造礁サンゴ骨格内に生息する微生物群に関する基礎研究

Endolithic microbes within calcium carbonate skeletons of reef-building corals

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Abstract

The establishment of obligate symbiotic relationship with photosynthetic dinoflagellate (zooxanthellae) is essential for reef-building corals in adaptation to natural oligotrophic environments. Breakdown of the symbiotic relationship with zooxanthellae leads to coral bleaching that is pronounced under stress conditions. Ecological studies have suggested that susceptibility to bleaching and the ability to recover from bleaching vary among coral species. However, it is yet unconfirmed what endogenous factors make such differences. Here I describe endolithic microbe within coral skeleton as a novel factor that may account for the diversity in response to environmental stimuli. Endolithic microbial communities were investigated in skeleton of the massive coral *Goniastrea aspera* that is one of the bleaching tolerant species. "*Halomicronema* sp.", a moderately halophilic and thermophilic cyanobacterium, was found in the skeleton of *G. aspera*. Comparative study of the endolithic microbial flora within skeletons of *G. aspera* showed that bacterial diversity was much higher in the coral skeletons collected from oligotrophic environments than those from eutrophic ones. The endolithic microbial community included sulfate-reducing bacteria and resembled microbial mats commonly found in harsh environments. To explore beneficial effects of endolithic microbes on coral physiology, the branching coral *Acropora digitifera* with and without endolithic algae were exposed to high irradiance of visible light. The presence of endolithic algae within the skeletons was found to suppress photoinhibition of photosynthesis in the host tissue. The results suggest that endolithic algae have a photoprotective role in the coral photosynthesis. The interactions between reef-building corals and endolithic microbes are discussed in terms of secondary or facultative symbiotic relationship.

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Chapter 1

General Introduction

Diverse reef-building coral species dominate tropical and subtropical coastal areas. Reef-building corals form obligate symbiotic relationship with zooxanthellae that enable them to adapt to oligotrophic environments. Their primary production contributes to the formation of coral reef ecosystem inhabited by diverse marine organisms. However, this ecosystem is in danger of destruction due to natural disturbances such as global warming as well as anthropogenic disturbances such as pollution. Corals harbor endolithic algae in addition to zooxanthellae. Endolithic algae are boring microorganisms that inhabit within coral skeletons. However, biological relationship between coral and endolithic algae is still obscure. This chapter introduces microbial consortia, reef-building corals and coral reef environment from the perspective of microscale to macroscale.

Keywords:

endolithic algae, oligotrophic, reef-building corals, symbiotic relationship, zooxanthellae

1.1 Tropical and subtropical oceans are oligotrophic environments

In general, tropical and substropical oceans are characterized by nutrient-poor waters whereas higher latitudinal oceans (temperate and polar regions) are known to be rich in nutrients as shown in Fig. 1.1 (Wada and Hattori 1991). This is due to the difference in the properties of thermocline. Thermocline is a layer of water column in the ocean that separate the warm surface water from the cold deep water. It is a transition zone where temperature decreases rapidly with depths. Factors affect the depth and thickness of thermocline include latitude, season, weather and local environment conditions (Fiedler and Talley 2006). Patterns of thermocline reflect the patterns of primary production in the world ocean because thermocline coincides with the nutricline, a portion of water column where nutrients increase with depth (Barber and Chavez 1991; Fiedler and Talley 2006).

In tropics and subtropics, differences in seasons are less distinct than those in temperate and cold areas and the weather is relatively constant throughout the year. Surface ocean water has a lower density compared to deeper water due to year-round heating of the sea. Thermocline is generally a permanent feature in tropical marine environment. Therefore, density stratification of waters is present throughout the year. Due to differences in density, the cold nutrient-rich water below the thermocline hardly mix with the warm nutrient-poor surface water without any turbulence such as storm or high wind (Sarmiento *et al*. 2003; Pennington *et al*. 2006). Since the thermocline is rarely broken up in tropics and subtropics oceans, the warm surface water remains nutrient-depleted. Although phytoplankton is present most of the time, their populations are limited by the lack of nutrients (Rivkin and Anderson 1997). Thus, clear, blue tropical waters are generally known as “desert of the sea” due to their low productivity except for coral reefs (Odum and Odum 1955).

The depth and thickness of thermocline in temperate oceans changes with seasons. In spring, daylight hours increase and surface water becomes warmer. Colder water sinks to

bottom and warm surface water overlies it. These conditions promote the development of thermocline. Thermocline becomes stable in the summer but recede in autumn. During winter season, windy and stormy weather facilitate mixing of deeper nutrient-rich water into surface water (Roden and Raine 1994). Plankton blooms in the spring as daylight hours increase and nutrients are abundant, an ideal conditions for productivity. The ability of vertical mixing to replenish surface waters with nutrients from deeper waters resulted in higher productivity in temperate oceans (Wafar *et al*. 1983; Kudela and Dugdale 2000).

Polar regions lack thermocline, hence the water column is isothermal, i.e. same temperature regardless of depth. This enables easy mixing of the deeper nutrient-rich water into the surface waters. Polar oceans are relatively high in productivity but due to limited sunlight, the period of productivity is short (Codispoti *et al*. 1991; Wang *et al*. 2006).

Most marine life exists where there is ample nutrients and sunlight. In recent years, anthropogenic nutrient enrichment has altered the nutrient levels in many coastal areas. This causes phytoplankton and algae bloom which subsequently has negative environmental impact on marine environments such as development of anoxic conditions, reduction in water quality, changes in benthic community structure (Smith *et al*. 1999; Tyrrel 1999).

1.2 Microbial consortia in ecosystems

Microorganisms contribute very important role in the global biogeochemical cycles of nitrogen, carbon, oxygen and other elements. These cycles are mediated by consortia of microbes consists of various functional groups having different metabolic requirements and physiological capacities (Rheinheimer 1991). Microbial consortia found in large scale ecosystems such as oceans and lakes as well as small scale ecosystems such as sediments, microbial mats and microbial biofilms exhibit many similarities. Vertical zonation of

microbes in these habitats corresponds with the vertical distribution of dissolved oxygen, irradiance, temperature, salinity, pH and redox potential (Sørensen et al. 1979; van Gemerden 1993; Ramsing et al. 2996; Kopylov et al. 2002). Although microbes are stratified accordingly, assemblages of different groups of microbes function as a cooperative consortium in a complex but coordinated manner. In a microbial consortium, metabolic products or wastes from one group of microbes serve as food or source of energy for another group of microbes (Paerl and Pinckney 1996).

Biogeochemical characteristics of both macroscale (stratified ocean/lake) and microscale (sediment/microbial mat) are typified by steep gradients and distinct microenvironments. These characteristics are due to both environmental factors such as light and temperature as well as metabolic activities of microorganisms (van Gemerden 1993; Hollibaugh *et al*. 2001). In general, the upper layer is characterized by higher oxygenic conditions which decrease with depth. A peak of oxidized nitrogen compounds can be found underneath or most of the time overlaps with the oxidized layer. This is followed by an anaerobic layer high in hydrogen sulfide at the bottom. It should be noted that this chemical gradient exhibits distinguish pattern between daytime and nighttime as a result of the combine activities of microbial metabolisms. This gradient system is the basis for the stratification of microbial consortia with a wide range of different functional groups (Paerl and Pinckney 1996; Ramsing *et al*. 1996; Nealson 1997).

Stratified microbial consortia are complex and dynamic ecosystems consist of diverse microbial groups involved in nutrient transformations in close proximity. Microbial consortium generally consists of several layers. The uppermost layers are dominated by oxygenic photosynthetic microbes such as cyanobacteria or eukaryotic algae, whereas the lower layers are dominated by anaerobic sulfate-reducing bacteria or methanogens (Paerl and Pinckney 1996; Ramsing *et al*. 1996). Anoxygenic photosynthetic microbes such as colorless

sulfur bacteria, purple sulfur bacteria and green sulfur bacteria dominate the oxic-anoxic zone in the mid-layers (Takahashi and Ichimura 1970; Paerl and Pinckney 1996; Ramsing *et al*. 1996).

Photosynthesis by cyanobacteria and eukaryotic algae act as the driving force for laminated microbial consortium. Oxygen produce as the by-product of photosynthesis creates an oxidize zone for processes such as aerobic respiration and nitrification. In addition, the organic products from cyanobacteia and eukaryotic algae serve as energy source for other microorganisms (van Gemerden 1993). In deeper anoxic, H_2S-rich layers which are produced due to decomposition of organic materials, sulfate-reducing bacteria produce sulfide by dissimilatory reduction of sulfate. Sulfide is then reoxidized to sulfate by colourless, purple and green sulfur bacteria. Processes occurring in anoxic zones include denitrification, nitrogen fixation, fermentation and methanogenesis by a wide range of anaerobic heterotrophic microorganisms (Paerl and Pinckney 1996; Kopylov *et al*. 2002; Visscher and Stolz 2005). A schematic diagram of stratified microbial consortium is represented in Fig. 1.2.

1.3 Corals have adapted to oligotrophic environments

Coral reefs are mainly located in coastal tropical and subtropical waters between latitudes 30°N and 30°S. Scleractinian or reef-building corals, the main component of coral reef ecosystems thrive in warm (18°C to 30°C), optically clear, oligotrophic waters characterized by low levels of inorganic nutrients (D'Elia and Wiebe 1990). They have adapted to oligotrophic environment through the formation of obligate symbiosis with symbiotic dinoflagellates from the genus *Symbiodinum*, or commonly known as "zooxanthellae" (Muscatine and Porter 1977). In other word, reef-building corals are symbiont that consists of the cnidarians host and symbiotic algae.

Zooxanthellea photosynthesize inside host coral and provide nutrients to the host by

translocating up to 95% of their photosynthetic products in the forms of amino acids, sugars and carbohydrates (Muscatine 1967; Muscatine 1990). Corals utilize these photosynthates provided by zooxanthellae for respiration, growth, calcification and reproduction. In return, corals supply zooxanthellae with essential nutrients in the form of ammonia, phosphate and carbon dioxide from the metabolism waste of corals (Trench 1979; Muscatine 1990). Nitrogen recycling occurs within this system in which amino acids are translocated to coral from zooxanthellae while ammonia is translocated to zooxanthellae by coral. Fig. 1.3 shows the mutalistic symbiotic relationship between reef-building coral and zooxanthellae. This is important in conserving nitrogen in nutrient depleted tropical oceans (Lewis and Smith 1971; Muscatine and Porter 1977). Mutualistic symbiosis between coral and zooxanthellae is crucial for the survival of both partners in water column that is normally poor in inorganic nutrients.

Symbiotic relationship between coral and zooxanthellae can be easily disrupted by environmental stresses. Increase in nutrient concentrations in seawaters has been suggested as a major cause of coastal reefs degradation. Anthropogenic nutrient enrichment causes negative impacts on coral reefs which resulted in the decline in corals health and degradation of coral reef ecosystems. Corals subjected to increase inorganic nitrogen concentrations showed reduction in growth, calcification, photosynthesis and reproduction (Marubini and Davies 1996; Ferrier-Pagès *et al*. 2000; Ward and Harrison 2000). In addition, corals exposed to increase inorganic nitrogen concentrations become more susceptible to other stressors (Bruno *et al*. 2003). This could eventually lead to a shift in community structure in reef ecosystem from coral-dominant to macroalgae-dominant ecosystem (Wielgus *et al*. 2004).

1.4 Coral bleaching phenomena

Coral bleaching is a phenomenon in which coral tissue loss its color due to partial or total elimination of the symbiotic dinoflagellates (zooxanthellae) or loss of the pigmentation within symbiotic dinoflagellates or both as shown in Fig. 1.4 (Glynn 1991; Brown 1997). In

general, any disruptions of the balance in coral-symbiont relationship will resulted in coral bleaching. In laboratory, several factors have been shown to trigger coral bleaching: changes in seawater temperatures, high irradiance, changes in salinity, prolonged darkness, heavy metals pollution and pathogenic microbes (Muscatine *et al*. 1991; Hoegh-Guldberg and Smith 1989; Kushmaro *et al*. 1997; Brown 2000). Large-scale, extensive coral bleaching around the world is believed mainly attributed to elevated sea surface temperature (SST), often combined with increase solar radiation (including ultra-violet radiation) (Brown and Suharsono 1990; Williams and Bunkley-Williams 1990). Other environmental stresses and pathogenic microbes may act synergistically with elevated SST and increase solar radiation. This can cause the lowering of temperature threshold at which coral bleaching occurs as well as reduce coral reef resilience (Douglas 2003).

Bleaching events have increase in frequency and severity. Since it was first described in the Pacific by Glynn in 1984, coral bleaching has been documented in the Caribbean, Indian, and Pacific Oceans on regular basis (Hughes 1994; Hoegh-Guldberg and Salvant 1995; Brown *et al*. 1996). In 1998, coral reefs around the world experienced the largest and most widespread mass bleaching event ever recorded. This bleaching event coincided with periods of high SST and was associated with the El Niño-Southern Oscillation (ENSO) (Wilkinson 1998; Kerr 1999). It was estimated that corals around the world suffered 16% mortality as the result of this mass bleaching event (Wilkinson 2000). Field studies and “Hotspot” program run by the US National Oceanic and Atmospheric Administration (NOAA) have shown that coral bleaching is tightly associated with the warmer-than-normal conditions, i.e. SST exceed their normal seasonal maximum (Jokiel and Coles 1990; Goreau and Hayes 1994; Winter *et al*. 1998).

Coral bleaching can be described as the breakdown in the symbiotic relationship between coral and zooxanthellae. Under stress conditions, host regulations on zooxanthellae as well as

signaling from the symbiont are disrupted resulted in the expulsion of zooxanthellae (Bhagooli and Hidaka 2004; Dunn *et al*. 2007). Recent study by Bouchard and Yamasaki (2008) implied that production of nitric oxide (NO) in zooxanthellae under heat stress is link to coral bleaching. Physiological responses to bleaching include degradation of zooxanthellae *in situ*, expulsion of zooxanthellae and expulsion of intact endodermal cells containing zooxanthellae (Brown *et al*. 1995). Decreases in photosynthesis and photosynthetic efficiency have also been measured in zooxanthellae associated with increased temperature and irradiance (Warner *et al*. 1996). Many studies to elucidate mechanisms involved in bleaching related to elevated temperature and irradiance have produced evidence that photoinhibition due to accumulation of oxidative stress at photosystem II (PSII) is a primary factor (Iglesias-Prieto *et al*. 1992; Warner *et al*. 1999). Heat stress suppresses Calvin cycle activity, over reduces the electron transport system (Jones *et a*l. 1998), causes the production of reactive oxygen species (ROS) in chloroplast (Lesser 2006) and inhibits PS II protein repair process (Takahashi *et al*. 2004) in zooxanthellae. All these mechanisms may occur sequentially or in parallel.

Interspecific and intraspecific variations in the degree of bleaching have been observed in the field. Differences in susceptibility to bleaching are the results of the genetic variation (ribotype) in *Symbiodinium* as well as the acclimatory response of the host animal (Brown et al. 2002; Rowan 2004). Branching corals from the genera *Stylophora*, *Acropora* and *Pocillopora* are highly susceptible to bleaching, whereas massive corals of the genera *Goniopora*, *Goniastrea*, *Galaxea* and *Cyphastrea* are more resistant to bleaching (Loya *et al*. 2001; McClanahan *et al*. 2004). In the case of *Symbiodinium*, ribotype C in *Montastreae* sp. has been shown to be less tolerance than ribotypes A and B to elevated SST and irradiance (Rowan *et al*. 1997). Recently, *Symbiodinium* ribotype D was found to be more stress tolerance compare to other ribotypes (Huang *et al*. 2006). Coral and their zooxanthellae are

able to acclimatize and adapt to changes in the environmental factors. Corals might be able to switch their zooxanthellae to more stress-resistance variant as proposed in the “Adaptive Bleaching Hypothesis” (ABH) by Buddemeier and Fautin (1993). Other mechanisms applied by host to reduce bleaching damage including the production of fluorescence pigments (FP) and mycosporine-like amino acids (MAA). Corals also have antioxidant systems and stress enzymes that can cope with oxidative stress (Baird *et al*. 2008). One of the examples of corals adaptation to extreme environment was reported by Nakamura *et al*. (2006) in which Acroporid corals are found growing over a methane-bubbling hydrothermal vent.

