Physiology and role of endolithic community in the massive coral Porites lutea

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塊状ハマサンゴ中の骨格に生存する微細藻類の生理的状態と役割

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Laddawan Sangsawang

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General abstract

Reef-building corals are the most important components of the coral reef ecosystems. They almost distribute in tropical areas of the world's oceans. Corals evolved to adapt to oligotrophic environments by establishing symbiotic relationships with a variety of microbes forming the so called "coral holobiont". In Addition to the symbiotic algae zooxanthellae, other associated organisms play crucial roles in environmental adaptation of the coral holobiont by providing organic carbon, inorganic carbon, organic nitrogen, and protecting the coral host from pathogen. Among associated microbes, the endolithic community is one of the less studied entities within the coral holobiont. Members of this community appear in remarkably high numbers in massive and encrusting corals, especially family Merulinidae and Poritidae. Translocation of organic matter from endoliths to the coral host was firstly mentioned by Fine and Loya (2002). However, they did not evaluate this process in terms of the organic matter synthetized and transferred. In this research I wished to determine how much the endolithic community fixes organic carbon and whether they are capable to fixing atmospheric nitrogen and translocating it to the coral host. Moreover, I would like to investigate how the endolithic community can provide photo-protection to the coral host. I focused my study in the massive coral *Porites lutea*, since it is a common coral in Okinawa reef and appear to be very resistant to environmental stressors as high temperature and strong illumination.

Firstly, I studied the chemical-biological interactions between the coral holobiont and surrounding seawater environment in healthy and bleached corals. Results showed

significant changes in water quality along the incubations of bleached corals: particularly nutrients and dissolved organic carbon (DOC) were released from bleached corals with parallel increase in bacterial abundance and decreased in dissolved oxygen (DO). This condition can promote coral disease and disturbances in the reef community. Following, I dedicated to the study the endolithic community composition and their pigments, photosynthetic performance (*Fv/Fm*) and physiological parameters as primary production, nitrogen fixation in endolithic community as well as in the coral tissues. I also tried to quantify how much organic matter can be translocated from endoliths to the coral tissues. Microscopic studies revealed the presence of the Chlorophyta *Ostreobium quekettii*, the cyanobacterium *Leptolyngbya terebrans* together with two fungal forms. For physiological studies, I exercised incubation experiments of *Porites lutea* with addition of ^{13}C and ^{15}N tracers to measure primary production and nitrogen fixation in coral tissues (Addition experiment) and direct injection of ^{13}C inside endolithic green band (Injection experiment) to evaluate translocation rates. After incubation period, *Fv/Fm* of endoliths showed similar values in healthy and bleached corals. These are indicating comparable photosynthetic performances in both conditions. Primary production of endolithic community was higher in bleached corals (3.4 µg C cm^{-2} day^{-1}) than in healthy corals (2.2 µg C cm^{-2} day^{-1}). In coral tissues there was an increase in primary production over the 24h incubation in spite of the respiration during the dark period from 8.7 µg C cm^{-2} day^{-1} in 12h light to 23 µg C cm^{-2} day^{-1} in 24h, revealing some external source of organic matter that could be related to translocation from endoliths. Nitrogen fixation was detected in both coral tissues and the endolithic community and translocation of nitrogen rich organic compounds was revealed by

the study of C/N ratio along incubations. The study of ^{13}C atom (%) incorporation in coral tissues during the Injection incubation revealed an important translocation pattern with 2 to 8% translocation from endoliths (higher in healthy corals). These indicate that endolithic community can support coral nutrition in normal as well as stressful conditions during bleaching.

To explore the responses of coral host and endolithic community to oxidative stress, H_2O_2 scavenging activity was measured in the coral tissue and the endolithic community under healthy and bleached *P. lutea*. Coral nubbins were incubated under two conditions of temperature (27 °C as normal temperature or 34 °C), two conditions of illumination (360 as normal and 680 µmol photons m^{-2} s^{-1}), and the combination of them during 6h. *Fv/Fm* of healthy coral tissue decreased from initial after exposure to light and high (34 °C) temperature stresses. However, the *Fv/Fm* in bleached coral tissue increased along or remained the same in all treatments. Endolithic algae showed the same pattern in both coral conditions with a decrease in *Fv/Fm* under high irradiance and the combined treatment. Corals showed a significant increase in H_2O_2 scavenging activity during light and thermal stresses, however scavenging activity was low under combined stresses. On the other hand, endolithic algae showed increase in scavenging activity at all treatments. These results show the capacity of rapid response of endolithic algae to environmental stresses, however in coral tissues, the stress levels in combined treatment overpassed the coral capacity to fight against oxidative stress.

The main conclusion is that the endolithic community plays a crucial role in supporting the coral holobiont. The rapid recovery of photosynthesis and pigments in endolithic algae illustrates their plasticity to acclimate to drastic environmental changes.

Chapter 1

General introduction

1.1 Corals in tropical and subtropical environment

As evidenced by fossil records, scleractinian corals first appeared during the mid-Triassic, about 250 million years ago and became the reef-building organisms around 25 million years later (Stanley, 2003). Coral reefs are important ecosystems in shallow coastal areas bearing one of the most marine biodiversity. They provide food, shelter, and nursery for numerous marine species, including fishes, mollusks, worms, crustaceans, echinoderms and sponges (Hoegh-Guldberg et al., 2007). In addition, includes income from tourism thus this generates enormous income to the countries have coral reef coastlines (Moberg and Folke, 1999). Recently it was found that, the chemical production from corals can be used as important sources of new medicines being developed to anti-inflammatory properties, anticancer properties, bone repair and neurological benefits (Cooper et al., 2014). Coral reefs grow in environments such as warm, clear water, salinity (33-36 ppt) and with seawater temperature relatively constant throughout the year. This environment can found in tropical and subtropical regions, no more than 30 degrees of latitudes above and below the equator (Goldberg, 2013). In addition, these areas are nutrients poor oligotrophic waters. Paradoxically, the reef-building corals can success in these oligotrophic waters with high gross primary production.

1.2 Coral bleaching

Coral bleaching phenomenon was firstly described to have impacted coral reef s in the Pacific Ocean by Glynn, 1984. Until now, bleaching frequency and severity have increased due to changes in environmental conditions as well as anthropogenic stressors (Hoegh-Guldberg, 1999; Hughes, 2003; Wilkinson, 2000). The cause of this phenomenon has been attributed to several environmental conditions as elevated or reduced sea surface temperatures (SST), solar radiation including UV radiation, reduction in salinity, sedimentation and bacteria infection (Fabricius, 2005; Higuchi et al., 2013; Kleypas et al., 1999). A combination of SST and increase solar radiation are believed the main cause of coral bleaching around the world (Brown and Suharsono, 1990; Williams and Bunkley-Williams, 1990). Many studies have been done to elucidate the mechanism of light and temperature stresses on coral physiology compare to other causative factors of bleaching. Oxidative stress caused by the effects of high irradiance and higher SST promotes the production of reactive oxygen species (ROS) in chloroplast and damage the photochemistry and carbon fixation in zooxanthellae (Lesser, 2006; Lesser and Farrell, 2004). The host and their holobiont possess defense mechanisms to prevent oxidative stress by utilization various of antioxidant tools such as enzymes: superoxide dismutase (SOD), catalase and glutathione peroxidase and non-enzymatic mechanism: ascorbic acid, Glutathione, α-tocopheral (vitamin E), carotenoids and mycosporine-like amino acids (MAAs) (Lesser, 2006). In addition to antioxidant systems, They are repair mechanisms aimed at repairing or removing damaged molecules and to replace them with new ones. However, these antioxidant systems might not be able to protect the organisms from all ROS produced especially under a long period under high temperature

and light effect. If these mechanisms cannot limit or suppress stress damage, living organisms will eventually die (Baird et al., 2008). However, some coral species such as massive coral (e.g., Merulinidae species and *Porites* species) have been reported to be more tolerant to thermal stress than branching coral (e.g., *Acropora* species) and may recover after bleaching events (Hongo and Yamano, 2013; Loya et al., 2001)

1.3 Coral holobiont

Corals had evolved establishing symbiotic relationships with a variety of microorganisms not only the zooxanthellae but filamentous algae, cyanobacteria, bacteria archaea, fungi, and viruses are associated with the coral. These microorganisms inhabited different sites inside the coral host. The outermost layers are dominated by oxygenic photosynthetic microbes such as the zooxanthellae (*Symbiodinium* spp.) and cyanobacteria. The inner layers (inside the coral skeleton) are dominated by facultative anaerobic microbes such as filamentous cyanobacteria (*Plectonema* spp.) (Berman-Frank et al., 2003) and filamentous green algae (*Ostreobium* spp.). Moreover, anoxygenic photosynthetic microbes are found in deep layers of coral skeleton such as colorless sulfur bacteria, purple sulfur bacteria, and green sulfur bacteria (Kim, 2015; Yang et al., 2016). The role of the microbial community has been found to be important for corals (Agostini et al., 2012, 2009; Sangsawang et al., 2017). This microbe can provide useful carbon and nitrogenous compound to the host and are also responsible for mounting antibiotic defenses against foreign organisms (Rohwer and Kelley, 2004). This agglomeration of the different organism within and on the surface of the corals was given the term “coral holobiont” (Rohwer et al., 2002).

