofluor stain and biovolume of fungi present was calculated using the Image analysis software. The fungal biovolume was converted to fungal biomass as described under 'Materials and methods'

pollution and pathogens are believed to be some of the causal factors. It will be worth examining the relationship between the extreme incidence of bleaching observed by us in July 1998 in Andaman and the SST during the summer of this year. Similar high incidences of bleaching have been reported this year from Seychelles, Maldives, and Sri Lanka reefs (Jason Rubens and Thomas Goreau, pers. commun.). A species of *Vibrio* has been described recently to be causing bleaching of the coral *Oculina patagonica* in the coast of Israel[13]. Disturbances in biodiversity and the removal of predatory animals may result in increase in population of *Acanthaster planci*, the predator of corals[14]. Corals grazed by the star fish could be distinguished from bleached colonies. While the former had the trails of predation and a bare skeleton without polyps, the bleached colonies did not show any trails of predation and the corals still retained their polyps. The star fish was always associated with the grazed colonies.

Silt deposition was observed to cause a high incidence of coral mortality in the Gulf of Kutch, Gujarat. Increased construction activities and altered tidal currents in the areas could be a major cause for this. The deleterious effects of quarrying and mining on Indian coral reefs have been described by Patel[15], Rashid[16], and Wafar[17]. Most corals in this area were almost buried under silt. Intercolonial space between coral colonies in Paga reef revealed silt deposition of few mm to two feet in thickness.

Thus, during our survey of two years, we observed various factors which result in the different disease symptoms of corals. Table 2 summarizes the causes of coral mortality in different locations.

Global reef monitoring in the name of 'Reef check 97' (ref. 6) was carried out in 31 countries during the Year of Coral Reefs in 1997. India was not a participant in the event. Such monitoring programmes should be given priority as these help in assessing reef health and evolve long-term strategies to preserve and protect them.

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