Depending on the duration of elevated SST and other environmental stresses as well as coral ability to acclimatize or adapt to these abiotic factors, bleached corals may survive or die (Coles and Brown 2003; Douglas 2003). Apart from coral mortality, bleaching affects coral populations by decreasing coral reproductive capacity (Szmant and Gassman 1990), reducing coral growth, calcification and repair capabilities (Goreau and Macfarlane 1990; Meesters and Bak 1993). These in turn reduce coral ability to compete for space with other organisms in coral reef ecosystems which eventually causes changes in community structure (Glynn 1993; Hughes 1994). With the current global climate change scenarios, future increases in SST are likely to cause more mass coral bleaching which will have a severe impact on coral reef ecosystem (Hoegh-Guldberg 1999). This situation is of great concern as highlighted in the recent report produced by the Intergovernmental Panel on Climate Change (IPCC) (Fichilin *et al*. 2007).

1.5 Endolithic microbes in coral skeleton

Scleractinian corals live in close association with a wide range of microorganisms including algae, fungi, bacteria, archaea and viruses. (Rosenberg *et al*. 2007). Coral skeleton provides a unique niche for the growth of endolithic microorganisms in which it is surrounded

by coral tissue, and with physical and chemical compositions significantly different from that of ambient water (Risk and Muller 1983; Shashar and Stambler 1992). Visible green, black, brown and red bands correspond to eukayortic algae, fungi, bacteria and cyanophyte have been observed inside the skeletons of diverse species of corals (Di Salvo 1969; Highsmith 1981; Bak and Laane 1987; Ralph *et al*. 2007). Table 1 shows a list of documented endolithic microorganisms in coral skeletons. Most of the coral species studied are massive type scleractinian corals. Endolithic filamentous green algae of the genus *Ostreobium* (Siphonales: Chlorophyta) are the most frequently described endolith in coral skeletons (Lukas 1974; Le Campion-Alsumard *et al*. 1995a). They form a dense green band or multiple layers of band underneath the coral tissue (Magnusson *et al*. 2007; Ralph *et al*. 2007). Fig. 1.5 shows a green layer of endolithic algae observed in the skeleton of *Goniastrae aspera*.

Species composition of endolithic eukaryotic algae and cyanobacteria as well as their boring activity are different between live corals and dead or denuded coral skeletons (Le Campion-Alsumard *et al*. 1995a). *Ostreobium quekettii* which bore from the inside outward was the dominant endolith in live colonies of *Porites lobata* whereas *Phaeophila dendroides* (Chlorophyta), *Mastigocoleus testarum* and *Plectonema terebran*s (cyanobacteria) colonized the surface and bored inward dead coral skeletons (Le Campion-Alsumard *et al*. 1995a).

A prominent feature of the coral skeleton habitat is the light microclimate. It was estimated that actual levels of photosynthetically active radiation (PAR) reaching the endolithic algae range from <0.01% to 2% of the incident irradiance at the coral surface. Approximately 90 to 99% of the incident irradiance was either absorbed by the symbiotic dinoflagellate in coral tissue or attenuated by the coral skeleton (Schlichter et al. 1997; Magnusson et al. 2007). Beside the extreme low light intensity, light quality reaching the coral skeleton is also different from the outside environment in which it is enriched in far-red wavelengths (Magnusson et al. 2007). *Ostreobium* spp. that inhabit in coral skeleton have adapt to such

environment with multiple strategies (Highsmith 1981; Fork and Larkum 1989; Koehne *et al*. 1999; Wilhelm and Jakob 2006). In some coral species, the distance of the green band from the skeletal surface has been shown inversely correlated with water depth (Highsmith 1981).

In addition to the extreme low light intensities, endolithic microorganisms living inside coral skeleton are exposed to drastic diurnal fluctuations in pH and oxygen levels (Bellamy and Risk 1982; Shashar and Stambler 1992). Shashar and Stambler concluded that this diurnal pattern was due to the photosynthesis in the day and respiration at night by the coral tissue. Using planar oxygen optodes and a luminescence lifetime imaging system, Kühl *et al*. (2008) were able to map the oxygen dynamics in endolithic photosynthetic communities in corals. Their results indicate that the internal oxygen level is determined not only by coral tissue layer but also by endolithic activity. Endolithic microorganisms utilize various enzymatic processes to adapt to this extreme environment in addition to their low metabolic activity rates (Shashar and Stambler 1992).

Endolithic microorganisms living inside coral skeletons are well known microborer that has destructive role in reef ecosystems (Le Campion-Alsumard *et al*. 1995a; Gracia-Pichel 2006). However, they are also of trophic importance (Odum and Odum 1995; Schlichter e*t al.* 1997; Tribollet *et al*. 2006). Ferrer and Szmant (1988) and Schlichter *et al*. (1997) suggested that nutrient generated by the endolithic communities are a potential nutrient source to the host corals. This is proven using radioisotopic method in which translocation of ^{14}C-labelled photoassimilates from the endoliths to coral tissue was observed (Schlichter *et al*. 1995). This alternative source of nutrient is especially important for the survivorship and recovery of corals during bleaching event (Fine and Loya 2002). Recent study shows that endolithic algae also have a photoprotective role for the host photosynthesis, i.e. zooxanthellae's photosynthesis during high light stress (Yamazaki *et al*. 2008).

1.6 Objective

The discovery of endolithic algae in coral skeleton covered with live tissue can be traced back to more than 100 years ago; nevertheless biological interaction between these 2 organisms remains unresolved. In the study of microorganisms associated with corals, many research focus on the microorganisms reside in coral tissue and coral mucus as but not within the coral skeleton. Coral skeleton covered with live tissue is presumed to create a hypoxic or anoxic conditions suitable for the growth and colonization of a wide range of microorganisms. Using molecular biological tools such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and sequencing analysis of specific functional genes, study of the population of endolithic microorganisms within coral skeleton is possible without conventional culture method. This study aims to characterize the endolithic microbes in coral skeletons and explore the beneficial role of endolithic microbes-coral association. Chapter 1 provides a brief introduction on coral reef environment, coral bleaching, microbial consortium as well as endolithic microorganisms found in coral skeleton. Chapter 2 described endolithic algae inhabit in *Goniastrea aspera*. Chapter 3 compared the endolithic microbial flora in coral skeletons from both oligotrophic and eutrophic waters. Chapter 4 explored the photoprotective role of endolithic algae to host coral. Finally in Chapter 5, I discussed potential symbiotic relationship between coral and endolithic microbes as well as the pros and cons of endolithic microbes on reef-building corals.

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Table 1. Endolithic microorganisms found in coral skeletons.

Endolithic microorganisms	**Coral species**	**Reference**
Fungi	*Porites lutea*, *Porites lobata*	Bak and Laane 1987 Kendrick *et al*. 1982 Priess *et al*. 2000
Siphonalean chlorophyte: *Ostreobium quekettii* *Ostreobium reineckei* *Ostreobium constrictum*	*Agaricia* sp., *Montastrea* sp., *P. lutea*, *Porites cylindrical*, *Favia pallida*, *Goniastrea retiformis*, *Platygyra lamellina*, *Pavona clavus*, *Oulophllia crispa*, *Astreopora myriophthalma*, *Montipora monasteriata*, *Cyphastrea serailia*, *Goniastrea australensis*, *Mycedium elephantotus*, *Leptoseris fragilis*	Lukas 1974 Highsmith 1981 Le Campion-Alsumard *et al*. 1995a Schlichter *et al*. 1997 Magnusson *et al*. 2007 Ralph et al. 2007
Chlorophyta: *Phaeophila dendroides*	*P. lobata*	Le Campion-Alsumard *et al*. 1995a
Cyanobacteria: *Mastigocoleus testarum* *Plectonema terebrans*	*P. lobata*	Le Campion-Alsumard *et al*. 1995a
Bacteria	*P. lobata*	Di Salvo 1969

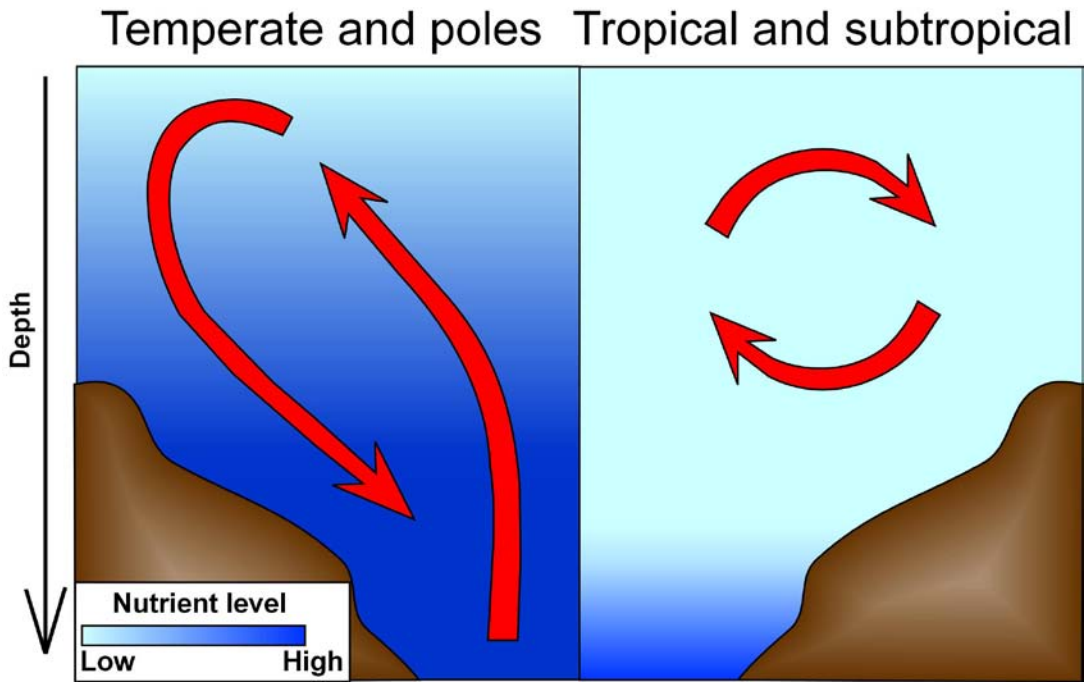

Fig. 1.1. Difference in nutrient level in temperate/poles oceans and tropical/subtropical oceans. The higher nutrient level in temperate and poles seawaters is due to the mixing of deep, cool nutrient-rich water with warm, nutrient-depleted surface water (left panel). Mixing between deeper water and surface water is restricted in tropical and subtropical oceans due to the presence of thermocline throughout the year (right panel).

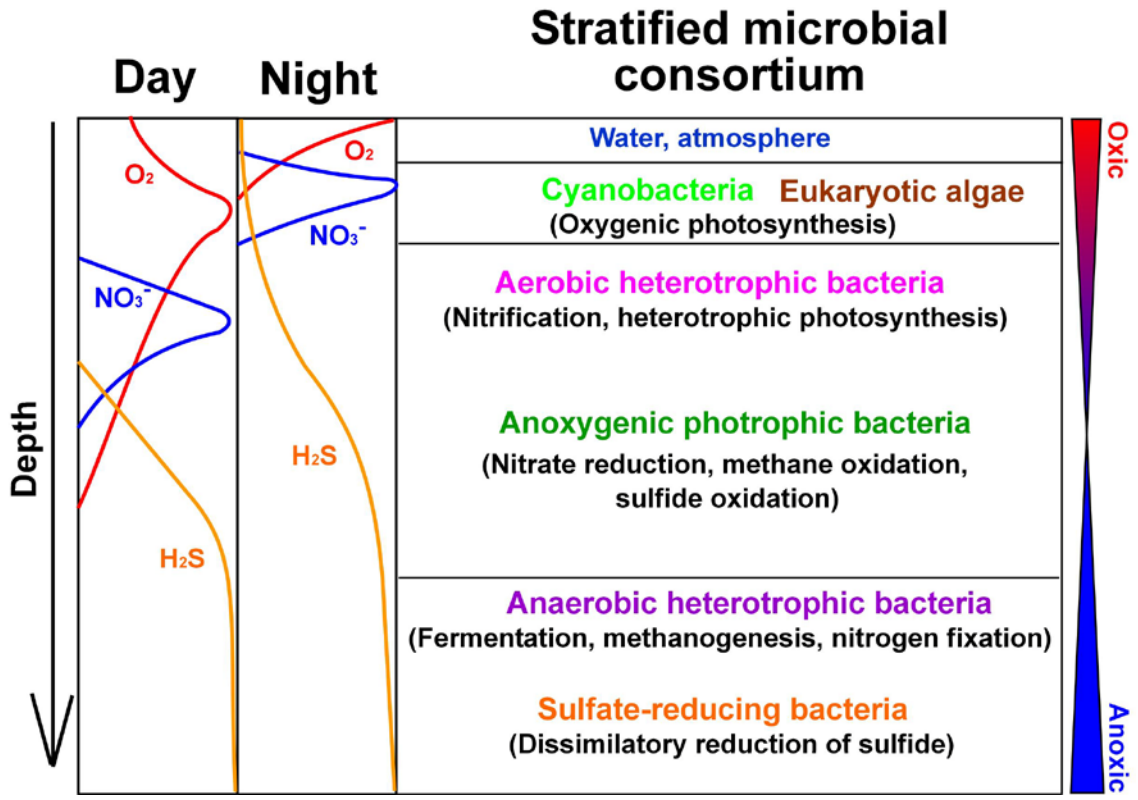

Fig. 1.2. Different functional groups of microorganisms and possible metabolic activities in a stratified microbial consortium. Such microbial consortium can be found in stratified lakes, oceans, sediments, microbial mats and microbial biofilm. Left panels show the temporal changes of oxygen, oxidized nitrogen and hydrogen sulfide concentrations with depth.