1.3.1 Interactions of the coral host, with zooxanthellae and the environment

The symbiotic dinoflagellate of the genus *Symbiodinium*, commonly named "zooxanthellae" are well known to live as endosymbiotic associated within gastrodermal cells of corals. More recently, molecular studies revealed that there are different types of *Symbiodinium* in the coral from clade A-D. The clades A and B has been show more tolerant to high temperature than types C, while clade C is the most dominant. (Rowan et al.1997; Baker 2003), Clade D seem to dominate under stressful environments such as high light intensity and temperature. (Baker 2003; Fabricius et al. 2004; Berkelmans and Van Oppen 2006). Zooxanthellae are translocating their photosynthetic products up to 95% in the form of sugars, carbohydrates and amino acid, to the corals that utilize these photosynthetic products for growth, survival, and reproduction. In addition, Corals also released as metabolic waste products (carbon compound, ammonia and phosphate) that may return to zooxanthellae (Muscatine, 1990; Muscatine and Porter, 1977; Rahav et al., 1989; Trench, 1979). Nitrogen cycling also occurs between coral and zooxanthellae. The corals and their symbiotic algae can uptake the dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON) from seawater. The sources of DIN includes ammonium (NH_4^+) and nitrate (NO_3^-) (Pernice et al., 2012), while, the sources of DON is amino acids such as glycine and alanine (Ferrier, 1991; Kopp et al., 2013; J. T. Wang and Douglas, 1997). Moreover, coral polyps can feed on small organisms such as phytoplankton, bacteria, and suspended particulate matter to balance their nitrogen requirements (Levinton, 1995). For the phosphorus, Corals holobiont also obtain phosphorus in form of dissolved organic phosphorus (DIP), by take up phosphate (PO_4^{3-})

from seawater and their preys. These interactions demonstrate that, nutrient recycling is an important feature to support the coral life in such oligotrophic waters.

1.3.2 Endolithic microorganisms in coral skeletons

The Coral skeleton provides a very specific but non-explored environment for living, therefore this was the reason for the limited characterization of endolithic microorganisms (Shashar and Stambler, 1992). The endolithic microorganism was firstly reported in coral skeleton by Duerden, 1902. They were found in various corals forming green, orange and magenta bands, which corresponded with eukaryotic algae, fungi, bacteria and cyanobacteria (Jeffrey et al., 1997; Le Campion-Alsumard et al., 1995; Titlyanov et al., 2009; Yang et al., 2016). The green band layer is almost found in both live and dead calcareous substrates as coral skeletons and encrusting coralline algae. The filamentous of the green algae genus *Ostreobium* are found dominant in the endolithic green band. In live corals, they are able to colonize the skeleton of live corals from their base as soon as the larvae settle, or enter through lateral fissures. Moreover, endolithic green layer also consist with Cyanobacteria *Plectonema terebrans* and *Halomicronema excentricum* (Le Campion-Alsumard et al., 1995; Yamazaki et al., 2008), and less frequently, the Conchocelis stages of Bangial rhodophytes (Tribollet and Golubic, 2011). Moreover, fungi are also observed in live corals, coral-associated fungal assemblage are divers, and in some case may represent a hazard to coral health (Amend et al., 2012; Bentis et al., 2000; Bourne et al., 2009; Wegley et al., 2007). The variation of cyanobacteria, fungi, and algae was correlated with the distance of green layer from coral skeleton surface (Ralph et al., 2007; Yang et al., 2016) and inversely correlated with water depth (Highsmith, 1981).

In addition, endolithic algae are adapted to live in an environment with limited exchange of gases and dissolved and particulate organic matter between the surrounding water column and the coral skeleton. Moreover they can acclimate to wide light intensity variations (Shashar et al., 1997). Changing pH and oxygen concentration are attributed to photosynthesis and respiration reactions in coral tissue and by zooxanthellae (Shashar and Stambler, 1992). The photosynthetically active radiation (PAR) available to endolithic organisms is reduced in both quantity and quality as light penetrates the coral skeleton. Light is almost absorbed and scattered by zooxanthellae, coral tissue, and the inorganic calcium carbonate skeleton. Estimated of PAR levels reaching the endolithic organisms at $\leq$ 2% of the incident irradiance at the coral surface (Halldal, 1968; Magnusson et al., 2007; Schlichter et al., 1997). The remaining light penetrating to the endolithic algae is extremely attenuated, and the photosynthetic productivity of the endolithic region is relatively low (Shashar and Stambler, 1992). Endolithic algae could produce less than 10% of the total oxygen production of a living coral colony, while the respiration of endolithic represented only 1.4% of the colony's total oxygen consumption (Schlichter et al., 1997). Recent studies indicate, the importance of endolithic communities as the process term bioerosion (Golubic et al., 2005; Islam et al., 2012), and beneficial for reef-building coral, for instance, contributing the photosynthesis activity (Odum and Odum, 1955). It might be a potential nutrient source by translocating significant amounts of carbon to the coral and therefore can be of importance for coral host survival (Fine et al., 2005, 2004; Fine and Loya, 2002; Sangsawang et al., 2017). The endolithic community also has a photoprotective role to the host corals since they

reduce the reflection from the white coral bone (Rodríguez-Román et al., 2006) and therefore reducing ROS produced as consequence of strong light. (Yamazaki et al., 2008)

1.4 Aims and objectives

The aim of this study was to understand the composition, physical characteristic, and role of endolithic community associated with massive corals. Using incubation experiments I focused on the elucidation of the responses of endoliths in healthy and bleached corals. Emphasis was put on the following point below:

- To identify the endolithic community of the green-pigmented layer within coral skeletons.
- To determine the capacity of endolithic algae for primary production and nitrogen fixation.
- To investigate the translocation of organic matter from endolithic algae to the coral tissue under healthy and bleached conditions.
- To investigate effects and responses inside the coral tissues and endolithic algae under high temperature and high light stress.
- To investigate possible photoprotection of corals by the endolithic community

Chapter 2

Changes of chemical-biological components in the seawater surrounding healthy and bleached *Porites lutea*

2.1 Abstract

Changes of chemical-biological components in the surrounding seawater of corals under healthy and bleached conditions was investigated by using *in situ* incubation at light (12 h) , light + dark (24 h) periods. Dissolved oxygen (DO), pH, dissolved organic carbon (DOC), nutrients, and microbial community were measured. The seawater around bleached corals shows lower DO and pH values than that around healthy corals. The decreasing of DO is about 60% between healthy and bleached corals, while the decreasing of pH is about 6% between both corals. This suggests that most of the decrease in DO concentrations are due to the loss of photosynthesis activities. DOC concentrations, nutrients (ammonium and phosphate) concentrations and bacterial abundance were higher than around healthy corals. The high concentrations of DOC in water of bleached coral are due to mucus released from coral. And the increasing of organic matter in the surrounding water leaded to increase of microbial abundance.

Keywords: *Porites lutea*, bleached coral, DO, dissolved organic carbon (DOC), bacterial abundance and nutrient

2.2 Introduction

Coral reefs are important ecosystems in shallow water areas that bear the highest biological diversity. The coral reef provides foods, shelter and nursery sites for numerous marine species, including fishes, mollusks, worms, crustaceans, echinoderms and sponges (Chumun et al., 2013; Hoegh-Guldberg, 1988). Most coral reefs are built in oligotrophic waters. Along with carbon and other nutrients, nitrogen and phosphate are an essential element in coral reef ecosystems, and are present at very low concentrations in the waters surrounding coral reefs. Many microorganisms associated with the coral surface mucus, tissue, and skeleton are essential for the coral host such as microalgae (zooxanthellae), bacteria, cyanobacteria, filamentous algae and fungi. The zooxanthellae is a major source of energy for the coral host and coral supply nutrients and shelter for coral symbionts. (Rohwer et al., 2010). Coral hosts gets more than 95% of photosynthetic organic products from zooxanthellae that are used in important processes like respiration, tissue growth and calcification (Muscatine, 1990). Moreover, bacteria and cyanobacteria provide an important part of the nitrogen requirements for the coral host by nitrogen fixation (Lesser, 2004; Shashar et al., 1994). Changing environmental factors can disrupt the balance of energy and nutrients translocation between corals host and their microorganism such as increased sea surface temperature, high irradiance, and low water quality lead to coral disease or coral bleaching (Higuchi et al., 2013; Hoegh-Guldberg, 1999; Wang and Douglas, 1997). Exchange of dissolved organic matters and nutriens between corals and surrounding water were observed (Haan et al., 2016; Godinot et al., 2011). Recent study has shown that zooxanthellae can be damaged under high nitrate and temperature conditions (Chumun et al., 2013; Pogoreutz et al., 2017).

In this study, we would like to investigate the changing of chemical–biological components in the surrounding water of the coral *Porites lutea* under healthy and bleached conditions using in situ incubation. Dissolved oxygen (DO), pH, dissolved organic carbon (DOC), nutrient, and abundance of microbial community were followed during these incubations.

2.3 Materials and Methods

2.3.1 Coral sampling

This study was carried out at Sesoko reef which is located at the Sesoko Island, Okinawa, Japan (26°39' N 127°51'.14 E) (figure 2.1). Sesoko reef is actually in a post bleaching phase with less than 10% of living coral coverage, 50% of sandy bottom, 30% of coral rubbles and the rest covered by macro-algae and turf algae (Casareto et al., 2008). The dominant corals in this area were the non-branching corals like *Porites* spp., *Goniastrea* spp. and *Favia* spp. (Cabaitan et al., 2012). Fragments of healthy colonies of *Porites lutea* were collected with permission from Okinawa Prefectural Government, No. 28 – 6, during low tide (depth 0.5 - 1 m). Coral samples were transported back to the laboratory and kept in aquarium with running natural seawater under ambient light and temperature. The environmental conditions as temperature, salinity at the time of sampling were registered using a multi-sensor Hydrolab MS5 Multiparameter Sonde (OTT Hydromet, Kempten, 2, Germany).

2.3.2 Incubation design

Healthy and partially bleached corals were selected for these incubation experiments. Fragments of the same coral colonies were divided into two parts: healthy coral fragments were acclimatized for three days under the ambient conditions with running seawater. The remaining coral fragments were induced to bleach at the seawater temperature (33 ºC). Three replicates of healthy and partially bleached coral fragments of approximately the same size were placed into individual polycarbonate Nalgene bottles (1,230 mL). Additionally, one set of experiment was set up to investigate seawater (only seawater) as control. The incubation bottles were filled with seawater, collected at the site of coral collection. Tracer natural isotope (^{13}C and ^{15}N were added to these incubation bottles to measure primary production and nitrogen fixation. These results will be presented in Chapter 3. The ^{13}C tracer solution (2.46 mL; $NaH^{13}CO_3$, 99.9% ^{13}C), prepared by adding 1 g of the salt to 100 mL deionized water, was added to the incubation bottle to achieve a final ^{13}C concentration of around 11.5% (a ^{13}C enrichment factor of 10.4), and subsequently 2.46 mL of $^{15}N_2$ gas (99.8 % ^{15}N, Shoko Co. Ltd, Tokyo, Japan) was added using a gas-tight syringe to obtain an enrichment of 6.8% in the seawater.

The incubation bottles containing the coral fragments were set-up *in situ* at a depth of around 5 m, with average illumination of 360 µmol photons m^{-2} s^{-1} and an average temperature of 26 ºC. The incubations were performed from 6:00 h to 18:00 h (12 h, light period) and from 6:00 h to 6:00 h on the next day (24 h, light and dark periods). The water temperature and light intensity during the incubations were monitored using *in situ* sensors (MDS-MkV/T and MDS-Mk/L, Alec Electronics, Kobe, Japan).

2.3.3 Measurements

Corals and seawater samples were sampled at the initial, after 12 h of light and after 24 h of light and dark periods for measurement of dissolved organic carbon (DOC), dissolved oxygen (DO), pH, bacteria abundance and dissolved inorganic nutrients analysis (nitrate; NO^{3-}, nitrite; NO^{2-}, ammonia; NH_4^+ and phosphate; PO_4^{3-}). Variations of these parameter (Δ values) during incubation were calculated by deducting the initial values from the final values.

2.3.4 Dissolved organic carbon (DOC), Dissolved oxygen (DO) and pH

40 ml of the seawater samples were collected at initial time and from bottles after each incubation period were filtered using a glass syringe connected directly to a filter holder containing GF/F filter (pre-combusted at 500 ºC for 4 hours). The filtrates were kept at -20º C in glass vials until analysis. DOC concentrations were analyzed following the methods of Suzuki et al. (1992) and Shinomura et al. (2005) using Shimadzu TOC-5000A analyzer. Average analytical error of DOC measurement was 0.95% as coefficient of variation. Dissolved oxygen (DO) and pH were measured with an Orion 4 star sensor with a polarographic DO Probe on a 5 ft Cable (Thermo Scientific, INC., Waltham, MA, USA). The pH (NBS scale) sensor was calibrated with NIST (NBS)-scaled buffer solutions (Mettler pH 9.228 and 6.880 buffers at 20° C) at 25°C. The accuracy of pH measurements was ± 0.005 pH units.

2.3.5 Bacterial abundance

The initial seawater and seawater after incubation was collected in 50 mL bacteria free bottles and preserved with 25% glutaraldehyde (1% final concentration). Bacteria were filtered on to 0.2 µm black polycarbonate filters by filtering 10 ml aliquots of seawater, stained with

4’6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980). Samples were mounted on slides and bacteria were counted under UV excitation using epifluorescence microscopy (Nikon; Eclipse/E6000, Japan).

2.3.6 Nutrients measurement

Seawater were collected in 125 ml polyethylene bottles and preserved at -20º C until analysis. Nutrients concentration were determined with an auto analyzer (TRAACS 2000; Bran and Luebbe, Norderstedt, Germany) in the laboratory according to (Hansen and Koroleff, 2007). Nitrate was determined by subtracting the values of nitrite from the values of nitrate + nitrite. Detection limit was estimated as 3σ, where σ is the standard deviations of the replicated (*n* = 5) analyses for 3.5% NaCl (blank) solution. Reproducibility (precision) of nutrient analysis was ±0.2% for NO^{3-}, ±0.5% for NO^{2-}, ±1.2% for NH_4^+ and ±0.8% for PO_4^{3-}.

2.3.7 Statistical analysis

ANOVA test was performed to determine the differences among experiment conditions at different times. Post hoc Tukey test was used to assess pairwise differences when ANOVA revealed statistically significant effects. The software MINITAB ver. 14 was used for all statistical analysis.

2.4 Results

2.4.1 Light intensity and temperature

Light intensity was highest around 13:00 pm with 900 µmol m^{-2} s^{-1}. The water temperature along the experiment varied between 24-28º C and the highest temperature was found around 10:00 am (figure 2.2).

2.4.2 DO and pH

The DO concentrations (figure 2.3) decreased from initial values along the light period (12 h) and 24 h in the seawater of bleached corals incubations, while for healthy corals, DO concentration is increased during light period from initial and slightly decreased during dark period due to respiration. The pH values (figure 2.4), in seawater of healthy coral did no changed, however a decrease (statistically significant) was observed after 24 h in bleached corals. The decrease of DO was about 60% between healthy and bleached corals, while the decrease of pH is about 6% between both corals. This suggests that most of reduced DO concentrations are due to the loss of photosynthetic activities rather than high respiration pattern.

2.4.3 DOC concentrations

The DOC concentrations are shown in (figure 2.5). The concentration of DOC was higher in bleached corals incubations. Increasing of DOC concentrations were found in all samples during the 12 h light period and after dark period (24 h). In healthy corals a significant increase of DOC production ($P < 0.05$) was observed during the day period but then it slightly decreased overnight. However, in bleached corals significantly increase ($P < 0.01$) in DOC production was observed at both, 12 h and 24 h of incubations with further increase during the dark period.

2.4.4 Bacterial abundance

The abundance of bacteria was higher during the dark period (24 h) than during light period (12 h) in health coral experiments but this difference was not statistically significant (figure

2.6). However, in bleached corals abundance of bacteria was highest (significantly difference ($P < 0.01$) compared with that in health coral along incubation periods and further increased from 12 to 24 h incubations. Increase of microbial abundance followed the same trend of DOC, indicating that microbial communities consume the DOC that was released as mucus from corals, and rapidly grow in the incubation bottles.

2.4.5 Nutrient dynamics

The initial concentrations of ammonium, nitrate, nitrite and phosphate are 0.9 ± 0.4, 0.81 ± 0.04, 0.09 ± 0.02 and 0.20 ± 0.2 µM ($n = 3$), respectively. Table 2.1 shows difference of nutrients concentration between initial and final in all samples. The ammonium and phosphate concentrations highly increased in the incubation bottles of bleached corals. These results suggest bleached corals released ammonium and phosphate resulting as waste from their metabolism particularly when the coral is under stresses (bleached condition).

2.5 Discussion

These results show that the coral *Porites lutea* under healthy and bleached conditions displayed different chemical-biological responses affecting the surrounding seawater along the incubation periods (12 and 24 h). The higher DO concentration in healthy corals during light shows the effect of photosynthesis due to phytoplankton present on the seawater and the symbiont zooxanthellae. Therefore, the lower DO concentration in bleached corals during light period is due to the loss of photosynthetic activities (decreased zooxanthellae abundance), because decreased of pH did not reflect was not comparable to the decrease in DO concentrations. While decrease of DO concentration during dark period (24 h) is due to

the respiration. This means that impact of stresses in bleached corals affected primarily the photosynthesis rather than respiration.

Under normal condition, corals release 6%−40% of the photosynthetically fixed carbon by the zooxanthellae as mucus in the form of DOM to the ambient seawater (Crossland, 1987; Ferrier-Pages et al., 1998; Muscatine et al., 1984). Coral mucus is content (protein, triglycerides and wax ester) serves as primarily energy for the corals. The coral mucus immediately dissolves in the sea water as DOC, and DOC support metabolism and growth of bacterial community (Meekaew et al., 2014; Wild et al., 2004). The DOC concentration in the incubation bottles of bleached corals shows remarkable increase at both periods 12 and 24 h. This suggests that bleached corals have more stress and exude large amount of mucus. The previous research found that the microbes on the corals with excess DOC grew ten times faster than normal coral condition (Kline et al., 2006). Our results show, the DOC values are a similar trend with a number of bacteria abundance, and a significant positive correlation ($r = 0.94$, $P < 0.01$, $n = 9$) between DOC and bacterial abundance is found. The rapid growths of bacteria consume also DO. The increases of bacteria in the bleached coral might have affected to nutrient cycles. The concentration of ammonium and phosphate in the water in bleached corals (table 2.1) was much higher than healthy corals. Under normal condition, symbiont algae and phytoplankton consume ammonium as the primary source of nitrogen as it can be easily taken up through passive diffusion. Zooxanthellae and cyanobacteria have the ability to convert nitrate to ammonium and subsequently assimilate it into amino acids (Sheppard et al., 2009). But rather than consumption, release from coral was important causing the higher concentration of ammonium and phosphate in the surrounding water.

These results indicate that chemical-biological components in surrounding water of corals under the stresses such as high temperature and strong light can be remarkably changed by three major processes: 1) loss of symbiont algae and changes in phytoplankton activities, 2) release of mucus and nutrients from coral response, 3) metabolisms of microorganisms. This study shows the dynamic response and interaction between corals surrounding seawater and its microbial community. To know the changes of chemical-biological components is an important factor for understanding mechanisms of coral response under the stresses inside the coral holobiont in both healthy and bleached corals conditions.