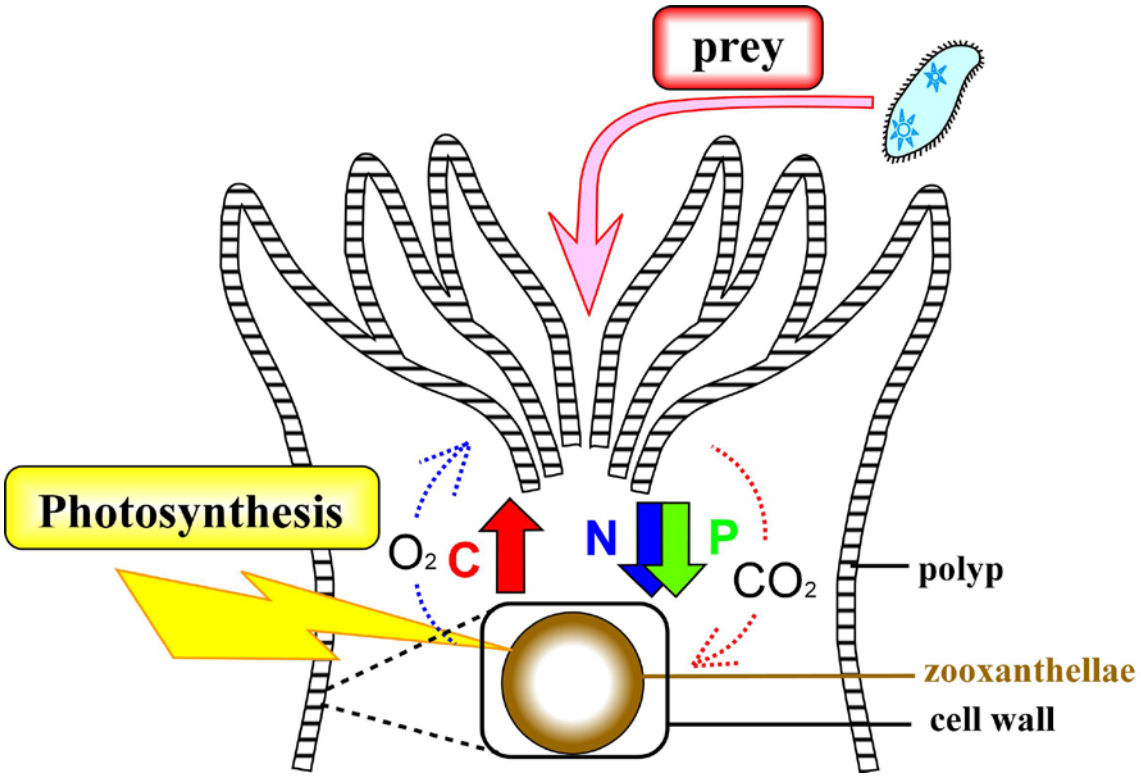

Fig. 1.3. Mutualistic symbiotic relationship between reef-building coral and symbiotic dinoflagellate (zooxanthellae). Zooxanthellae reside in the endoderm layer of coral polyp. Besides obtaining nutrient via heterotrophic activity, reef-building coral also obtain nutrient in the form of fixed carbon from zooxanthellae. In return, host coral translocate nitrogen and phosphorus compounds to zooxanthellae.

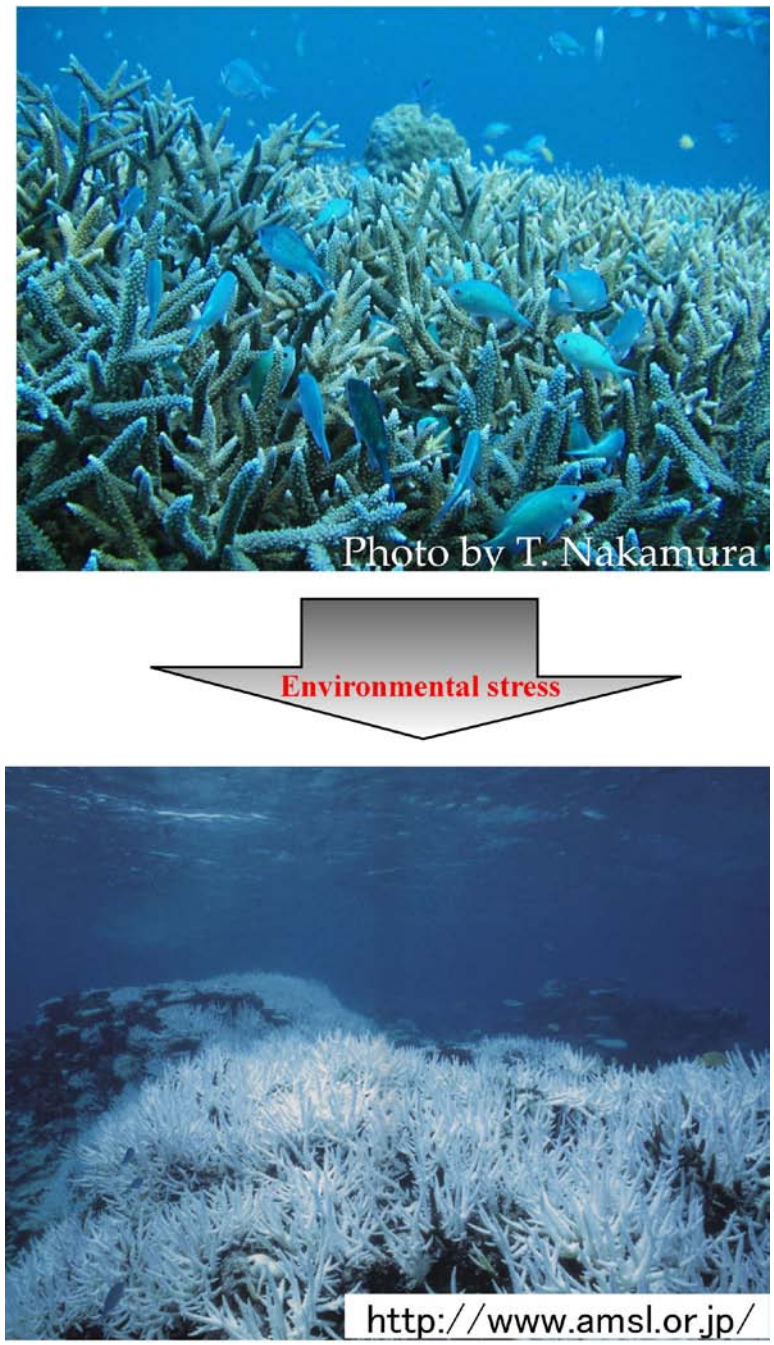

Fig. 1.4. Corals expel their zooxanthellae or lost their pigmentation resulted in coral bleaching under environmental stress conditions. Upper panel shows healthy coral reef whereas lower panel shows bleached reef.

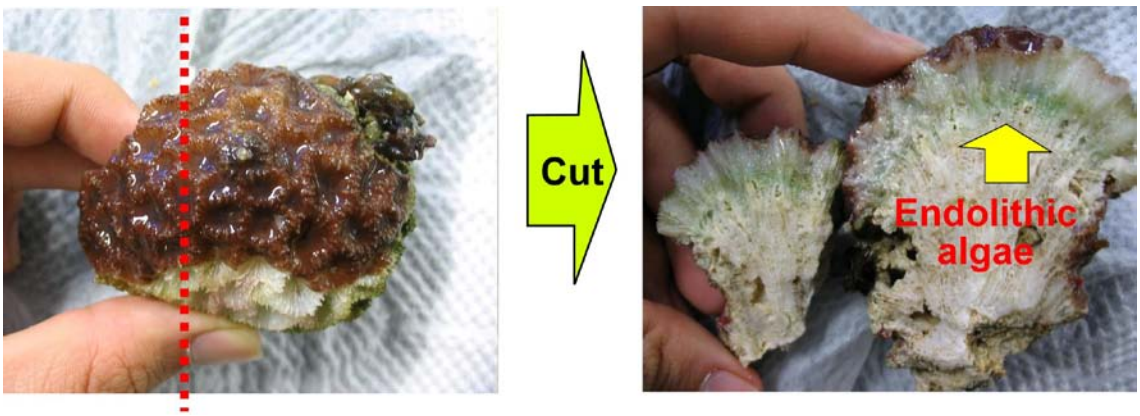

Fig. 1.5. Endolithic algae in massive coral *Goniastrea aspera*. A layer of green band was observed in the skeleton of *G. aspera* that was cut vertically. This green layer mostly consists of endolithic algae (as indicated in yellow arrow).

Chapter 2

Endolithic algae inhabit in Goniastrea aspera

Reef-building corals harbor diverse communities of cyanobacteria, eukaryotic algae, bacteria and fungi within their skeleton. In spite of their potential significance, the interaction between these microbes and host corals is still obscure. Here we report a novel cyanobacterium within the skeleton of *Goniastrea aspera*, a massive reef-building coral that is predominantly found in shallow reef habitats. Characteristics of this cyanobacterium are: (1) non-branching filaments having a 1 μm diameter, (2) lack of heterocysts, (3) hormogonia formation, and (4) chlorophyll *a* as the sole chlorophyll pigment. Consistent with these phenotypic traits, 16S rDNA sequence analysis showed a close association with this cyanobacterium to *Halomicronema*, a recently identified genus that includes species found in benthic microbial mats of hypersaline ponds. A possible interaction between *Halomicronema* sp. and the host coral is discussed in terms of stress tolerance.

Key words:
coral skeleton, cyanobacteria, endolithic algae, *Goniastrea aspera*, *Halomicronema* sp.

2.1 Introduction

The obligate symbiotic relationship between reef-building corals and dinoflagellates (zooxanthellae) is well recognized (D'Elia and Wiebe 1990). In addition to this well-established relationship, coral tissue and coral skeleton are also known to harbor diverse microbial consortia (Rohwer *et al*. 2002; Le Campion-Alsumard *et al*. 1995a). Until recently, however, these coral-microbial interactions remain obscure (Le Campion-Alsumard *et al*. 1995b).

The presence of endolithic microorganisms in skeleton of corals was first discovered in 1902 by Duerden (Duerden 1902). Visible green bands usually observed in the skeletons of massive corals comprised of cyanobacteria, fungi, bacteria, red and green algae (Le Campion-Alsumard *et al.* 1995a; Schlichter *et al*. 1997). Among endolithic microbes, siphonalean chlorophyte, *Ostreobium* spp. are frequently found in coral skeletons (Lukas 1974).

The function of bioerosion that contributes to the geochemical and sedimentological importance in the reef was documented (Kobluk and Risk 1977). Also, endolithic microorganisms have been considered to be one of the major primary producers in coral reef environments (Tribollet *et al*. 2006). Recent findings have shown that endolithic algae transfer photoassimilates to coral host (Fine and Loya 2002; Lesser *et al*. 2007). This serves an alternative nutrient source especially during bleaching events. More recently, endolithic algae were reported to possess a photoprotective role in the coral-algal photosynthesis during high-light stress (Yamazaki *et al*. 2008).

Coral skeleton covered by living coral tissue is a harsh environment for the growth of many organisms (Shashar and Stambler 1992). This environment partly shares a similarity with that for microbial mats that harbor diverse microbial communities (Fourçans *et al*. 2006). In the intertidal reefs in Okinawa, Japan, massive coral *Goniastrea aspera* is constantly being exposed to a strong sunlight and high salinity during low tides. In this study, we report an

endolithic community found within the skeleton of *G. aspera* .

2.2 Material and Methods

Colony of massive coral *Goniastrea aspera* (approximately 5-6 cm in diameter) was collected in June 2004 from a shallow intertidal pool of Bisezaki, Okinawa Japan. Coral tissue was removed using a WaterPik (EW170, National, Japan) (Johhannes and Wiebe 1970) and coral skeleton was crushed to small fragments (approximately 1 cm in diameter) with a chisel. Skeletal fragments were then trimmed off with anatomical scissors to small pieces < 2 mm in length). The crushed skeletal pieces of *G. aspera* containing green bands were investigated utilizing culture method, microscopic observations, pigments analysis and molecular techniques.

Small pieces of coral skeleton were incubated in a liquid A medium (Mitsui and Cao 1988). Nitrogenous compounds were omitted from this medium to prevent the growth of undesirable microorganisms. The culture medium was modified to include sterile coral tissue extraction. Repeated subcultures of colonies were carried out on agar plates to obtain a pure culture of cyanobacterium. All cultures were incubated at 28°C under a 12h dark : 12h light (30 μmol photons m^{-2} s^{-1}) photoperiod. The cultured cyanobacterium was used for pigment analysis and DNA analysis.

For microscopic observation, pieces of trimmed coral skeleton were ground in sterilized seawater using mortar and pestle. The samples were observed under a fluorescence microscope.

Pigments extracted from the green band and the cultured cyanobacterium were analyzed by thin layer chromatography analysis (TLC). Coral tissue was removed with a WaterPik as the described above. The tussue-removed skeletons were crushed in 90% acetone at 4°C, and were centrifuged at 10,000 x *g* for 2 min and the supernatant was used for TLC analysis. The cultured cyanobacterium was collected by centrifugation (15,000 x *g*, 3 min). The supernatant

was then discarded and the cyanobacterial pellet was homogenized in 90% acetone at 4°C, and it was centrifuged at 15,000 x *g* for 3 min and the supernatant was used for TLC analysis. All procedures were carried out under a dim light. TLC analysis was carried out on a reverse phase C_{18} plate (MERCK) with 100% MeOH as the developer. The spots of chlorophyll pigments were visualized under a blacklight (UVP UVL-56, UVP, USA).

Total genomic DNA of the cultured cyanobacterium was extracted using the UltraClean Soil DNA Kit (MoBio, Solana Beach, CA). PCR was performed using the primers of fD1 (5'-AGAGGATGATCAGCCACACTG-3') and rP2, which were designed for eubacterial 16S rDNA (Weisburg et al 1991). The reaction mixture (50 µl) contained 0.15 mM deoxynucleotides (Takara, Tokyo, Japan), 0.2 µM forward primer, 0.2 µM reverse primer, 2µl of the PCR template and 0.05 U of recombinant *Taq* DNA polymerase (Takara) per µl in PCR buffer (Takara). The temperature program for 30 cycles of PCR was 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, and 72°C for 5 min as the final extension after the last cycle. The amplified DNA fragments were purified by gel percolation. The sequence was determined in opposite orientations using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with a DNA Sequencer (ABI 3100 Avant; Applied Biosystems, USA). The obtained nucleotide sequence has been deposited in DNA Data Bank of Japan (DDBJ) with an accession number AB257773. Related sequences were aligned with CLUSTAL X 1.83 software for multiple sequence alignment, and bases of ambiguous alignment were corrected or removed manually. Tree topology was constructed with the neighbor joining method with MEGA 3.0 software (Kumar *et al*. 2004).

2.3 Results

Fig. 2.1A shows the localization of green bands observed in the transverse section of *G. aspera*. It exhibits two distinct layers of green band within the skeleton. Green bands were

observed just beneath and in deep of the skeleton that were separated by a light green or whitish zone. The green bands were primarily composed of photosynthetic organisms having non-branching, narrow filament approximately 1 μm in diameter as shown in Fig. 2.1B and C. *Ostreobium* sp. was observed on the fragments of the coral skeletons. The green alga showed repeatedly branching, non-septating 3 μm diameter filaments having knobby surface irregularities (Fig. 2.1D and E). In addition to *Ostreobium* sp., coccoid photosynthetic organisms with less than 1 μm in diameter were also observed in the suspension of the green band and on the surface of skeletal fragments. These could be attributed to the deposits during the process of tissue removal (data not shown).

A cyanobacterium was successfully isolated and cultured with the modified A medium. This cultured cyanobacterium was non-branching, non-heterocyst forming, had narrow filaments around 1 μm in diameter and formed motile hormogonia (Fig. 2.2). All these characteristics can be found in cyanobacteria belonging to the order Oscillatolariales.

Analysis of chlorophyll pigments using TLC revealed that both green band from coral skeleton and the isolated cyanobactrium contained chlorophyll *a* (Fig 2.3). However, chlorophyll *b* was only found in the extract obtained from the green band in the coral skeleton (Fig 2.3, lane A). Similar results were obtained with HPLC analysis (data not shown).

A phylogenetic tree constructed by the neighbor joining method is presented in Fig. 2.4. A BLAST homology search of the determined sequence showed the closest similarity to a 16S rDNA region of "*Halomicronema excentricum* (AB257773)".

2.4 Discussion

To the best of our knowledge, this is the first report of a cyanobacterium closely related to "*Halomicronema excentricum*" inhabiting in the skeleton of a live coral. Species of the genus *Halomicronema* are one of the dominant cyanobacateria found in hypersaline microbial mat.

They are characterized moderately halophilic and thermophilic (Abed *et al*. 2002; Fourçans *et al*. 2006). Their presence in *G. aspera* skeleton suggests that the internal environments of this coral skeleton could be similar to the habitats for hypersaline microbial mats.