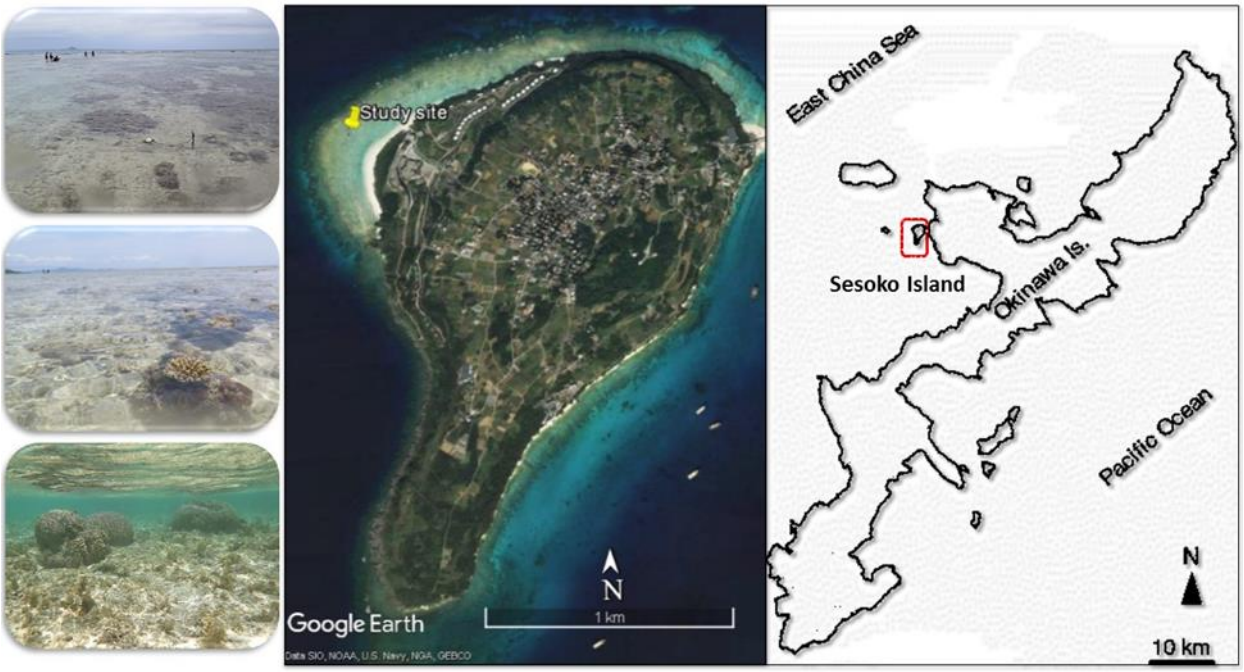


Figure 2.1 Study area at Sesoko, Okinawa, Japan

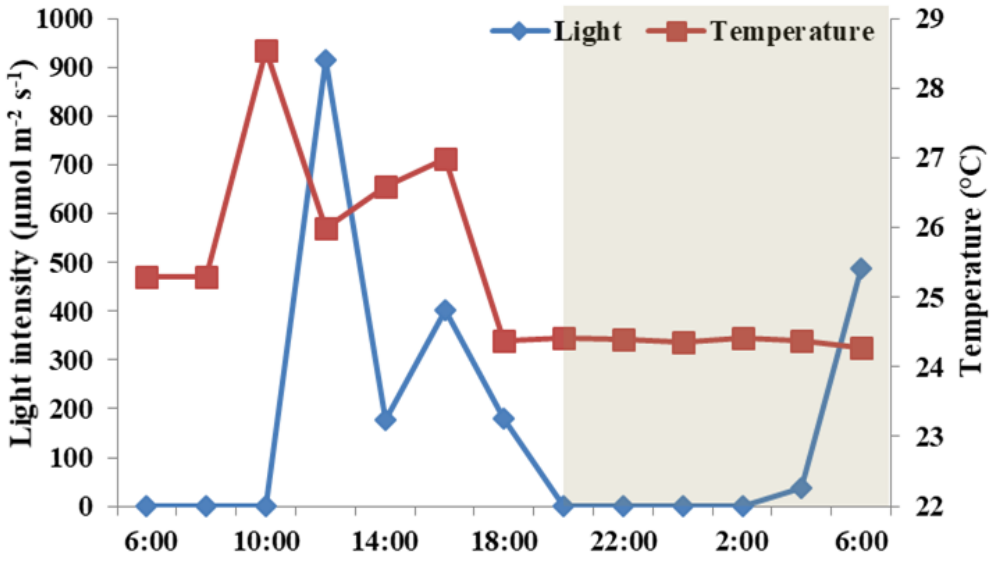


Figure 2.2 Time course of light intensity and temperature during the incubation time.

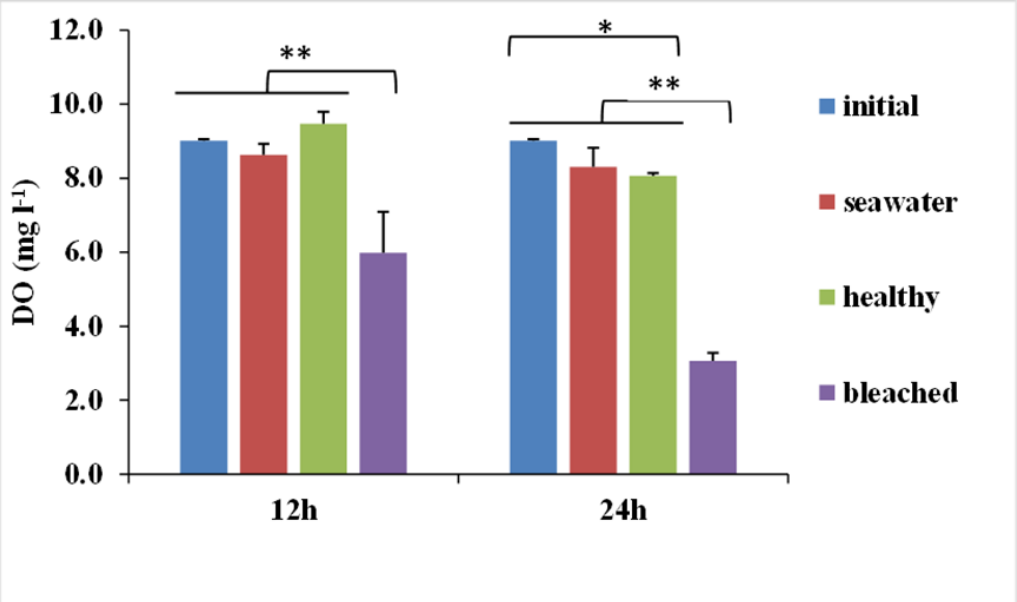


Figure 2.3 Dissolved oxygen (DO ± standard deviation) concentration in the seawater of healthy and bleached corals incubation bottles during 12 h and 24 h incubation time.

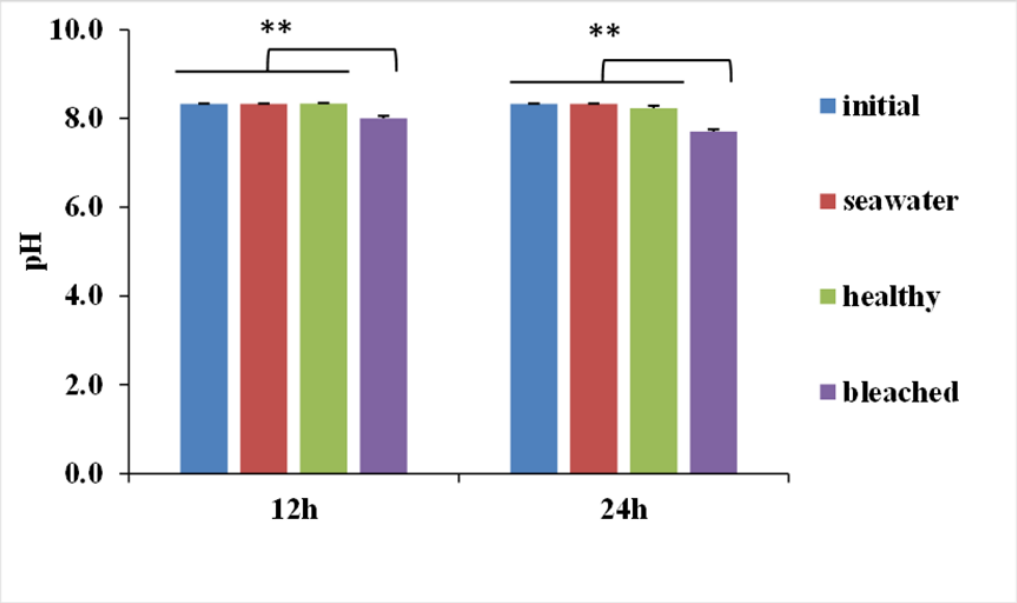


Figure 2.4 pH in the seawater of healthy and bleached corals incubation bottles during 12 h and 24 h incubation time.

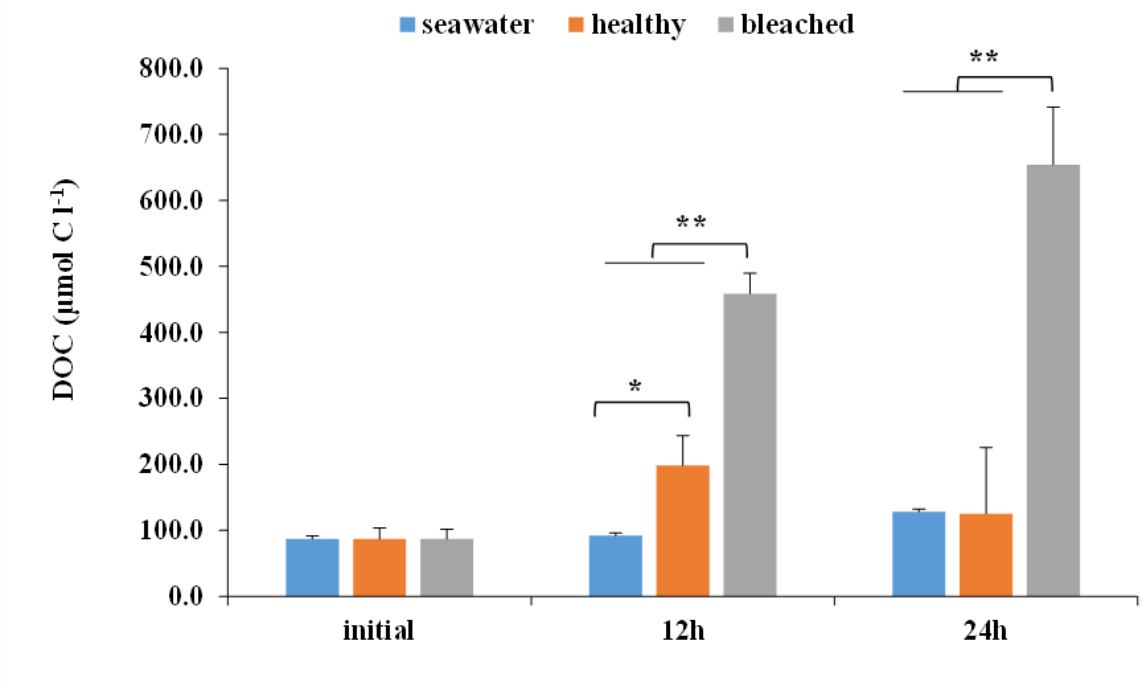


Figure 2.5 Dissolved organic carbon (µM; means ± standard deviation) in the seawater of healthy and bleached corals incubation bottles during 12 h and 24 h incubation time.