Shallow intertidal pools in coral reefs are harsh environments that accompany daily and seasonal changes in salinity and water temperature with large extents (Brown *et al*. 1994). The massive coral *G. aspera* can be dominantly found in such harsh habitats. It is interesting to note that even after the mass bleaching event in 1998, many *G. aspera* survived and increased in abundance around Okinawa Island (Loya *et al*. 2001). It appears that *G. aspera* is relatively stress tolerant. Endolithic algae found in coral skeletons have been reported to facilitate the host recovery from a bleached condition (Fine *et al*. 2002). "*Halomicronema*" was reported to have halophilic and thermophilic characteristics (Abed *et al*. 2002). Recently, it has been detected in the tissue and mucus of bleached *Oculina patagonica*, a scleractinian coral found in the Mediterranean Sea off the coast of Israel (Koren and Rosenberg 2008).

High temperature and high salinity conditions in a shallow intertidal pool may allow the colonization of "*Halomicronema*" within *G. aspera* skeleton. The presence of such stress-resistant microbes would contribute to the overall stress tolerance of *G. aspera*.

In addition to "*Halomicronema*", eukaryotic algae of the genus *Ostreobium* were also present in the skeleton of *G. aspera* as indicated in the pigments in TLC analysis as well as microscopy observation. Since we did not quantify the number of either "*Halomicronema*" or *Ostreobium* in the skeleton, major constitute of the green bands could not be determined.

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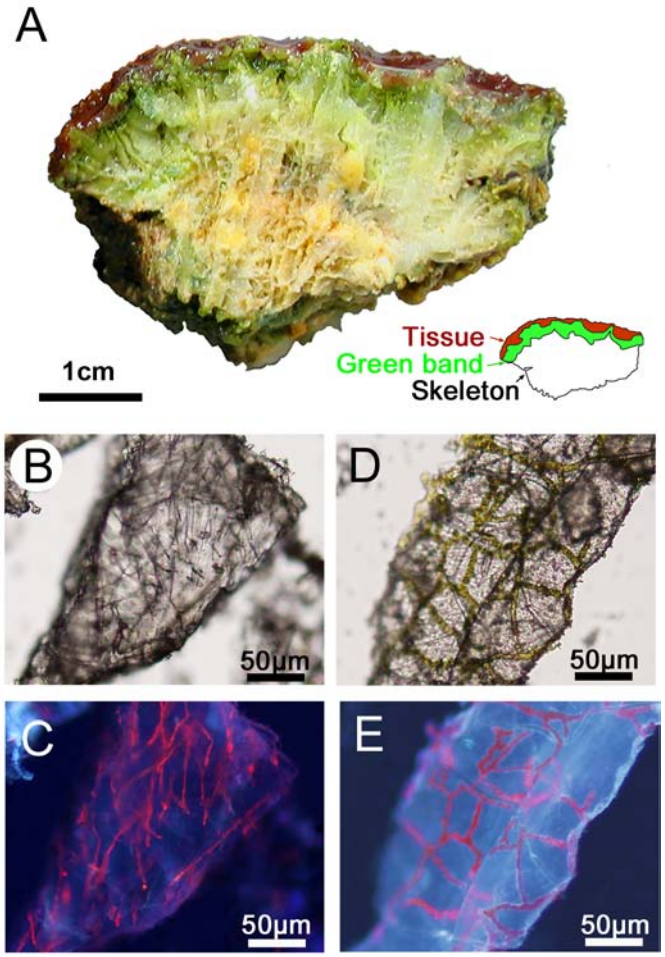

Fig. 2.1. Layer of green band observed within the skeleton of *Goniastrea aspera*. (A) Dissected transverse section of *G. aspera* skeleton, (B and C) Photosynthetic organism found in skeletal fragments, (D and E) *Ostreobium* sp. (C) and (E) are epifluorescence images of (B) and (D), respectively.

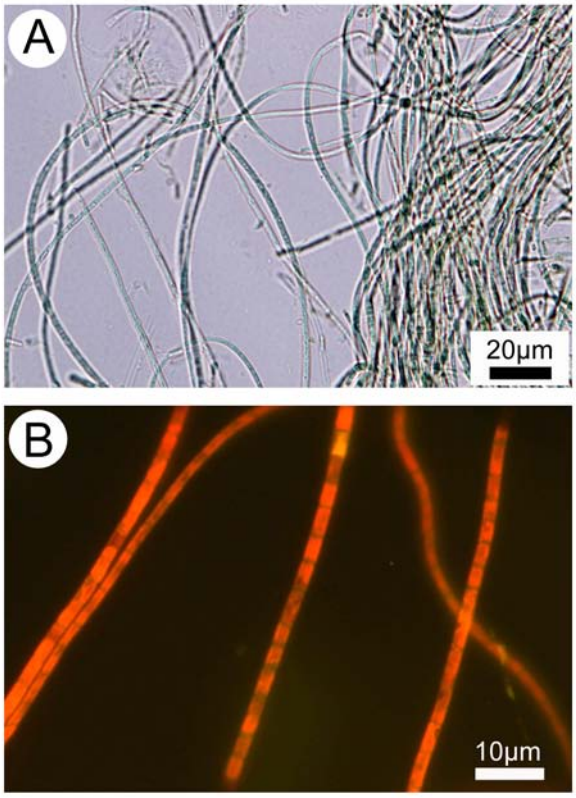

Fig. 2.2. Morphology of the cultured filamentous cyanobacterium isolated from the green band in *G. aspera* skeleton. (A) Light micrograph of the filamentous cyanobacterium (B) Epifluorescence image of the filamentous cyanobacterium.

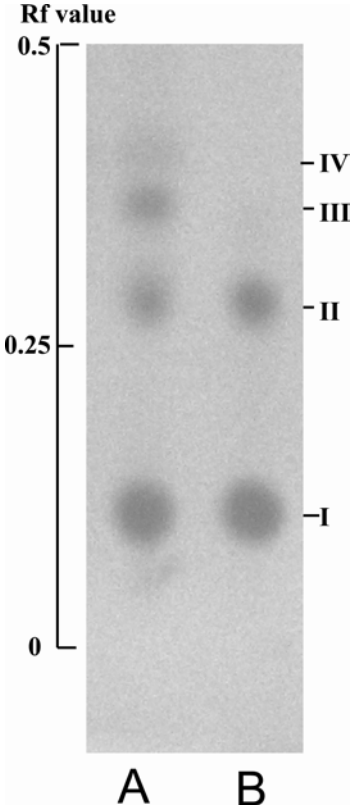

Fig. 2.3. TLC chromatogram of chlorophyll pigments. Lane A, pigments extracted from the green band found in *G. aspera* skeleton. Lane B, pigments extracted from the isolated filamentous cyanobacterium. (I) Pheophytin *a* (II) Chlorophyll *a* (III) Pheophorbide *a* (IV) Chlorophyll *b*.

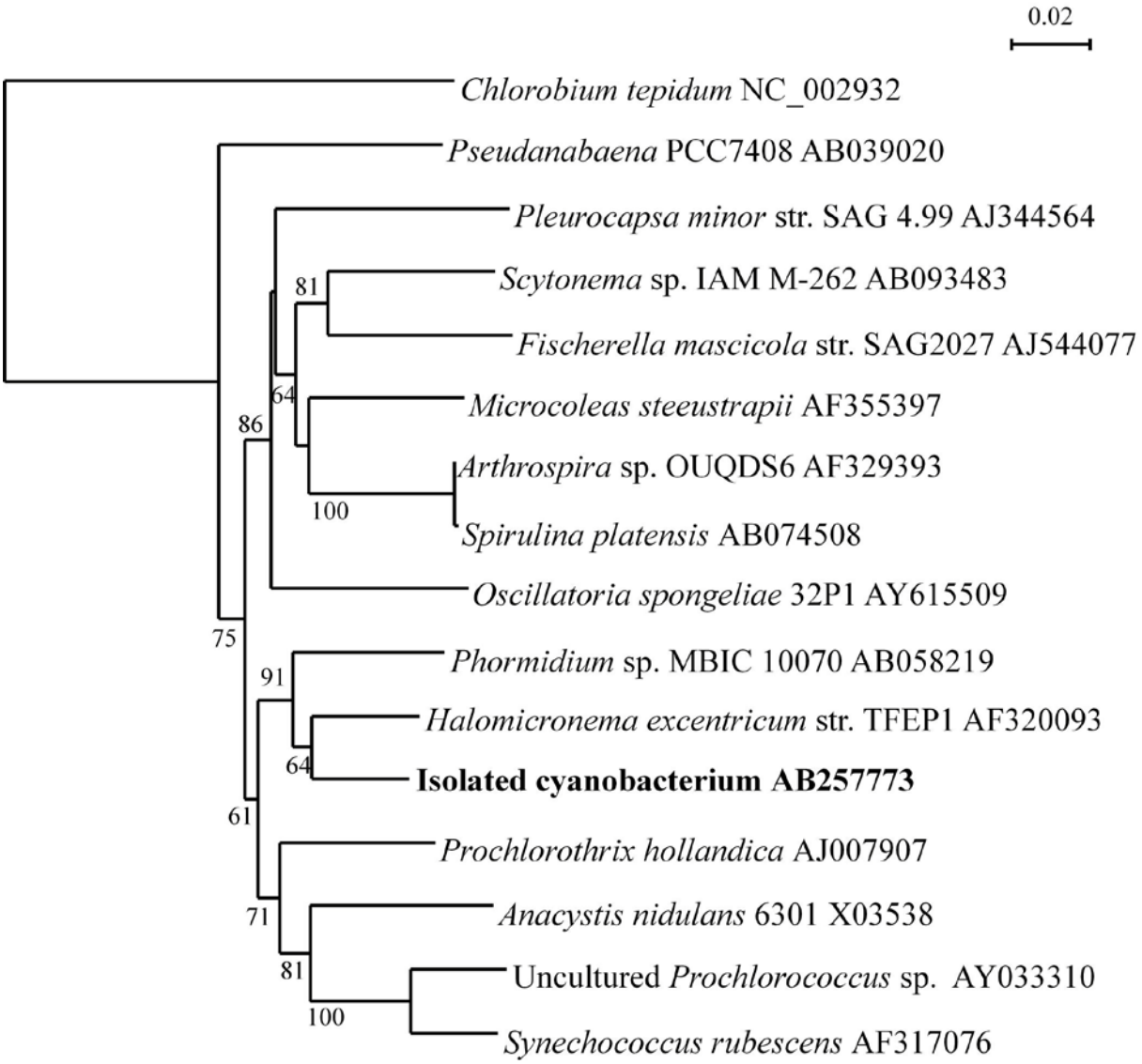

Fig. 2.4: A phylogenetic tree constructed by the neighbor-joining method on the basis of 1029 bases 16S rDNA partial sequence. The sequence of *Chlorobium tepidum* was used as an outgroup. The scale bar represents a 0.02 substitution per nucleotide position. Numbers indicate the percentage of bootstrap support out of 1000 resampling data from the neighbor-joining method.

Chapter 3

Comparison of endolithic microbial flora between oligotrophic waters and eutrophic waters

Coral skeletons are known to harbor diverse endolithic microorganisms. Beside cyanobacteria and eukaryotic microalgae, the endolithic community is poorly understood. Although reef-building corals have evolved and adapted to oligotrophic waters, many coastal reefs are constantly exposed to anthropogenic nutrient enrichment. Nevertheless, the effects of increase nutrient levels on coral endolithic community remain unknown. Here we report the difference in diversity of bacterial community in the skeleton of massive coral *Goniastrea aspera* collected from both oligotrophic waters and eutrophic waters. Higher bacterial diversity was found in coral skeletons collected from oligotrophic waters compared to those from eutrophic waters. Moreover, anaerobic bacteria were only found in the coral skeleton from oligotrophic area. This finding suggests that the community of endolithic microorganisms in coral skeleton is more complex than previously thought. Our findings imply that changes in seawater nutrient levels can have an impact on the endolithic microbial community.

Keywords:
aerobic, anaerobic, coral skeleton, endolithic bacterial community, eutrophic, oligotrophic

3.1 Introduction

Healthy corals associate with a large, diverse and specific population of microorganisms (Rosenberg *et al*. 2007). These microorganisms which include eukaryotic algae, bacteria, archaea and viruses can be found living in the mucus layer, skeleton and tissue of corals (Ducklow and Mitchel 1979; Campion-Alsumard *et al*. 1995; Rohwer *et al*. 2001; Baker 2003; Wegley *et al*. 2004). Following the discovery of endolithic algae in coral skeleton by Duerden 1902, many studies were then carried out to characterize this group of algae as well as the symbiotic relationship between the coral and the endolithic algae (Jeffrey 1968; Le Campion-Alsumard *et al*. 1995; Fine and Loya 2002). Filamentous green algae of the genus *Ostreobium* (Siphonales: Chlorophyta) and endolithic cyanobacteria are the dominant endolithic community described (Lukas 1974; Le Campion-Alsumard *et al*. 1995).

Endolithic microorganisms were discovered in coral skeleton more than 100 years ago. Except *Ostreobium* and cyanobacteria, very little is known of this microbial community composition and biodiversity. Studies of endolithic microorganisms in rock, coral skeleton and crustose coralline algae carbonate substrate have focused mainly on the oxygenic phototrophs, *i.e.* cyanobacteria and green algae by the use of culture and microscopic methods for identification (Le Campion-Alsumard *et al*. 1995; Tribollet and Payri 2001; Horath and Bachofen 2009). However, many of the endolithic microbes are not readily cultured. Using sequence analyses of the small subunit ribosomal ribonucleic acid gene (SSU rRNA gene), a diverse heterotrophic endolithic population consists of many different taxa was discovered in dolomite rock (Horath and Bachofen 2009). In the case of coral, a novel cyanobacterium that is closely related to extremophile "*Halomicronema excentricum*" was discovered in the skeleton of *Goniastrea aspera* inhabiting shallow intertidal pool recently (Yamazaki *et al*. 2009). So far there is only one report on endolithic bacteria associated with skeletons of *Porites lobata* (Di Salvo 1969). With the advancement of molecular biological tools, coral microbiology has been gaining a lot of attention from coral reef biologists and coral reef ecologists alike to understand the coral-microbial association in terms of coral health, disease and evolution (Rosenberg *et al*. 2007).

Reef-building corals have evolved and adapted to oligotrophic ocean characterized by low levels of inorganic nutrients through the development of obligate symbiosis with dinoflagellate (zooxanthellae) (D'Elia and Wiebe 1990). Anthropogenic nutrient enrichment has been suggested as a major cause of localized coral reef degradation in direct and indirect manner. Negative effects of nutrient enrichment on corals and coral reefs include reduction in

growth, calcification and reproduction of corals; increase corals susceptibility to disease and bleaching (Marubini and Davies 1996; Ward and Harrison 2000; Bruno *et al*. 2003) as well as alteration of benthic community structure in coral reefs (Wielgus *et al*. 2004). However, there are evidences that reef-building corals can thrive in relatively high-nutrient waters either in the aquarium, the laboratory or in the field (Atkinson *et al*. 1995; Koop *et al*. 2001; Yuen *et al*. in press). It should be noted that in both cases; i.e. oligotrohic and eutrophic, the effect of nutrients in coral-endolith association remains unknown.

The objective of this study was to describe the diversity of endolithic bacterial community present in the massive coral *Goniastrea aspera* in regards to inorganic nutrient levels in seawater. Using specific primer sets that target the partial 18S rDNA, 16S rRNA genes and functional genes of a wide variety of microorganisms, here we report the presence of diverse endolithic microorganisms in *G. aspera* skeleton.

3.3 Materials and methods

3.3.1 Sample collection

The massive, shallow-water reef-building coral *Goniastrea aspera* colonies were collected from 2 coral reef sites: Nelly Bay, Magnetic Island (19°S, 146°00′23″E) and Pioneer Bay, Orpheus Island (18°S, 146°29′49″E) in the Great Barrier Reef, Australia in January 2007 (Fig. 3.1). These 2 sites were chosen because of the differences in their trophic conditions. Concentrations of ammonium and phosphate in Nelly Bay were almost 8 times and 2 times higher compared to in Pioneer Bay, respectively (Jompa and McCook 2002; Muslim and Jones 2003).Ten individual samples (5 – 7 cm in diameter) were collected from each site at the depth of 2 – 4 m using hammer and chisel while skin diving. Samples were kept in an outdoor tank in James Cook University for a day before processing.

3.3.2 DNA extraction and PCR

Approximately 3 cm^2 of the tissue from the top part of *G. aspera* was removed with filtered seawater using a Waterpik (Johannes and Wiebe 1970). It was then frozen in dry ice for around 5 min for the ease of later processing. Using a hammer and a chisel, denuded coral skeleton was separated from the frozen sample and the green layer of the skeleton was then trimmed off with a nipper (Fig. 3.2). Small pieces of these skeleton fragments were

mechanically broken with mortar and pestle on ice. DNA was extracted from the crushed skeleton using the UltraClean™ SoilDNA Isolation Kit (Mo Bio Lab. Inc., USA) according to the manufacturer's instructions.

The target regions of the 16S rRNA (cyanobacteria, green non-sulfur bacteria, green sulfur bacteria), 18S rDNA (eukaryotic algae), *dsrB* gene (sulfate-reducing bacteria) and *nifH* gene (nitrogen-fixing bacteria) were amplified using the TaKaRa Ex Taq™ Hot Start Version kit (TaKaRa Bio Inc., Japan). Primers used in this study are showed in Table 1. PCR amplification was carried out with 25 µl reaction mixtures containing 2.5µl of 10X Ex Taq buffer, 1.5µl of dNTP mixture, 1.0µl of forward primer, 1.0µl of reverse primer, 2.0µl of DNA template, 0.25µl of Taq DNA polymerase and 17.0µl of distilled water. Amplification was performed with the PCR thermal cycler ASTEC, model PC707 (ASTEC, Japan). The thermal cycling program was as follow: initial denaturation at 94°C for 1 min, annealing and then primer extension at 72°C for 3 min each. A touchdown annealing protocol was performed: the annealing temperature started 2 to 7°C above the optimum temperature (depending on the primers used), decreasing by 0.5°C for each second cycle. The number of this cycle was different for each primer sets. A final extension at 72°C for 10 min was then performed. Nested PCR was carred out for *nifH* gene. PCR products were confirmed by electrophoresis on a 2% agarose gel and UV tranillumination (BioDoc-It™ Imaging System, UVP, Inc., Canada) after ethidium bromide staining.

3.4 Results

The 2 sampling sites, Pioneer Bay and Nelly Bay have very different characteristics. As shown in Fig. 3.1C and D, Pioneer Bay has much higher corals coverage and coral diversity compared to Nelly Bay. Dominant coral species are the branching *Acropora* spp. in this site. Fish diversity and density are also higher in Pioneer Bay. Nelly Bay is mainly covered with turf algae and brown alga *Padina* sp. with only few massive coral species such as those from the genera *Goniastrea*, *Porites* and *Faviates*. Fewer fish diversity and number were observed in Nelly Bay. Based on visual observation, sedimentation was higher in Nelly Bay resulted in turbid water conditions compared to Pioneer Bay.

A green layer inside *G. aspera* skeleton was observed in all samples collected. This green band is not located directly underneath the live coral tissue. The distance between the green band and the live coral tissue is approximately 5 mm (Fig. 3.2E). In addition to this green

band, we have also observed black bands in the skeleton of some *G. aspera*, especially those collected from Pioneer Bay (Fig. 3.2B). These black bands were also located approximately 5 mm underneath live coral tissue. During samples processing, hydrogen sulfide smell was detected from black-band samples but absence from green-band only samples.

PCR products corresponding to 16S rRNA for cyanobacteria, green nonsulfur bacteria and green sulfur bacteria, 18S rDNA for eukaryotic algae, *dsrB* gene for sulfate reducing bacteria and *nifH* gene for nitrogen-fixing bacteria were successfully detected in the DNA isolated from the green layer as well as from the black layer of the skeletons (Fig. 3.3). Diversity of microorganisms was higher in Pioneer Bay than in Nelly Bay as shown in Fig. 3.4. All targeted genes were detected from colonies collected from Pioneer Bay. In contrast, only 4 out of 6 targeted genes were detected from colonies collected from Nelly Bay. Genes detected were the 18Sr DNA for eukaryotic algae, *nifH* gene and 16S rRNA for green non-sulfur bacteria and cyanobactera; whereas both 16S rDNA gene of green sulfur bacteria and *dsrB* gene of sulfate-reducing bacteria were not detected.

In addition, the frequency of targeted genes was higher in Pioneer Bay samples compared to Nelly Bay samples except in the case of eukaryotic algae (Fig. 3.4 and 3.5). Cyanobacteria and green non-sulfur bacteria were detected in all the samples collected from Pioneer Bay, however only 40% and 60% of Nelly Bay samples contained cyanobacteria and green non-sulfur bacteria, respectively. Nitrogen fixing bacteria was detected in 90% of Pioneer Bay samples but only 50% of Nelly Bay samples (Fig. 3.5). The detection of *dsrB* gene in black bands samples collected from Pioneer Bay indicates that sulfate-reducing bacteria was indeed presence in *G. aspera* skeleton and they produce hydrogen sulfide gas.

3.5 Discussion

3.5.1 Oligotrophic versus eutrophic

Nelly Bay reef is typical of eutrophic inshore reefs on the Great Barrier Reef and support seasonally abundant stands of macroalgae (Muslim and Jones 2003). In contrast, Pioneer Bay is typical of midshelf reefs and macroalgae is less abundant (Jompa and McCook 2002). Endolithic microbial community in coral skeleton was different between the 2 sites with higher diversity and frequency in samples collected from oligotrophic site. This may be attributed to the different trophic conditions of the studied sites. Schneider and Torunski (1976) and Campbell (1984) found that community composition of endolithic microorganisms

changed and decreased under the influence of pollution. In a study to evaluate the potential usage of endolithic microorganisms as a sensitive mean of biological monitoring of pollution, Ghirardelli (2000) reported that higher diversity of endolithic cyanophyta and chlorophyta as well as number of individual were found in coralline algae collected from relatively good water quality environment. Whereas in area subjected to petrochemical pollution and eutrophication, species of endolithic flora was very few or sometimes absent.

Reef-building corals have adapted to living in oligotrophic waters. Therefore, changes in inorganic nutrient levels of coral reef environment not only affect coral itself but also microbes associated with the coral (Rosenberg *et al*. 2007). Eutrophication is believed to enhance coral disease by unknown mechanisms (Voss and Richardson 2006). It is plausible that eutrophication changes bacterial flora inside the coral skeletons. The shift of bacterial flora may contribute to the occurrence of coral disease as a result of floral changes from non-pathogenic to pathogenic microbial communities

3.5.2 Microbial communities

Like lime stone or rock, the porous coral skeleton provides shelter for a wide variety of microorganisms against solar radiation, oxygen tension as well as grazer (Le Campion-Alsumard *et al*. 1995; Shashar *et al*. 1997). Massive coral skeleton of *G. aspera* provides microenvironments that support a wide diversity of autotrophic and heterotrophic species. These microorganisms conduct a wide range of metabolic processes that take place in close proximity. Since measurement of oxygen tension in *G. aspera* skeleton is not feasible, a wide range of microbes with different tolerance of oxygenated conditions was used as an indicator to assess the environment inside coral skeleton. Three categories of microbes were used, i.e. aerobic, microaerophilic and anaerobic microbes. Eukaryotic algae and cyanobacteria are aerobic microbes that can photosynthesize using water as electron donor and release oxygen as a waste product. Green non-sulfur bacteria are facultative aerobic microbes that can carry out photosynthesis but do not produce oxygen in the process. A wide range of anaerobic and microaerophilic microbes have the ability to fix nitrogen from the atmosphere but can only carry out the process when oxygen is absent. Both green sulfur bacteria and sulfate-reducing bacteria are obligate anaerobic microbes. Green sulfur bacteria are photoautotrophic microbes that can use sulfide ions, hydrogen ions or ferrous ions as electron donor during photosynthesis process. Sulfate-reducing bacteria are chemoautotrophic microbes that oxidize sulfate into sulfide (Madigan *et al*. 2000). All these microorganisms can

be found in microbial mats or microbial biofilms (Paerl and Pinckney 1996). Microbial consortium in *G. aspera* skeleton resembles those in microbial mat, biofilm, stromatolites or planktonic microalgal-bacterial assemblages (Paerl and Pinckney 1996; Visscher and Stolz 2005). Further studies should emphasize on the spatial and temporal heterogeneity of this dynamic and complexity ecosystem of coral skeleton.

3.5.3 Anoxic inner space in coral skeleton

Based on their metabolic relationships to oxygen, endolithic microorganisms detected in *G. aspera* skeleton can be grouped into obligate aerobic microbes, microphilic microbes, facultative anaerobes microbes as well as obligate anaerobes microbes as shown in Fig. 3.5. The detection of obligate anaerobic green sulfur bacteria and sulfate reducing bacteria in the skeleton indicate that the inner space of the *G. aspera* skeleton could be anoxic (Achenbach *et al*. 2001; Geets *et al*. 2006). It should be noted that both obligate anaerobic bacteria were only detected from samples collected in oligotrophic waters. In addition, *nifH* gene was detected in higher number of oligotrophic samples compared to eutrophic samples. This further support the idea that the inner space of *G. aspera* skeleton inhabiting oligotrophic waters is anoxic since nitrogen fixation can only occur under anoxic conditions (Canfield *et al*. 2005). Further study is required to elucidate this phenomenon.

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Table 1. Primer sequences and PCR annealing temperature used in this study.

Primer names	Target gene or mRNA	Sequence (5' to3')	Annealing temperature	Reference
Cyanobacteria	16S rRNA		60°C	Nübel *et al*. (1997)
-CYA359F		-GGG GAA TYT TCC GCA ATG GG		
-CYA781R(a)		-GAC TAC TGG GGTATC TAA TCC CAT		
-CYA781R(b)		-GACTAC AGG GGT ATC TAA TCC CTT		
Green nonsulfur bacteria	16S rRNA		55°C	Achenbach *et al*.
-CFX856F		-TGC CTT AGC TCA CGC GGT AA		(2001)
-CFX1240R		-GCA ACG CAT TGT CGT GGC CA		
Green sulfur bacteria	16S rRNA		55°C	Achenbach *et al*.
-GS619F		-GGG GTT AAA TCC ATG TGC T		(2001)
-GS1144R		-CAG TTC ART TAG AGT CC		
Eukaryotic algae	18 S rDNA		62°C	van Hannen *et al*.
-18SF1427		-TCT GTG ATC CCC TTA GAT GTT CTG GG		(1998)
-18SR1616		-GCG GTG TGT ACA AAG GGC AGG G		
Nitrogen-fixing bacteria	*nifH*		55°C	Zani *et al*. (2000)
-nifH1		-TGY GAY CCN AAR GCN GA		
-nifH2		-AND GCC ATC ATY TCN		
-nifH3		-ATR TTR TTN GCN GCR TA		
-nifH4		-TTY TAY GGN AAR GGN GG		
Sulfate reducing bacteria	*dsrB*		55°C	Wagner *et al*.
-DSRp2060F		-CAA CAT CGT YCA YAC CCA GGG		(1998)
-DSR4R		-GTG TAG CAG TTA CCG CA		Geets *et al*. (2006)

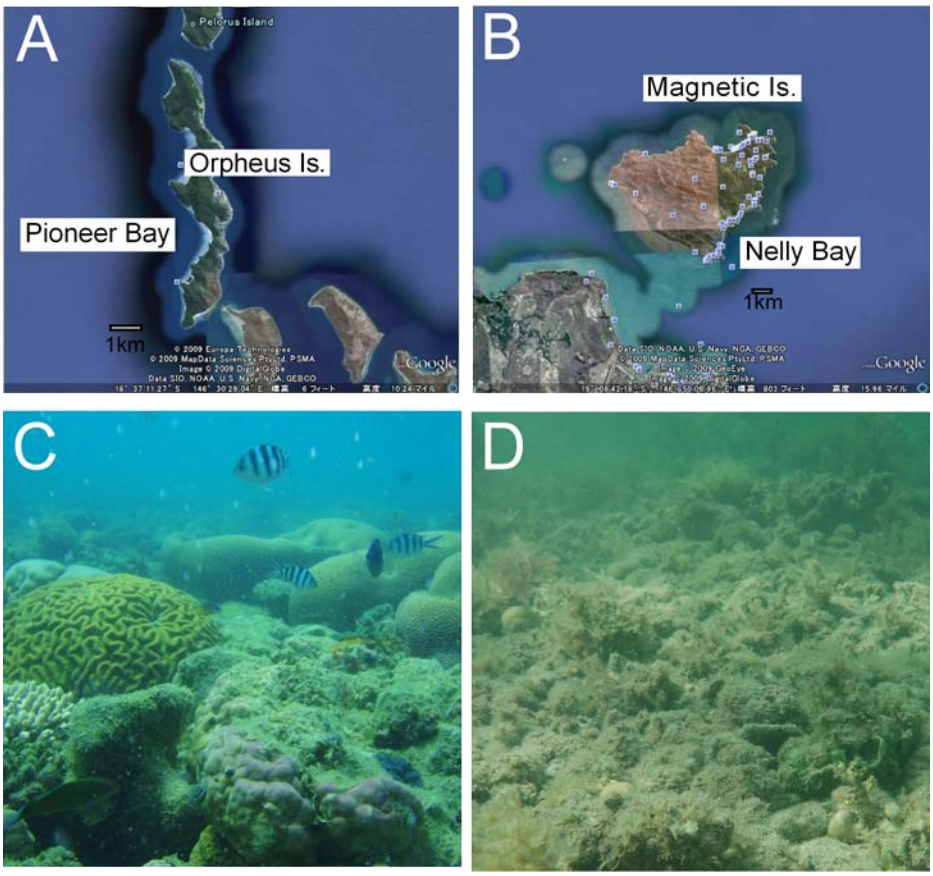

Fig. 3.1. Sampling sites of *G. aspera*. (A and B) are map showing the location of sampling sites in Pioneer Bay, Orpheus Island and Nelly Bay, Magnetic Island, respectively. (C and D) shows the characteristics of the sampling sites. Pioneer Bay has higher diversity of coral species (C) whereas Nelly Bay is mostly covered by turf algae with low diversity of coral species (D).

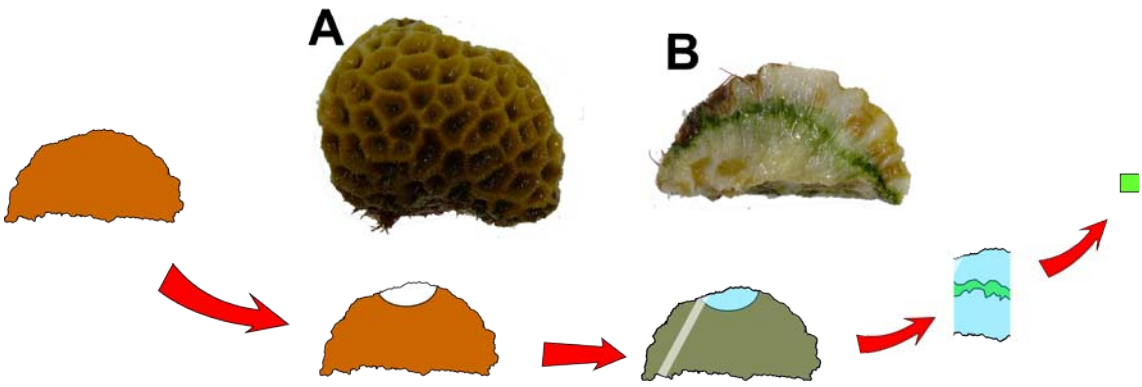

Fig. 3.2. Procedure for the preparation of coral skeleton sample before DNA extraction. (A) Top view of *G. aspera*, (B) Cross-section of *G. aspera* skeleton. A layer of green band can be observed inside the skeleton parallel to the coral tissue.