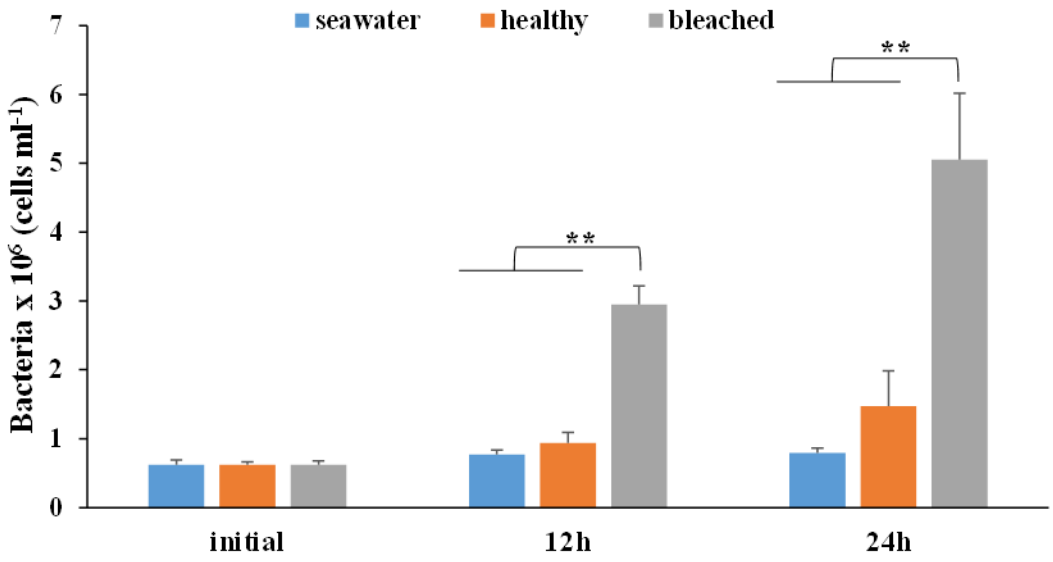


Figure 2.6 Bacteria abundance (cells ml^{-1}) in the seawater of healthy and bleached corals incubation bottles during 12 h and 24 h incubation time.

Table 2.1 Change (Δ) in concentrations (µM; means ± standard deviation) compared to initial seawater of nitrate (NO_3^-), nitrite (NO_2^-), ammonium ($NH4^+$), and phosphate (PO_4^{3-}).

condition	nutrients (µM)							
	ΔNO_3^-		ΔNO_2^-		ΔNH_4^+		ΔPO_4^{3-}	
	12 h	**24 h**	**12 h**	**24 h**	**12 h**	**24 h**	**12 h**	**24 h**
sea water	-0.11±0.00	-0.25±0.07	-0.03±0.01	-0.03±0.00	-0.17±0.11	-0.46±0.15	-0.47±0.01	-0.47±0.02
healthy	-0.39±0.04	-0.69±0.07	0.09±0.04	-0.02±0.00	-0.49±0.01	-0.46±0.12	-0.37±0.14	-0.40±0.04
bleached	-0.18±0.09	-0.86±0.03	0.09 ±0.02	0.07±0.00	3.96±0.01	3.94±0.01	3.76±0.88	3.93±0.02

Chapter 3

^{13}C and ^{15}N assimilation and organic matter translocation by the endolithic community in the massive coral *Porites lutea*

3.1 Abstract

Corals evolved by establishing symbiotic relationships with various microorganisms (the zooxanthellae, filamentous algae, cyanobacteria, bacteria, archaea, fungi, and viruses), forming the "coral holobiont". Among them, the endolithic community is the least studied. Its main function was translocation of photo-assimilates to the coral host, particularly during bleaching. Here, we hypothesize that 1) endolithic algae may show similar primary production rates in healthy or bleached corals by changing their pigment ratios, and therefore that similar production and translocation of organic matter may occur at both conditions; and 2) diazotrophs are components of the endolithic community; therefore, N_2 fixation and translocation of organic nitrogen may occur. We tested these hypotheses in incubation of *Porites lutea* with ^{13}C and ^{15}N tracers to measure primary production and N_2 fixation in coral tissues and endoliths. Assimilation of ^{13}C atom (%) was observed in healthy and bleached corals when the tracer was injected in the endolithic band, showing translocation in both conditions. N_2 fixation was found in coral tissues and endolithic communities with translocation of organic nitrogen. Thus, the endolithic community plays an important role in supporting the C and N metabolism of the holobiont, which may be crucial under changing environmental conditions.

Keywords: Endolithic algae, Primary production, Nitrogen fixation, Translocation, *Ostreobium quekettii*, *Porites lutea*

3.2 Introduction

Stony corals or reef-building corals are the most important components of the coral reef ecosystems that distribute in tropical areas of the world's oceans. Corals evolved to adapt to oligotrophic environments by establishing symbiotic relationships with a variety of microbes forming the so called "coral holobiont" (Bourne et al., 2009; Koren and Rosenberg, 2006; Rohwer et al., 2002; Rosenberg et al., 2007). In Addition to the well-known symbiotic algae zooxanthellae, other associated organisms play crucial roles in environmental adaptation of the coral holobiont by providing organic and inorganic carbon (Rosenberg and Zilber-Rosenberg, 2011) and organic nitrogen (Lesser et al., 2004; Shashar et al., 1994), and protecting the coral host from pathogens (Ritchie, 2006; Shnit-Orland and Kushmaro, 2009; Thompson et al., 2015).

Recent changes in environmental conditions, such as increased sea surface temperature, high irradiance, and low water quality, are promoting coral bleaching and disease. However, the coral holobiont has the capacity to acclimate (to some extent) to these environmental changes by rapidly altering the population of associated microbes and their functioning in a dynamic way. The "coral probiotic hypothesis" was proposed to explain this concept (Reshef et al., 2006) .

Among associated microbes, the endolithic community is one of the less studied entities within the coral holobiont. Members of this community appear in remarkably high numbers in massive and encrusting corals (Fine et al., 2004; Highsmith, 1981). Previous studies had shown that the endolithic community consists of filamentous algae, especially chlorophytes of the genus *Ostreobium*, cyanobacteria, and fungi (Golubic et al., 2005; Lukas, 1974; Titlyanov et al., 2009). More recently, molecular studies revealed the presence of several bacteria, such as Firmicutes, Actinobacteria, Proteobacteria, and Chlorobi, including

nitrogen fixers and green-sulphur bacteria (Yang et al., 2016). Several functions were attributed to this community, such as providing potential nutrient sources (Fine and Loya, 2002) and photoprotection for the coral host (Schlichter et al., 1997; Yamazaki et al., 2008), particularly during bleaching events. Fine and Loya (2002) showed increasing ^{14}C activity with time in coral tissues after addition of ^{14}C inside the endolithic band of bleached corals. However, they did not evaluate this process in terms of the organic matter synthetized and transferred.

In this study, we wished to determine how much the endolithic community fixes organic carbon and whether the endolithic community is capable of fixing atmospheric nitrogen and translocating it to the coral host. We also wanted to know what percentage of the synthetized organic C is translocated to the coral tissue under the healthy and stressful condition, and what the responses are of photosynthetic pigments of the endolithic community under higher illumination when the coral is bleached.

We hypothesized that 1) endolithic algae keep their photosynthetic performance at similar levels in healthy or bleached corals; therefore, translocation may occur similarly under both conditions. 2) Cyanobacteria and other N_2 fixers are components of the endolithic community; therefore, N_2 fixation may occur in this layer and organic nitrogen-rich compounds may be transferred together with organic carbon. 3) The endolithic community may acclimate to changes of illumination under bleached coral conditions by rapid changes of their pigments and pigment ratios.

In this study, we provide a quantification of the primary production and nitrogen fixation of the endolithic community and the coral tissues in terms of organic carbon and nitrogen; we also evaluated the proportion of translocated photo-assimilates under both normal and bleached conditions. We also evaluated the acclimation capacity of the endolithic

community by studying their pigments composition and their temporal changes during short-term incubations.

Our result shows that similar or higher amount of organic carbon was translocated in healthy corals when compare to bleached corals. Similarly, we found comparable photosynthetic performances of endoliths under healthy and bleached coral conditions during the illuminated period of our incubations. This showed that photosynthetic performance of endolithic algae was not affected by high illumination under the bleached coral tissues, in spite of some decrease in the concentrations of Chl *a*. This means that endolithic community can still support the coral holobiont for some period of time, even under stressful conditions.