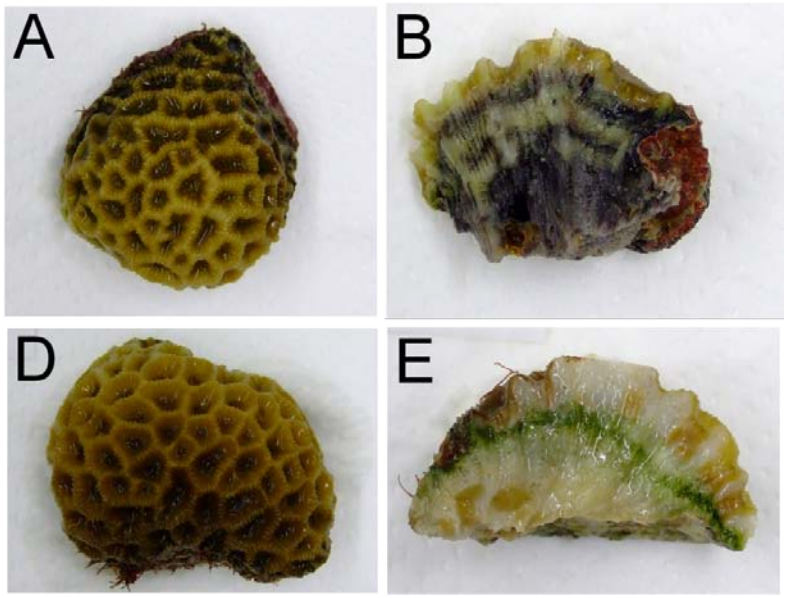

Fig. 3.3. Comparison between the cross-section of the *G. aspera* skeletons collected from both Pioneer Bay (A and B) and Nelly Bay (C and D). Black bands were observed in the coral skeleton from samples collected from Pioneer Bay (B) whereas green band was observed in the coral skeleton from samples collected from Nelly Bay (C).

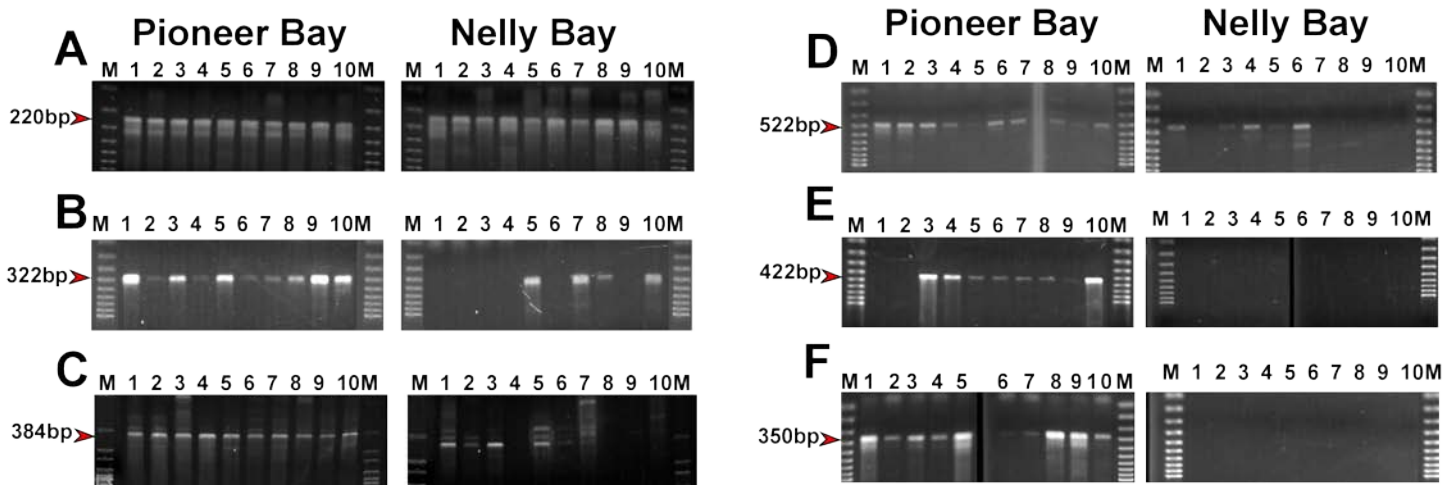

Fig. 3.4. Agarose gels of amplification products from the coral skeletons using specific primer sets. (A) Eukaryotic algae; (B) cyanobacteria and chloroplast; (C) green non-sulfur bacteria; (D) nitrogen fixing bacteria; (E) green sulfur bacteria; (F) sulfate-reducing bacteria. Lanes 1 to 10 correspond to 10 different individual samples; lane M, marker (100-bp ladder).

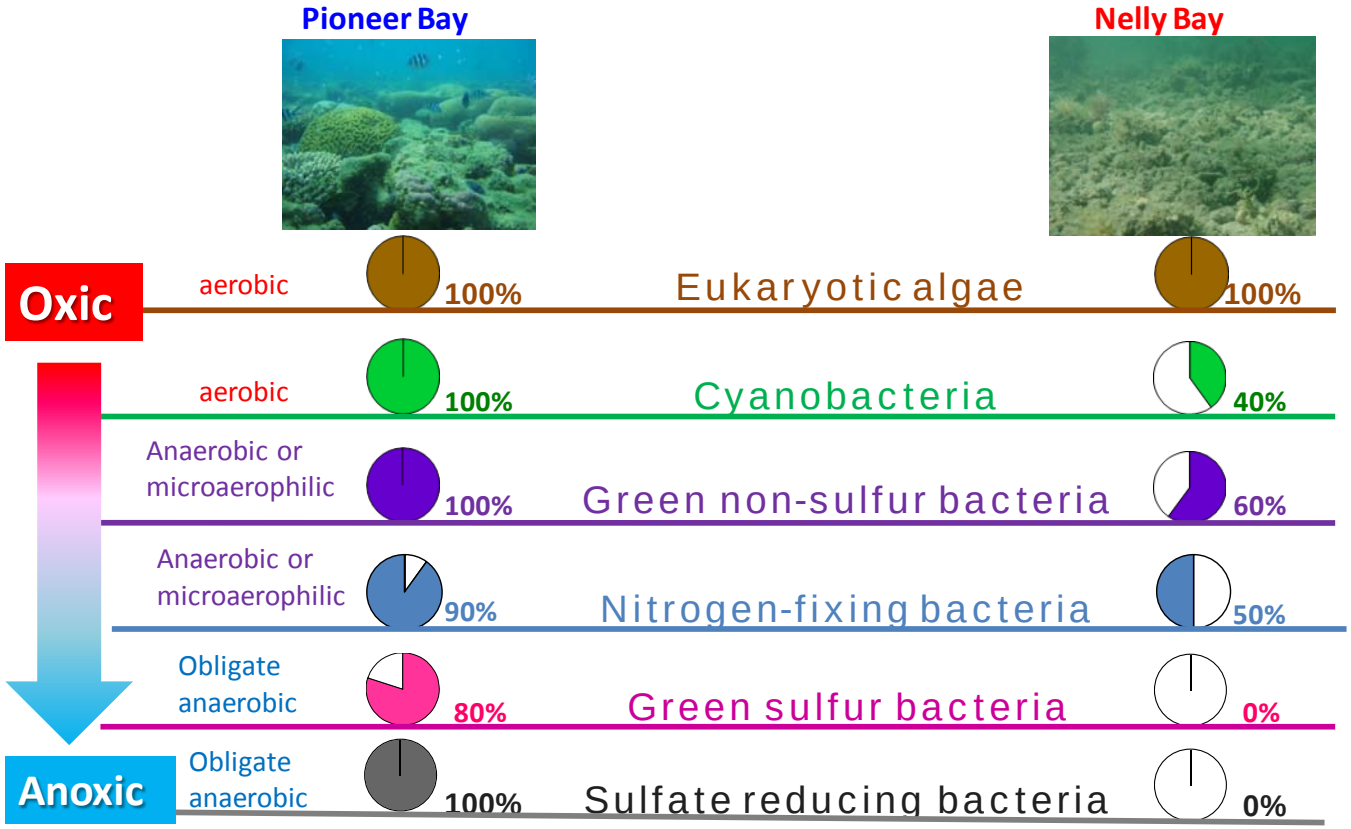

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Fig. 3.5. Comparison of endolithic microorganisms in individual *G. aspera* skeletons collected from both Pioneer Bay (oligotrophic reef) and Nelly Bay (eutrophic reef). Pie charts indicate the percentage of individual coral skeleton detected with the targeted endolithic microorganisms.

Chapter 4

Photoprotective role of endolithic microbes to host coral

Reef-building corals have established an obligate symbiosis with dinoflagellates that is advantageous to survive in oligotrophic coral reef environment. Although corals can thrive in low nutritional conditions by utilizing photosynthetic products assimilated by their symbionts, the coral-alga symbiotic relationship is susceptible to environmental stress and its disruption leads to the coral bleaching phenomenon, a major impact of global warming. Because corals are important primary producers in tropical ocean, solutions are urgently needed to protect corals from the impacts of global climate changes. Here I report that endolithic algae colonizing the skeleton of corals may help to protect coral photosynthesis from high-light intensity stress. Using the PAM chlorophyll *a* fluorescence technique, I compared the photosynthetic activity of endolithic alga-infected reef-building coral *Acropora digitifera* to that of a non-infected group. Short-term lab experiments (7 h) showed that the infected group maintained a higher maximal quantum yield of PSII (*Fv/ Fm*) compared to the non-infected group under photoinhibitory stress conditions at normal growth temperature. Similar results were obtained during the course of a long-term monitoring (6 months from summer to winter) during which the infected and non-infected coral groups were exposed to ambient light conditions. Results from this study suggest that colonization of endolithic algae within the coral skeleton provides beneficial effects on coral photosynthesis in terms of high-light tolerance.

Keywords:
endolithic algae, *Fv/Fm*, high-light stress, photoinhibition, photoprotective effect, reef-building coral

4.1 Introduction

Global climate change has threatened many species of plants, algae and animals inhabiting from terrestrial to aquatic environments. Reef-building corals, primary producers in tropical and subtropical oceans, are no exceptions. Indeed, the mass scale decline of reef-building corals reported worldwide has been associated with climate change (Wilkinson 1999, Hughes *et al*. 2003). To ensure their survival in oligotrophic environments, reef-building corals have evolved by acquiring the capability of carbon and nitrogen assimilation through the establishement of an obligate endosymbiotic relationship with dinoflagellates referred to as "zooxanthellae". This symbiotic relationship, however, is susceptible to high-light stress and its disruption can lead to "coral bleaching". The coral bleaching phenomenon can be induced by the photoinhibition of the symbiont photosynthesis, a process which itself can be exacerbated by high water temperature (Lesser 1997; Takahashi *et al*. 2004).

Reef-building corals can be categorized into two types, namely, massive-type corals and branching-type corals. Massive-type coral species are generally more tolerant to environmental stress than branching-type coral species (Marshall and Baird 2000, McClanahan 2000, McClanahan *et al*. 2001, Loya *et al*. 2001). In addition to the zooxanthellae found in their tissue, massive-type corals are characterized by the presence of endolithic algae found within their skeleton as a green band. The microbial community found within this green band includes green algae, cyanobacteria and fungi that are together dissolved in the calcium carbonate structure (Lukas 1974, Le Campion-Alsumard *et al*. 1995). We hypothesized that presence of endolithic algae may protect the coral-algae symbiotic system from high-light stress. In this study I report that novel branching-type corals harboring endolithic algae have a higher tolerance against high-light stress than branching-type corals harboring no endolithic algae.

4.2 Materials and Methods

The branching-type coral *Acropora digitifera* was collected in May 2005 from a shallow intertidal area of Bisezaki, Okinawa, Japan. After 2 weeks pre-acclimation in a lab tank under weak light around 200μmol, chlorophyll *a* fluorescence from the symbiotic algae within the corals was assessed using a DIVING-PAM under water fluorometer (Walz, Germany). In order to assess the recovery from photoinhibition, short-term experiments were performed during which corals were exposed to high-light (400 μmol photons $m^{-2}s^{-1}$) for 5 h followed by a 2-h exposure to low-light (20 μmol photons $m^{-2}s^{-1}$). To determine the light intensity to be used for the high-light treatment, a rapid light curve (RLC) was drawn with the DIVING-PAM and the light intensity at which photosynthesis saturated, i.e., 400 μmol photons $m^{-2}s^{-1}$, was selected as the high-light treatment. The maximum quantum yield of PSII (*Fv/Fm*) was measured after 15 minutes of dark adaptation. Seawater temperature was controlled with a thermominder system (SM-05R, TAITEC, Japan) and maintained at 26, 28, 30 and 32 ºC.

Long-term experiments were also performed in order to assess the differential capabilities to sustain exposure to ambient light conditions in endolithic alga-infected and non-infected coral groups. Corals were separated into endolithic alga-infected group and non-infected group by observing the presence or absence of green coloration at the base of the branches that were sectioned. For these experiments, twelve samples of both endolithic alga-infected branches and non-infected branches were cultured in an outdoor tank supplied with running seawater. *Fv/Fm* was measured weekly after sunset for a 6 month period. Light intensity and water temperature were monitored using a HOBO 2K light intensity data logger (Onset Computer Corporation, USA) and a TidbiT temp data logger (Onset Computer Corporation, USA), respectively.

4.3 Results

PAM chlorophyll *a* fluorescent technique is a powerful tool for coral photosynthesis research because it is a non-destructive method that allows the monitoring of *in vivo* photosynthetic activity (Takahashi *et al*. 2004). Similarly to land plants, coral exposure to high-light intensity or excessive irradiance can lead to photoinhibition of photosynthesis, a phenomenon frequently assessed by a decline in the *Fv/Fm* parameter. Figure 1 shows declines in *Fv/Fm* during the high-light intensity treatment and its subsequent recovery from photodamaged conditions upon exposure to low light conditions. Decline in *Fv/Fm* was more pronounced in non-infected colonies (Fig. 4.1a). Because coral photosynthesis is temperature-sensitive, four temperatures were tested to examine the effect on infected and non-infected colonies. Temperatures tested were 26 (optimum growth temperature), 28, 30 and 32 ºC and although there was no substantial differences in the initial *Fv/Fm* values between infected and non-infected colonies, upon the onset of high-light intensity (400 μmol photons $m^{-2}s^{-1}$), *Fv/Fm* values rapidly decreased under all temperatures tested but to a greater extent under 32 ºC (Fig.1d). In all cases, the decreased *Fv/Fm* value recovered after switching from high to low light intensity (20 μmol photons $m^{-2}s^{-1}$).

Infected colonies including endolithic algae within the skeleton maintained significantly higher *Fv/Fm* compared with non-infected colonies at optimum temperature ($P<0.05$ t-test) (Fig. 4.1a). In contrast, such differences between infected and non-infected colonies disappeared when water temperature was elevated (Fig. 4.1c and d). Overall, results from these short-term experiments suggest that, at optimal temperature, coral colonies containing endolithic algae are more tolerant to high-light intensity stress (Fig. 4.1a and b). We further tested the effect of colonization of endolithic algae on coral photosynthesis during a long-term monitoring of infected and non-infected coral colonies kept in an outdoor tank under natural light conditions. *Fv/Fm* of both infected and non-infected colonies showed a seasonal variation over the 6 month study period with a generally lower *Fv/Fm* during the summer

period followed by gradually increasing *Fv/Fm* during the autumn-winter period (Fig. 4.2). During this long-term monitoring the presence of endolithic algae inside the coral skeleton of the infected group helped maintaining higher *Fv/Fm* values compared to the non-infected group (Fig. 4.2). These results are in agreement with those obtained during the short-term experiments and suggest a protective effect of the presence of endolithic algae inside the coral skeleton.

4.3 Discussion

Results from both short-term (Fig. 4.1) and long-term (Fig. 4.2) experiments reveal that the extent of high-light intensity induced photoinhibition is smaller in infected colonies than in non-infected ones. Results of Fig. 1 suggest that the presence of endolithic algae is beneficial for the branching-type coral *A. digitifera* in terms of suppression of photoinhibition. This photoprotective role was supported by the long-term experiments (Fig. 4.2). One might argue that the photosynthetic response of endolithic algae might be superimposed in PAM measurements. However, it was estimated that only 0.1 % of photosynthetically active radiation (PAR) is transmitted into the coral skeleton (Halldal 1968). It can thus be assumed that chlorophyll detected originated from zooxanthellae and that interference from endolithic alga chlorophyll emission could be negligible.

The mechanism of photoprotective effect of the colonization of endolicthic algae within the coral skeleton remains unresolved. It has been suggested that endolithic algae assist the host coral in bleaching recovery by providing their photosynthetic products (Fine and Loya 2002). Recently, Rodriguez-Roman *et al*. (2006) have proposed that endolithic algae may contribute to photoprotection of coral photosynthesis from excessive radiation by reducing the reflectivity of white coral skeleton.

Production of harmful reactive oxygen species (ROS) is known to be involved in the

mechanism of oxidative damage induced by phoinhibition. In addition to ROS, reactive nitrogen species (RNS) such as nitric oxide (NO) have been suggested to cause nitrosative stress which potentially disturbs metabolism (Yamasaki 2000). We consider it plausible that endolithic microbial community would function as a sink for such harmful reactive species produced during coral exposure to high-light stress conditions. Shashar and Stambler (1992) reported that the endolithic algae may protect the algae from the damaging effects of ROS.

The presence of calcium carbonate skeleton is a unique feature of the photosynthetic system in reef-building corals. Coral tissue including zooxanthellae covers the surface of the skeleton and makes the inside space hypoxic. Owing to the sealing effect, there seems a gradient of redox potential towards the core of the skeleton. A micro-diversity in internal oxygen tension, as well as redox potential, would allow corals to harbor a range of microbial communities from aerobic to anaerobic microbes. The disappearance of photoprotective effects at high temperature observed in the present study (Fig. 4.1) could suggest an increased thermal sensitivity for the microbial community compared to that of the host symbiosis (Fine *et al*. 2005).

Different species of reef-building corals are known to exhibit different sensitivity to light ad thermal stress (Nakamura *et al*., in press). Higher stress tolerance in massive-type corals may be ascribed to the colonization of endolithic algal community that forms mutual metabolic network with the coral-alga symbiotic system. The present study is, to our knowledge, the first report on the beneficial role of endolithic algae in branching-type coral. Protective effects of endolithic algae in both massive and branching types of coral could be indicative of a novel "secondary or facultative symbiosis" in addition to the obligatory symbiosis with zooxanthellae.

One might argue that the ability to tolerance high-light intensity under ambient temperature is attributed to the coral harboring stress tolerance *Symbiodinium* phylotypes or the phylotypic changes in the *Symbiodinium* community (Baker 2003; Rowan *et al*. 1997). Indeed,

seasonally changes in the community of *Symbiodinium* phylotypes were observed in *Acropora palifera* and *Acropora valida*. In the case of *A. valida*, sun-adapted and shade-adapted surfaces of the coral were found to harbor different *Symbiodinium* phylotypes (Chen *et al*. 2005; Ulstrup *et al*. 2008). *Acropora digitifera* from Okinawa harbors only *Symbiodinium* phylotype C3 (LaJeunesse *et al.* 2004). *Symbodinium* phylotype C has been shown to be more stress-sensitive compare to other phylotypes (Rowan *et al*. 1997). In this study, corals were acclimatized for more than 2 weeks with 1 side of the branch facing upward. During the experiment, the same side of the corals was subjected to high-light intensity treatment to exclude any sun/shade-adapted effects. In addition, the shuffling of genetically different *Symbiodinium* types in corals in response to changes or disturbances in the environment may takes months or years (Thornill *et al.* 2006) whereas the short-tem experiment in this study was conducted in less than 8 hours. Therefore, we concluded that the high-light intensity stress tolerance of *A. digitifera* is attributed to the endolithic algae instead of the *Symbiodinium*.

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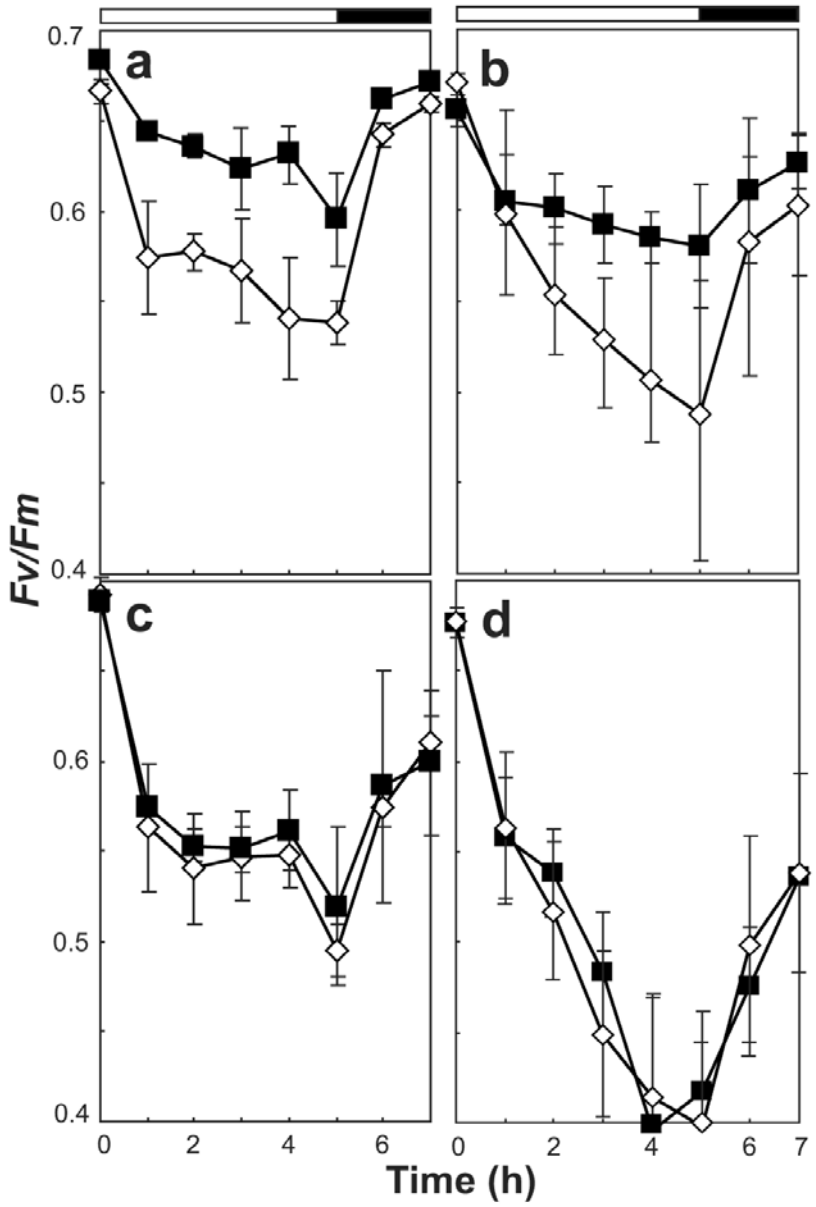

Fig. 4.1. Photoinhibition induced by high-light in the coral *Acropora digitifera*. *Fv/Fm* value is compared between endolithic alga-infected colonies (black square) and non-infected colonies (white diamond) of *A. digitifera* at 26 (a), 28 (b), 30 (c) and 32 ºC (d). Corals were exposed to 400 μmol photons $m^{-2}s^{-1}$ for 5 h (white bars) followed by an exposure to 20 μmol photons $m^{-2}s^{-1}$ for recovery (black bars). Values are means ± SD of 3 replicates.

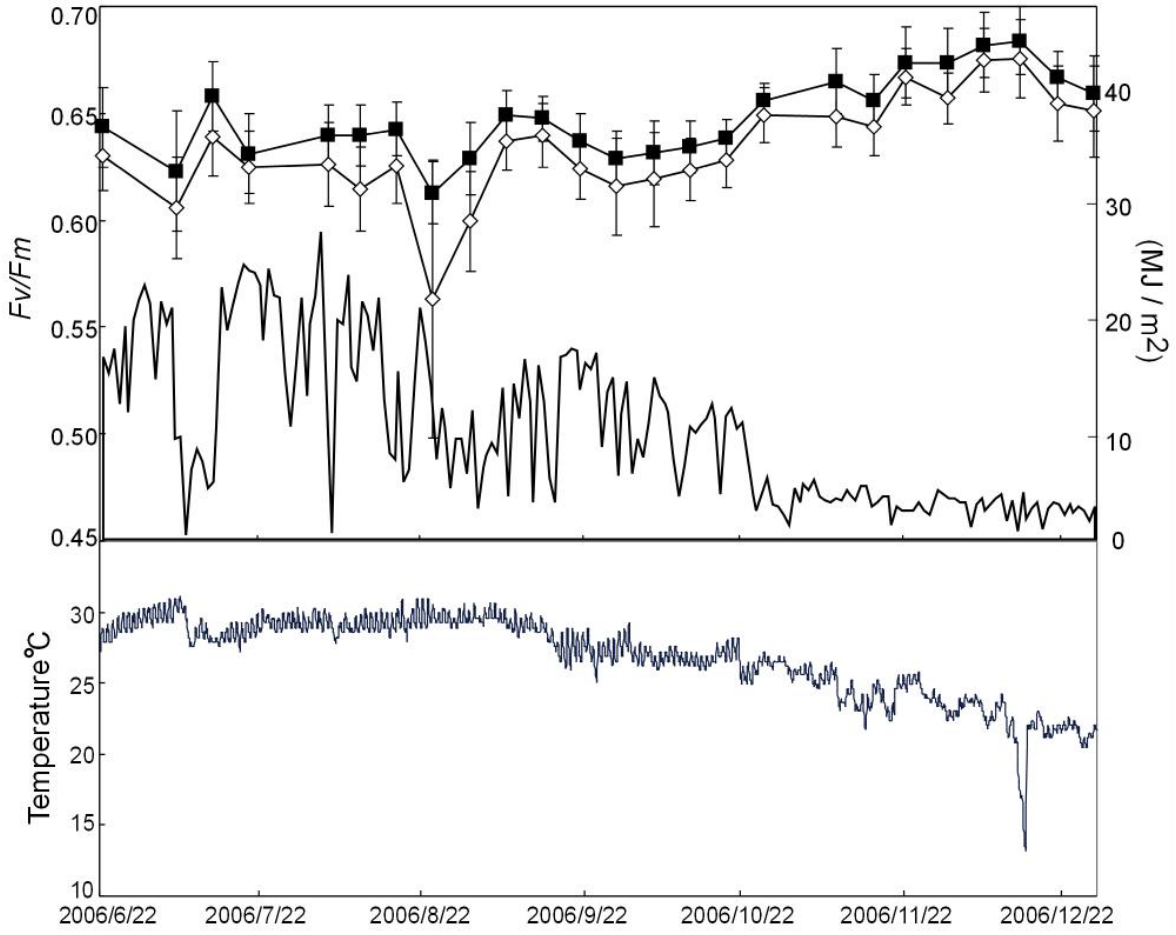

Fig. 4.2. Long-term monitoring of photosynthetic efficiency in the coral *A. digitifera*. The upper panel shows the *Fv/Fm* values of infected colonies (black square) and non-infected colonies (white diamond) cultured under natural sunlight. The solid black line with no symbols shows changes in integrated daily irradiance measured in the outdoor tank. Values are means ± SD of 12 replicates. The lower panel shows the water temperature.

Chapter 5

General discussion

Coral tissue covers the surface of coral skeleton and can create a hypoxic environment. Endolithic microbes such as cyanobacteria, fungi, algae and bacteria have been found to inhabit this unique microenvironment. However, the community of endolithic microbes remains poorly understood. Although both beneficial and negative effects of endolithic microbes have on the host corals have been reported, it seems that the importance of endolithic microbes in coral survival is still underestimated by coral reef scientists. In the present study, endolithic microbial community within coral skeleton consists of a wide range of different functional groups of microbes with varieties of metabolic requirement. Here we propose a novel "secondary or facultative symbiosis" between scleractinian corals and endolithic microbes in addition to the obligatory symbiosis with zooxanthellae. This potential symbiotic relationship could provides benefits to the corals via different mechanisms including transfer of photosynthetic products to host corals, protection of symbiotic dinoflagellates from excessive light by reducing the reflectivity of the skeleton as well as acting as a sink for harmful reactive species produced during coral exposure to stress conditions.

Key words:
coral skeleton; endolithic microbes, microbial consortium, photoprotection; secondary or facultative symbiosis

In this study, endolithic microbial community resembles the stratified microbial community found in hypersaline microbial mats was detected in the skeleton of *Goniastrea aspera* residing in oligotrophic waters. Although the effects of endolithic microbes on the physiology of corals are still under debate, the current study suggests that endolithic microbes confer benefits to their host corals by various mechanisms including photoprotection, photoassimilation as well as nutrient recycle.

5.1 *Goniastrea aspera* and endolithic microbes

It is a consensus that reef-buildings corals thrive best in clear, warm waters with few environmental conditions changes. However, some coral species can be found in harsh environments with extreme conditions such as in shallow intertidal pools. Marine organisms living in such areas are exposed to daily and seasonally changes in salinity, irradiance and temperature that can be quite large. The massive shallow-water coral *Goniastrea aspera* is one of the dominant coral species commonly found in intertidal areas (Brown *et al*. 1994; Brown *et al*. 1999). *G. aspera* is a relatively stress tolerant coral species that can withstand even under high sedimentation and is less susceptible to bleaching (Loya *et al*. 2001; Brown *et al*. 2002). This could be attributed to the animal acclimatory mechanisms as well as stress tolerance of the *Symbiodinium* clade.

Besides endolithic algae, cyanobacteria and fungi, skeleton of *G. aspera* harbors a wide range of bacteria. In this study, the different functional groups of microbial found in the skeleton of healthy *G. aspera* resemble those in microbial mats (Chapter 3, Fig. 3.4). Although oxygen tension was not determined with depth, based on the discovery of different functional groups of aerobic and anaerobic microbes inside the coral skeletons, one can deduce that the microenvironment inside a massive coral skeleton show chemical gradients that are similar to those in microbial mats (Paerl and Pinckney 1996). The surface oxic layer is represented by

the live coral tissue that covers the skeleton and cyanobacteria and microalgae that colonize the layer underneath coral tissue. The deeper inside anoxic layer is represented by the presence of green sulfur bacteria and sulfate-reducing bacteria. An oxic-anoxic interface can be found in between the oxic layer (surface) and anoxic layer (inside) in which green non-sulfur and nitrogen fixing bacteria are present (Chapter 3, Fig. 3.5). A massive skeleton of *G. aspera* represents a microscale ecosystem that harbor stratified microbial consortium. Both coral and microbial metabolisms affect the spatial and temporal heterogeneities of oxygen tension and other chemicals inside the skeleton (Kühl *et al*. 2008).