3.3 Materials and methods

3.3.1 Coral sampling

Observations were made in May 2016 at Sesoko coral reef lagoon located on the western side of Sesoko Island, Okinawa, Japan (26°39′N, 127°51′E). Live coral coverage was approximately 8% and was dominated by non-branching corals (mainly *Porites* spp., *Goniastrea* spp., and *Favia* spp.) (Cabaitan et al., 2012). Fragments of healthy colonies of *Porites lutea* were sampled with permission from the Okinawa Prefectural Government, No. 28 - 6 for incubation experiments and identification of the endolithic community. Sampling was performed during low tide (depth 0.5–1 m). Environmental conditions at the time of sampling were registered using a multi-sensor Hydrolab MS5 Multiparameter Sonde (OTT Hydromet, Kempten, 2, Germany). The temperature and salinity of the seawater were 27.5 °C and 34 ppt, respectively. Coral samples were immediately transported to the laboratory and kept in an aquarium with running natural seawater.

3.3.2 Taxonomic identification of endolithic algae

The soft tissue layer of corals was separated from the skeleton using a handsaw. The coral skeleton containing the green band layer formed by the endolithic community was decalcified in 1 N HCl until complete dissolution, and the emerging endolithic algae were fixed in formaldehyde-buffered solution (4% final concentration). Aliquots of endolithic algae were mounted on glass slides and observed at 4X, 20X, 40X, and 100X magnification under a light microscope (Nikon-ECLIPSE/80i). Algae were identified using key morphological characteristics as described in taxonomic references (Berger and Matthias J, 1992; Golubic et al., 2005; Le Campion-Alsumard et al., 1995; Lukas, 1974; Ravindran et al., 2001).

3.3.3 Incubation design

A natural isotope tracer technique using ^{13}C and ^{15}N was applied to measure the primary production and nitrogen fixation rates. The following two incubation experiments were performed in Addition: corals were incubated in seawater with Addition of ^{13}C and ^{15}N gas to assess the primary production and N_2 fixation in the coral tissue and the endolithic community; and Injection: corals were incubated in the same manner but ^{13}C solution was directly injected into the endolithic green band. Healthy and partially bleached corals were subjected to these two incubation experiments. For this, fragments of the same coral colonies were divided into two parts: healthy coral fragments were acclimatized for three days under the ambient conditions with running seawater, and the remaining coral fragments were induced to bleach at a seawater temperature of 33 ºC under dark conditions.

In the Addition incubation, three replicates of healthy and partially bleached coral fragments of approximately the same size were placed into individual polycarbonate Nalgene bottles (1,230 mL). The incubation bottles were filled with seawater collected at the

site of coral collection. The ^{13}C tracer solution (2.46 mL; $NaH^{13}CO_3$, 99.9% ^{13}C), prepared by adding 1 g of the salt to 100 mL deionized water, was added to the incubation bottle to achieve a final ^{13}C concentration of approximately 11.5% (a ^{13}C enrichment factor of 10.4). Subsequently, 2.46 mL of $^{15}N_2$ gas (99.8 % ^{15}N, Shoko Co. Ltd, Tokyo, Japan) was added using a gas-tight syringe to obtain an enrichment of 6.8% in the seawater. The incubation bottles containing the coral fragments were set up *in situ* at a depth of approximately 5 m, with an average illumination of 360 µmol photons m^{-2} s^{-1} and an average temperature of 26 ºC. The incubations were performed from 6:00 h to 18:00 h (12 h light period) and from 6:00 h to 6:00 h on the next day (24 h incubation). The water temperature and light intensity during the incubations were monitored using *in situ* sensors (MDS-MkV/T and MDS-Mk/L, Alec Electronics, Kobe, Japan).

In the Injection incubation, the ^{13}C solution was added directly to the green band using a syringe after opening a narrow hole (< 2 mm in diameter) inside the coral skeleton that was sealed after the Injection. Three replicates each for the 12 h illumination and 24 h (12/12 light-dark) incubation were set up in an indoor incubation system on the premises of the Tropical Biosphere Research Center of the University of Ryukyus, Sesoko Island, Okinawa. The incubation was performed at 27 ºC and 300 or 0 µmol photons m^{-2} s^{-1} light intensity.

3.3.4 Treatment of samples

After incubation, the coral fragments were separated into coral soft tissue and the endolithic green band layer. The latter was ground using a pestle and mortar and homogenized; the homogenate was acidified with 1N HCl to remove carbonates, filtered through a pre-combusted (500 ºC) Whatman GF/F filter (47 mm), and finally stored at -20 ºC until subsequent treatments. Samples on the filters were dried at 60 ºC in an electric oven until they attained constant weight. Particulate organic carbon (POC), particulate organic nitrogen

(PON), and ^{13}C and ^{15}N content were measured using a mass spectrometer (DELTA plus Advantage, Thermo Finnigan Co., USA equipped with an elemental analyzer EA1110). The primary production was calculated according to Hama et al. (Hama et al., 1993) and N_2 fixation was calculated using a modified method as described by Casareto et al., 2008, which was based on the method of Montoya et al. (Montoya et al., 1996).

3.3.5 Maximum quantum yield (*Fv*/*Fm*) of endolithic algae and symbiotic zooxanthellae

Chlorophyll *a* fluorescence was measured at the start and after 12 h and 24 h of incubations using a pulse-amplitude-modulated (PAM) fluorometer (JUNIOR PAM, Walz, Germany) according to the method of Schreiber et al. (Schreiber et al., 1998). The maximum quantum yield was calculated as *Fv/Fm*, where $Fv = Fm - Fo$. *Fo* is the initial fluorescence after dark adaptation and *Fm* is the maximum fluorescence after dark adaptation (Krause and Weis, 1991). Coral samples were kept for 15–30 min in the dark for adaptation before the fluorescence measurements were obtained. The fluorescence data were registered from three to six different points in both coral tissues and the endolithic green band, from which averages and standard errors were calculated.

3.3.6 Pigment composition

Pigments were analyzed at the start and after 12 h and 24 h of incubation in coral tissues and the endolithic green band subsampled from the incubated corals. Measurements were obtained using high-performance liquid chromatography (HPLC) (LC-30AD; Shimadzu, Kyoto, Japan). The HPLC system was equipped with a ZORBAX Eclipse Plus C8 Column (2.1 x 50 mm; Agilent Technology, Santa Clara, CA, USA). The subsamples of coral tissues and endolithic algae were ground with mortar, filtered through a GF/F filter, and preserved at -20 ºC until the measurements were obtained. The pigments were extracted with 2 mL of

N, N-dimethylformamide and maintained overnight in the dark at -20 ºC. The extract was filtered through a syringe filter (0.2 µm, Millex-LG; Millipore, Bedford, MA, USA) to remove cell debris. The pigment extract (1 mL) was mixed with 0.2 mL of ultrapure water (Milli-Q, Millipore) and each 10 µL of the mixed solution was injected into the HPLC system using an auto-sampler (SIL-30AC, Shimadzu). The pigments were eluted at a flow rate of 0.3 mL min^{-1} at 25 °C using a programmed binary gradient. All samples were prepared under subdued light. The solvents used were as follows: (A) methanol: acetonitrile: 0.25 M aqueous pyridine solution (50:25:25, vol/vol/vol); (B) methanol:acetonitrile:acetone (20:60:20, vol/vol/vol). The separated pigments were detected spectrophotometrically using a photodiode array detector (SPD-M30AD; Shimadzu) with an optical resolution of 1.2 nm over a wavelength range from 320 to 720 nm. Each peak was identified by comparing HPLC retention times with the absorption spectra of standards and the data obtained from photodiode array detection. The photosynthetic pigment concentrations in the coral tissue and endolithic algae were normalized to the coral surface area (cm^2).

3.3.7 Statistical analysis

MINITAB ver.14 software was used for statistical analysis. ANOVA was performed to determine the significant differences among factors and Turkey's post hoc test was used to assess the pairwise differences when ANOVA revealed statistically significant effects ($P < 0.01$ and $P < 0.05$).

3.4 Results

3.4.1 Composition of the endolithic community

The endolithic community forming a green band in the coral skeleton underneath the coral soft tissue is shown in a cross-section of *Porites lutea* (figure 3.1). Two groups of algae (two species of Chlorophyta and Cyanophyta) and a fungus dominated the community.

Among the Chlorophyta, *Ostreobium quekettii* was largely dominant, showing main branches (3–10 µm in diameter) (figure 3.2*a*), ultimate branches (0.5-1.5 µm in diameter) (figure 3.2*b*), and swellings similar to sporangia (approximately 20–300 µm in diameter) (figure 3.2*c*). Dasycladales (holdfast) were present in low abundance, showing irregular branches (30–100 µm in diameter) (figure 3.2*d*). The cyanobacterium *Leptolyngbya terebrans* was present in low abundance, showing non-heterocystous filaments (0.8–1.3 µm in diameter) (figure 3.2*e*). Two fungal forms were found in low abundance with red and hyaline thallus, 2–5 µm and 1–2 µm in diameter, respectively (figure 3.2*f*).

3.4.2 Photosynthetic activity

The maximum photosynthetic quantum yields (*Fv/Fm*) of zooxanthellae in coral tissues and endolithic algae in both, healthy and bleached colonies are shown in figure 3.3. The *Fv/Fm* in the healthy coral tissues was almost stable or decreased slightly after 24 h during Addition incubation; however, in bleached coral tissues, the initial value of *Fv/Fm* was significantly lower than that of healthy corals and varied significantly ($P < 0.01$ and $P < 0.05$) after exposure to light for 12 h in both, the Addition and Injection incubations. The initial *Fv/Fm* values for the endolithic algae (Addition incubation) in healthy and bleached corals were similar, and after 12 and 24 h, the *Fv/Fm* values differed significantly ($P < 0.01$) from the initial values. In healthy corals, the *Fv/Fm* of the endoliths significantly decreased ($P < 0.01$) after 12 and 24 h, showing a slight recovery; however, in bleached corals, the *Fv/Fm* of endoliths showed a continuous decrease after 12 and 24 h ($P < 0.01$). Similar patterns were observed during Injection incubation, but no significant differences were observed. In spite of that, overall the *Fv/Fm* values of the endoliths after the incubations showed similar values in healthy and bleached corals, indicating comparable photosynthetic performance in healthy and bleached conditions.

3.4.3 Photosynthetic pigments and changes in their composition during the incubations

The pigment concentrations in the coral tissues and endolithic algae before the start of the incubations are shown in table 3.1. Pigments identified included Chl *a*, *b*, C_2 , 13^2,17^3-cyclopheophorbide *a* enol (cPPB-*a*E), and carotenoids (peridinin; tracer pigment of dinoflagellate, diadinoxanthin, and β carotene), which were identified together with zeaxanthin (tracer pigment of cyanophytes). Moreover, Chl *a* spectra could be separated into three peaks by HPLC elution profiles: Chl *a* allomer, Chl *a*, and Chl *a* epimer (Zapata et al., 2000). From these data, the Chl *a*:carotenoids, Chl *a*:Chl *b* and allomer:total Chl *a* ratios were calculated.

In the healthy coral tissues, Chl *a*, peridinin and diadinoxanthin concentrations were significantly ($P < 0.01$) higher than in the bleached corals and the Chl *a*:carotenoids ratio in the bleached corals was significantly ($P < 0.01$) lower than that in the healthy corals. Allomer:total Chl *a* was slightly higher in bleached corals but did not show a significant difference.

In the endolithic green band, the concentrations of Chl *a*, *b* and the Chl *a:*Chl *b* ratio did not show a significant difference between healthy and bleached corals. The Chl *a*:carotenoids ratio was significantly lower in the green bands of the healthy corals than in those of the bleached corals. Allomer:total Chl *a* was slightly higher in bleached corals but did not show a significant difference. Allomer: total Chl *a* was higher in coral tissue than in endoliths, denoting a more oxidative state of Chl *a* in coral tissues. The concentration of zeaxanthin was higher in coral tissues, denoting an important association with cyanobacteria. Changes in the pigment percentage of endoliths during the incubations are shown in figure 3.4 and table 3.3. The percentage of Chl *a* in endoliths of bleached corals was higher than

that in healthy corals and increased during 12 h of incubation from 68 to 73% (bleached) and 52 to 61% (healthy). However, after 24 h of incubation, the values decreased in both coral conditions. The percentage of Chl *b* in endoliths of healthy corals was higher than that in the bleached corals and increased along incubations in both coral conditions. From these results, the percentage of Chl *a* decreased with an important increase in Chl *b* and carotenoids, and this resulted in a drastic decrease in the Chl *a*: Chl *b* and Chl *a*: carotenoid ratios.

3.4.4 Primary production and nitrogen fixation

The results for primary production and N_2 fixation are shown in table 3.2, and the changes in POC, PON, and POC:PON ratio measured during the two incubations in healthy and bleached *P. lutea*, are shown in table 3.4. During the Addition incubation, primary production in the healthy coral tissues reached a value of approximately 40 µg C cm^{-2} during the illuminated hours and decreased slightly after 24 h due to respiration during the night. However, in the bleached coral tissues, primary production during the illumination period was lower than 1 µg C cm^{-2} and a slight increase (2.5 µg C cm^{-2}) was observed after 24 h of incubation, which may indicate some external source of fixed organic matter. In contrast, the endolithic algae in the healthy corals showed a very low (0.23 µg C cm^{-2}) primary production rate during the 12 h light period, and the rate increased after 24 h of incubation. The primary production rate of the endolithic algae in the bleached corals decreased slightly compared to the production in the healthy corals and decreased after 24 h of incubation, revealing higher respiration than in the endoliths of healthy corals.

During the Injection incubation, the trend of primary production in healthy and bleached coral tissues was consistent with the results obtained during the Addition incubation. However, primary production values during the 12 h light period were approximately two

times higher for the healthy coral tissues (70 µg C cm^{-2}) and one order of magnitude higher (~ 9 µg C cm^{-2}) for the bleached coral tissues when compared to the values obtained during the Addition incubation. The values for primary production in the endolithic algae were also higher than those obtained during the Addition incubation. Approximately three times more carbon was fixed by the endolithic algae present in the healthy corals after 24 h of incubation. As in the Addition incubation, consumption (respiration) of organic carbon by endolithic algae in the bleached corals was observed after 24 h of incubation.

Nitrogen fixation was detected in both coral tissues and the endolithic community. In healthy coral tissues, approximately 30 ng N cm^{-2} was fixed during the 12 h light period and more than 50% was consumed during the night after 24 h. However, in the tissues of the bleached corals, N_2 fixation was lower (around 8 ng N cm^{-2}) during the 12 h light period and increased by 1.6-fold during the night period after 24 h of incubation. Nitrogen fixation in the endolithic community was slightly lower (~25%) in the bleached corals (1.6 ng N cm^{-2}) when compared to that in the healthy corals (2.12 ng N cm^{-2}). In both healthy and bleached conditions, N_2 fixation of endoliths decreased after 24 h. This pattern may also indicate some consumption and/or translocation of N-rich organic matter in the coral tissue. In coral tissues, the POC:PON ratio (table 3.4) increased after 12 h and 24 h of incubation, indicating an increase in the C-rich organic matter. In contrast, the POC:PON ratio in the endolithic algae increased during the illuminated period (12 h) and decreased slightly or remained the same after the 24 h incubation.

3.4.5 Translocation

Incorporation of ^{13}C atoms (%) in the coral soft tissues and in the endolithic community during Addition and Injection incubations for both healthy and bleached corals is shown in figure 3.5 and table 3.5. During Addition incubation, incorporation of ^{13}C atoms (%) in

healthy coral tissues increased (5.5 %) after 12 h of incubation ($P < 0.01$) and slightly decreased to 4.5% after 24 h. However, during the Injection incubation, the incorporation of ^{13}C atoms (%) was higher, and it increased by 8.8% during the 12 h light period ($P < 0.01$) and by 12.4% after the 24 h incubation period ($P < 0.01$). In the endolithic community of healthy corals, the incorporation of ^{13}C atoms (%) was almost undetectable during Addition incubation; however, during Injection incubation it was highly increased (7.1 %) ($P < 0.01$) during the first 12 h and increased by 14.3% ($P < 0.01$) after 24 h. In the bleached coral tissue, ^{13}C atoms (%) were almost not incorporated (< 1.0 %) throughout the Addition incubation; however, an increase of 2.1% after 12 h and of 6.9% ($P < 0.01$) after 24 h was observed during the Injection incubation. As in the case of healthy corals, incorporation of ^{13}C atoms (%) in the endolithic algae of bleached corals was not detected during the Addition incubation. However, it was high during the first 12 h of Injection incubation, with an increase of 11.5 % ($P < 0.01$), and this result was consistent with the primary production rates. Some of the incorporated ^{13}C atom (%) (2.1 %) ($P < 0.01$) were lost (respired and/or utilized) after the 24 h incubation.

3.5 Discussion

3.5.1 Coral acclimation to stressful conditions

Associated microorganisms in the coral holobiont show the capacity to rapidly acclimate to environmental changes by modifying their population growth and physiological responses. During our incubations, the pigment concentrations in coral tissues showed a pattern of rapid acclimation to changing environment (table 3.1 and table 3.3). The proportion of Chl *a* in the healthy coral tissues was much higher than that in the bleached corals, and during our incubations, the proportion of carotenoids increased in the bleached corals with a concomitant reduction of the Chl *a*: carotenoid ratio. The photo-protective and antioxidant

functions of carotenoids, particularly their importance in the xanthophyll cycle and β carotene variations, are well-known (Ambarsari et al., 1997; Arsalane et al., 1994; Young and Frank, 1996). In our study, the decrease in the Chl *a*: carotenoid ratio during 12-h light incubation of bleached corals resulted from extensive decrease in Chl *a* relative to carotenoids, showing how zooxanthellae respond to stressful conditions.

3.5.2 Photosynthetic pigments of endolithic community

The endolithic community that lives under the coral tissue layer is adapted to photosynthesis in the dark environment shaded by the zooxanthellae living inside the coral tissues (Kühl et al., 2008). In the present study, *O. quekettii* largely dominated the endolithic photosynthetic community of *P. lutea*. Other studies have also found *Ostreobium* spp. as the dominant endolithic algae in living and dead corals (Jeffrey, 1968; Tribollet et al., 2006). In particular, *Ostreobium* spp. is known for its capacity to harvest light under low light intensity because of its exclusive photosynthetic pigment composition, which includes Chl *b* and far red-absorbing Chl *a* that act as antennae under lower illumination conditions (Halldal, 1968). The Ch *a*:Chl *b* ratio in plants is widely used as an indicator of tolerance in shaded areas (Maina and Wang, 2015). The normal shade-adapted ratio in higher plants is approximately 3, whereas highly shade-adapted *Ostreobium* spp. may reach a ratio of 1.26–2.8 (Jeffrey, 1968; Ralph et al., 2007). In this study, the Chl *a*: Chl *b* ratio of endoliths varied from 1.6 to 2.9 (healthy corals) and 1.9 to 3 (bleached corals) (table 3.3 and figure 3.4). Figure 3.4 shows that in the endoliths of healthy corals; after 12 h light and 24-h light plus dark incubations, the proportion of Chl *b* increased, and the Chl *a*: Chl *b* ratio decreased after 24 h. In contrast, Chl *b* decreased and Chl *a* increased during the 12 h light incubations in the endoliths of bleached corals, showing a rapid response to the better illumination condition under the bleached coral tissues. The increased proportion of Chl *a* in bleached condition resulted in a

higher Chl *a*: carotenoid ratio. However, after 24 h of incubation, the drastic drop in this ratio shows that the endolithic algae might be affected to some extent by oxidative stress after being exposed to higher illumination. This pattern can be also observed from the increase in allomer:total Chl *a* along the incubation, as this ratio can be used to estimate the oxidative state of Chl *a* (Dove et al., 2006). These rapid variations in the pigments of endolithic algae illustrate their plasticity to acclimate to drastic environmental changes. Moreover, our measurements of photosynthetic quantum yield (*Fv/Fm*) of endolithic algae living in healthy and bleached corals (figure 3.3) showed that Chl *a* fluorescence measurement of the endolithic community was similar in these two conditions. This result reveals comparable photosynthetic performance under both coral conditions, which is in agreement with previous observations (Fine et al., 2005). Nevertheless, a slight decrease in Chl *a* fluorescence measurement after 24 h of incubation showed that some stress under non-shading conditions might decrease the photosynthetic performance with time. This result is in agreement with the important decrease in Chl *a*: carotenoid ratio.