Biogeochemical characteristics, such as the physical and chemical properties of substrates (rock, sediment, coral skeleton) as well as environmental conditions (nutrients, salinity, temperature etc.) influence the specific microbial consortium of endolithic communities (Carreiro-Silva *et al*. 2005; Tribollet 2008a). Microbial consortium inside is a relatively isolated and closed ecosystem that are efficient in nutrients recycle (Carreiro-Silva *et al*. 2005; Tribollet 2008a). This may explain the complexity of the microbial consortium found in *G. aspera* skeleton from oligotrophic waters where nutrients in the environment are scarce. On the other hand, the lower microbial diversity in *G. aspera* skeleton from eutrophic waters suggests that microbes obtain nutrients from the outside environment instead of recycling nutrient within the skeleton. Fig. 5.1 represents a hypothetical scheme of the difference in nutrients acquisition of endolithic microbes in oligotrophic and eutrophic waters.

5.2 Potential symbiotic relationship between coral and endolithic microbes

The establishment of obligate endosymbiotic relationship between scleractinian corals and *Symbiodinium* dinoflagellates is the success of this group in oligotrophic waters during the last 200 million years (Muscatine and Porter 1977). However, in the past few decades, breakdown of this endosymbiotic relationship leads to coral mortality through coral bleaching

has been documented (Hoegh-Guldberg 1999; Douglas 2003). The discovery of endolithic microorganisms within coral skeletons and their ability to serve as an alternative nutrient source to host corals support the idea that a potential symbiotic relationship between scleractinian corals and endolithic microbes exists.

Under favorable conditions (non-stress conditions), scleractinian corals obtain their nutrients mostly (> 80%) from their phototrophic symbiotic dinoflagellates (Muscatine 1967; Muscatine 1990). In these conditions the contribution of photoassimilates from endolithic algae to host corals could be negligible. In contrast under environmental stress conditions where most of the symbiotic dinoflagellates are expelled or digested by host coral, photosynthesis products from endolithic algae become an important nutrient source to the host coral until its symbiotic dinoflagellates population recovers (Schlichter *et al*. 1995; Fine and Loya 2002). The discovery of a similarity between microbial consortium in *G. aspera* skeleton from oligotrophic waters and microbial consortium in microbial mats suggests that there may be nutrient exchange between *G. aspera* and endolithic microbes. Microbial consortium such as those found in microbial mats is very efficient in conserving nutrients via nutrients recycle (van Gemerden 1993; Paerl and Pinckney 1996). Thus, the stress tolerance characteristics of *G. aspera* can be, at least in part ascribed to the presence of endolithic microbes within their skeleton.

The ability of endolithic algae and bacteria to utilize longer wavelengths effectively in addition to PAR (photosynthetically Active Radiation) for photosynthesis is an important feature in their survival in cryptic environments such as coral skeleton (Takahashi and Ichimura 1970; Ralph *et al*. 2007). Multiple scattering of the highly reflective white calcium carbonate coral skeleton plays an important role in regulating the light environment for optimum photosynthesis of symbiotic algae (Enríquez *et al*. 2005; Stambler and Dubinsky 2005). However, under multiple scattering by coral skeleton may have negative effects on

coral under stressful conditions, i.e. high-light irradiance, high temperature (Enríquez *et al*. 2005). Endolithic microbes benefit host corals under high-light conditions by the absorption of excessive radiation produced by the reflectivity of coral skeleton (Rodriguez-Roman *et al*. 2006). This photoprotective role is very important especially during coral bleaching to reduce the photoinhibition on remaining symbiotic dinoflagellate population, so that they have a better chance to recover (Enríquez *et al*. 2005; Rodriguez-Roman *et al*. 2006).

Microbial consortium is a complex community consists of various species of microorganisms that are able to endure extreme environmental conditions (Paerl and Pinckney 1996; Rothschild and Mancinelli 2001). It is plausible that this ability could benefit the host coral during environmental stress conditions by acting as a sink for harmful substances produced such as reactive oxygen species. However, among the beneficial roles of endolithic microbes have on corals, this has yet to be proven. The potential beneficial roles of endolithic microbes on host corals are summarized in Fig. 5.2.

Recent studies have implied that there may be a potential symbiotic relationship between endolithic microbes and scleractinian corals. Despite the discovery of endolithic algae in coral skeleton over 100 years ago, studies on the endolithic microbial community and their potential beneficial effects on corals are still in its infancy. Extensive study on the coral and endolithic microbial association will be necessary for understanding the acclimation, adaptation and evolution of scleractinian corals and ultimately prevent the destruction of coral reefs. The health of coral reefs worldwide is declining due to both natural disturbances and anthropogenic disturbances. Corals that harbor endolithic microbes inside their skeleton can have the upper hand in surviving these less favorable conditions.

5.3 Disadvantages of harboring endolithic microbes

Although mutualistic relationship between endoliths and live corals assist in the survival

of corals under environmental stress conditions, many of these endoliths (cyanobacteria, eukaryotic algae and fungi) are also known to cause bioerosion of coral skeletons (Highsmith 1981; Tribollet 2008b). Species compositions of boring microflora vary between live and dead corals' skeletons. Dominant endoliths in live corals are *Ostreobium quekettii*, *Plectonema terebrans* and fungi whereas dead corals are colonized first by *Phaeophila dendroides*, followed by *Mastigocoleus testarum* and *Plectonema terebrans* and then finally dominated by *O. quekettii* (Le Campion-Alsumard *et al.* 1995a).

Boring microflora (cyanobacteria, eukaryotic algae and fungi) play an important role in the recycle of calcium carbonate ion in coral reef ecosystems through bioerosion and sedimentation processes. In general, spatial and temporal variabilities in species composition, abundance, distribution, and bioeroding activity of endoliths were investigated in experimental block of massive coral skeletons instead of in live corals (Tribollet *et al.* 2002; Tribollet 2008b), therefore the damage to corals caused by boring endolithic microbes remains unknown. So far, only the activity of endolithic fungi has been associated to the formation of skeletal growth tissue anomalies in corals (Bentis *et al.* 2000; Domart-Coulon *et al.* 2006). To prevent fungi from penetrating into their live tissue, corals are known to form pearl-like deposits of carbonate around fungal hyphae (Le Campion-Alsumard *et al*. 1995b). Based on these studies, one can deduced that coral skeletons harboring endolithic boring microbes might be more fragile compare to those without endoliths.

Many species of massive corals harbor endolithic microbes inside their skeletons in comparison to branching corals. One reason might be due to the decrease in skeleton strength when endolithic microbes are present since many of these endolithic microbes are boring microorganisms. Compare to massive corals, branching corals are more susceptible to physical disturbances such as wave or typhoon (Highsmith 1982; Dollar and Tribble 1993). Therefore, the presence of endolithic microbes in branching corals could further increase the

frequency of skeleton breakage during typhoon or hurricanes. In Bisezaki reef, *Acropora digitifera* harboring endolithic microbes are found along the shore. In the channel-like structure between the small island and the shore in which the water flow is strong, *A. digitifera* do not harbor endolithic microbes (Yamazaki 2007). Water flow plays an important role in facilitating the recovery of bleached corals as well as reducing photoinhibition of photosynthesis in corals (Nakamura *et al.* 2003; Nakamura *et al.* 2005). On the other hand, endolithic microbes facilitate the recovery of bleached corals and provide photoprotection to corals inhabiting areas with slow water flow such as lagoon or back reef (Yamazaki *et al.* 2008).

Recent study has demonstrated that inorganic nutrient enhanced bioerosion rates in carbonate substrate by microborer such as cyanobacteria and eukaryotic green algae (Carreiro-Silva *et al.* 2009). This suggests that under eutrophic conditions, corals harboring endolithic microbes might have disadvantage compare to those without endolithic microbes. Changes in the environmental factors such as sedimentation and rising atmospheric pCO_2 could have an effect on endolithic microbes composition and activity which will eventually affect their host corals (Tribollet 2008a). Thus, more attention should be paid to the study of mutualistic and parasitic relationships between endolithic microbes and corals in regards to the changes in environmental conditions.

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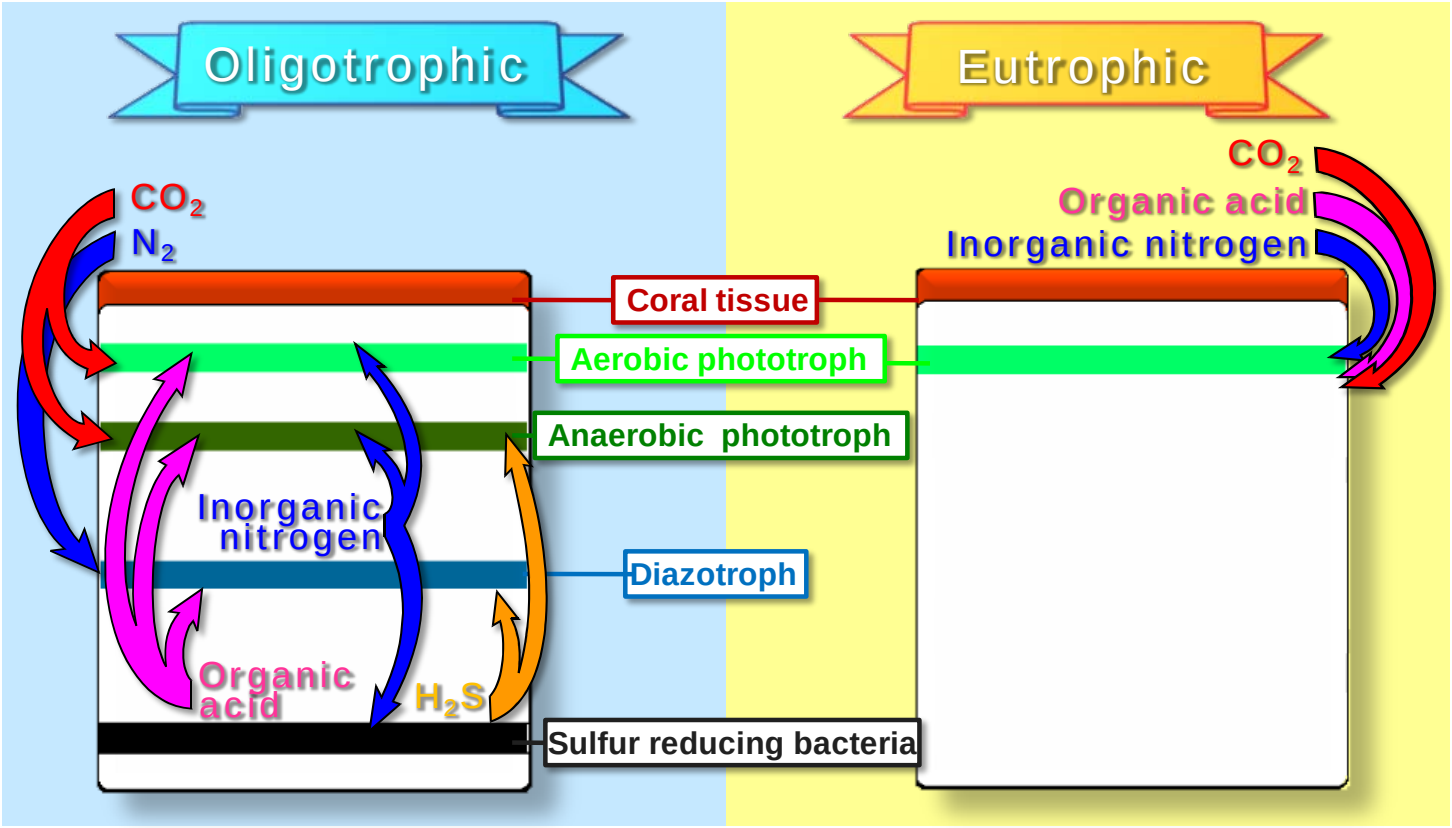

Fig. 5.1. A hypothetical scheme showing the difference in nutritional acquisition by endolithic microbes inside the skeletons of *G. aspera* from oligotrophic (left) and eutrophic (right) waters. In oligotrophic waters, efficient nutrients recycle occurs inside the coral skeleton due to the presence of different functional groups of microbes. Whereas in eutrophic waters, diversity of endolithic microbes is low and they obtain nutrients required from the seawaters.

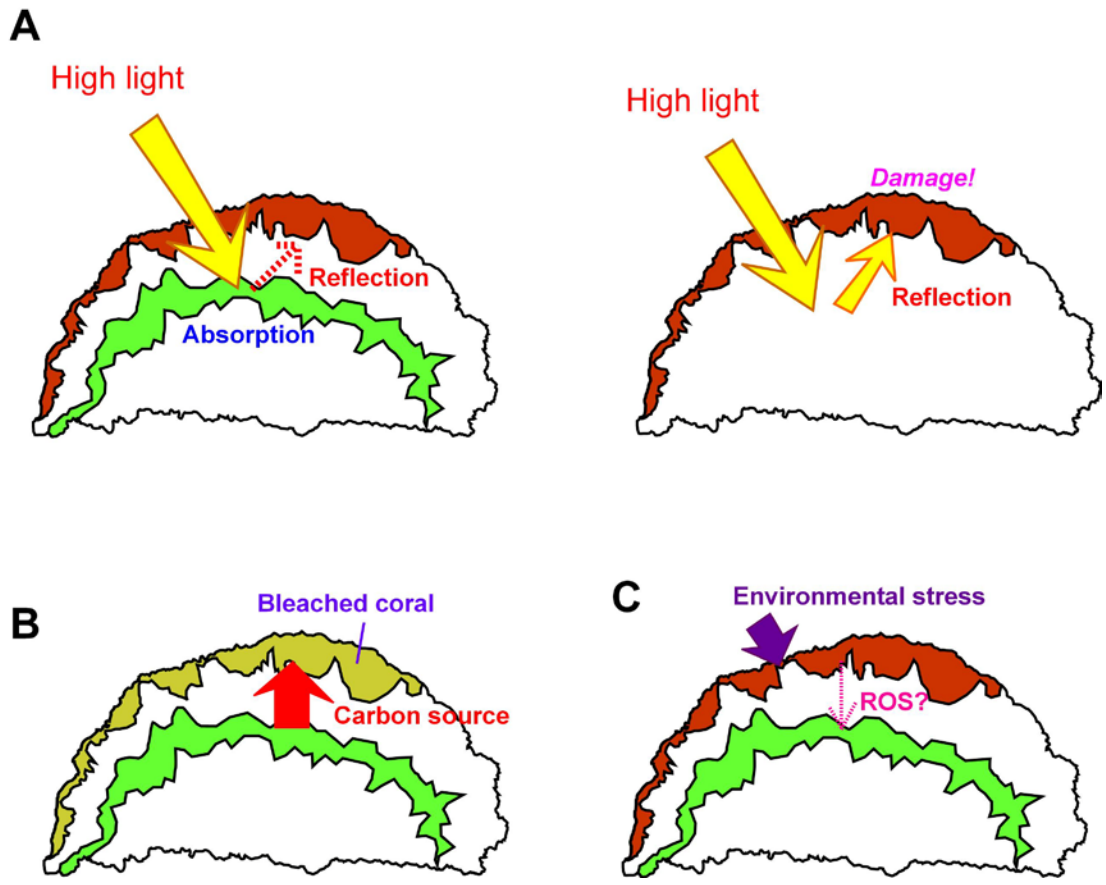

Fig. 5.2. Proposed beneficial effects of endolithic microbes on host corals under environmental stress conditions. (A) Endolithic microbes absorb excessive light that are otherwise reflect by the white calcium carbonate skeleton to the symbiotic dinoflagellates that can cause photodamage. (B) Endolithic microbes serve as an alternative nutrient source to bleached coral. (C) Endolithic microbes may act as a sink to remove reactive oxygen species produced by coral and symbiotic dinoflagellates during stress conditions.