3.5.3 Carbon fixed by endoliths

The endolithic community has been proposed to assist in coral survival when the coral is bleached (Fine and Loya, 2002); however, there are no available data on the amount of organic matter (in terms of carbon and nitrogen) synthetized by the endolithic community. The most notable result in this study is the quantitative measurement (in terms of organic C and N) of primary production, nitrogen fixation, and translocation from the endolithic community. These measurements were obtained under both normal and bleached conditions. Our incubation design allowed us to measure the primary production of zooxanthellae in coral tissues (Addition incubation) and that in the endolithic community (Injection incubation). Moreover, we calculated the proportion of carbon photo-assimilates that the

endolithic community could transfer to the coral tissues under both healthy and bleached conditions. In the Addition incubation, ^{13}C was added to the water in incubation bottles containing coral pieces; therefore, uptake of ^{13}C by zooxanthellae and their primary production rates were well measured. However, ^{13}C did not reach the endolithic zone in a direct and timely manner, and therefore, the primary production of endoliths was highly underestimated. In the Injection incubation, ^{13}C solution was directly added inside the green band of the endoliths; therefore, primary production of the endolithic algae was clearly revealed (table 3.2) and the translocation could be estimated by following the increase of ^{13}C atoms (%) in coral tissues (figure 3.5). The difference in ^{13}C atom content (%) between the Injection and Addition incubations allowed us to calculate the proportion of translocated assimilates from endoliths.

Our incubations revealed that translocation of organic matter occurred in both healthy and bleached conditions. Moreover, our data show that translocation in the healthy condition was higher than that in the bleached condition; this pattern differs from the results of a previous study by Fine and Loya, 2002 (Fine and Loya, 2002). Even if the endolithic community also showed some stress signals under the bleached coral condition, the Chl *a* fluorescence data showed that the photosynthetic performance was comparable between the two conditions; therefore, the endolithic community was also able to support the coral host under stressful conditions.

Another important feature revealed by the Addition incubation is that endoliths in the healthy corals may fix carbon over a longer period owing to the increase in their primary production during the dark hours. This is due to their capacity to harvest light even under low illumination conditions (Kühl et al., 2008), and therefore might surpassing our incubation time set-up of 12 h light. Moreover, the primary production of endoliths in the

bleached corals was lower than that of endoliths in healthy corals. This pattern agrees with the lower Chl *a* concentration (electronic supplementary material, table S1) and can be interpreted as a high respiration rate of endoliths in the bleached corals owing to oxidative stress. This can be attributed to the effect of high illumination resulting in inhibition of the photosystem of the algae because they are more adapted to photosynthesize under low light conditions. The lower primary production of endoliths in bleached corals (0.11 µg C cm^{-2} day^{-1} under the bleached condition vs. 0.54 µg C cm^{-2} day^{-1} in the healthy condition) and the concomitant increase of primary production in coral tissues (0.64 to 2.51 µg C cm^{-2} day^{-1}) exemplify the translocation pattern from the endoliths toward the bleached coral tissues during the dark period. However, ^{13}C atom (%) incorporation showed that translocation in healthy corals was higher, meaning that corals in the healthy condition may have higher respiration (consumption) rates. Variations in the POC: PON ratio over the period of incubation (table 3.4) also showed that the ratio increased along the incubations in the coral tissues, revealing an increase in C-rich organic matter.

3.5.4 Nitrogen fixed by endoliths

N_2 fixation is an important function to take into account when considering the entire metabolism of the holobiont. Our measurements of N_2 fixation during the Addition incubation revealed that this process occurred in both the coral tissues and endolithic band. Cyanobacteria are known as major diazotrophs (nitrogen-fixing organisms) together with other bacteria such as the alpha-proteobacteria. In our study, the presence of zeaxanthin, a tracer pigment for cyanobacteria (Stivaletta et al., 2010), in the coral tissue and in the endolithic green band confirms the presence of cyanobacteria at both sites. Lesser et al. (Lesser et al., 2004) also described the presence of N_2 fixer unicellular cyanobacteria in the tissue of *Montastraea cavernosa*. In the endolithic band, we found the presence of the

cyanobacterium, *L. terebrans*. *Plectonema* spp. (synonym of *Leptolyngby*a) are known to fix nitrogen at low oxygen levels, and they are not able to fix it in the anoxic environment, similar to most diazotrophs (Berman-Frank et al., 2003). In previous genetic studies on microbes associated with *P. lutea* collected from the same sampling area, nitrogen-fixing cyanobacteria and alpha-proteobacteria were identified in both the coral tissue and coral skeleton (Kim, 2015).

Our measurements showed that N_2 fixation rates in the coral tissues were 4 times higher in the healthy corals than in the bleached corals. However, the N_2 fixation rates of the endoliths in the healthy and bleached corals were similar. Moreover, N_2 fixation was higher during the first 12 h of the illuminated period, except in the case of bleached corals in which N_2 fixation was increased, and this pattern was like that of primary production. This result suggests that some nitrogenous compounds can be also translocated from the endolithic layer toward the coral tissue.

3.6 Conclusions

Overall, based on the results of this study, we can conclude that the endolithic community plays a crucial role in supporting the coral holobiont under normal as well as stressful conditions. Rapid responses by changing their pigments to different illumination conditions and extended photosynthetic period are important acclimation features of the endolithic community to rapid environmental changes. Not only under stressful but also under normal conditions, photo-assimilates from the endoliths are translocated to the coral tissues together with some nitrogen-rich compounds. Our study is the first to provide quantification of the primary production, N_2 fixation, and translocation of organic matter from the endolithic community toward the coral tissues in both the normal and bleached coral scenarios. Further studies are necessary to understand how photo assimilates are transported during

translocation. Moreover, photo-protection by the production of antioxidants in the endolithic community needs to be investigated.

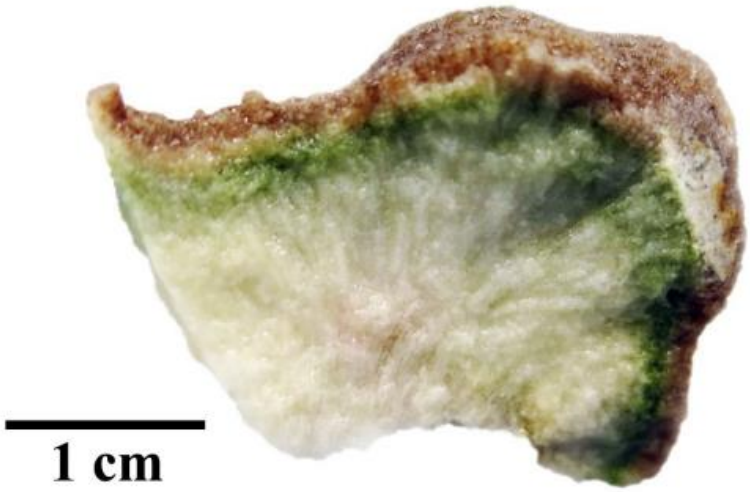


Figure 3.1 Distinct green layer formed by the endolithic community in the skeleton of *Porites lutea*.

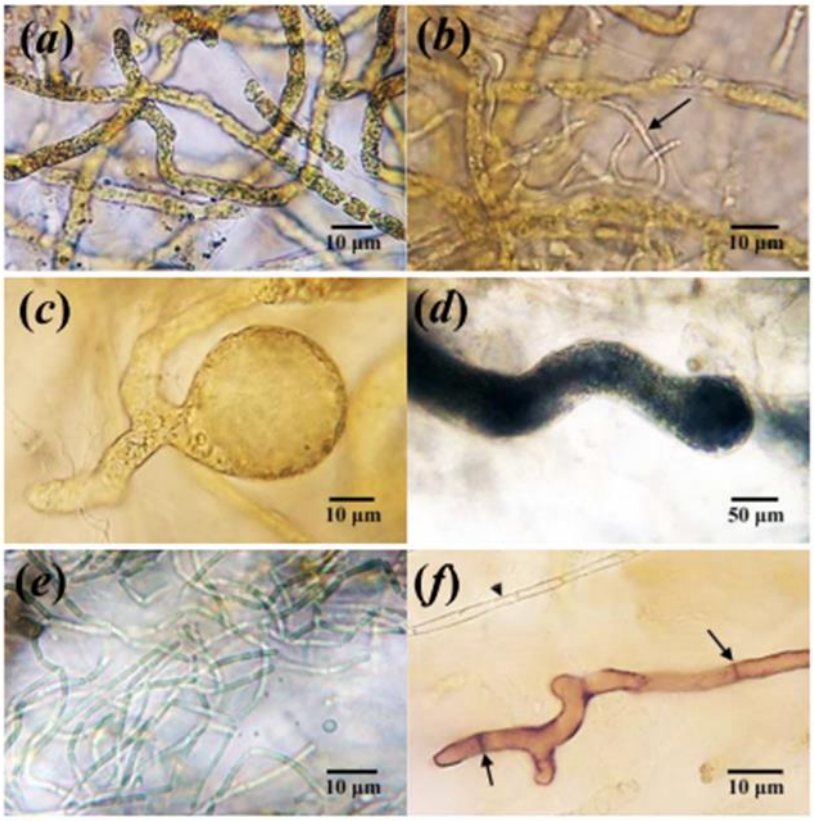
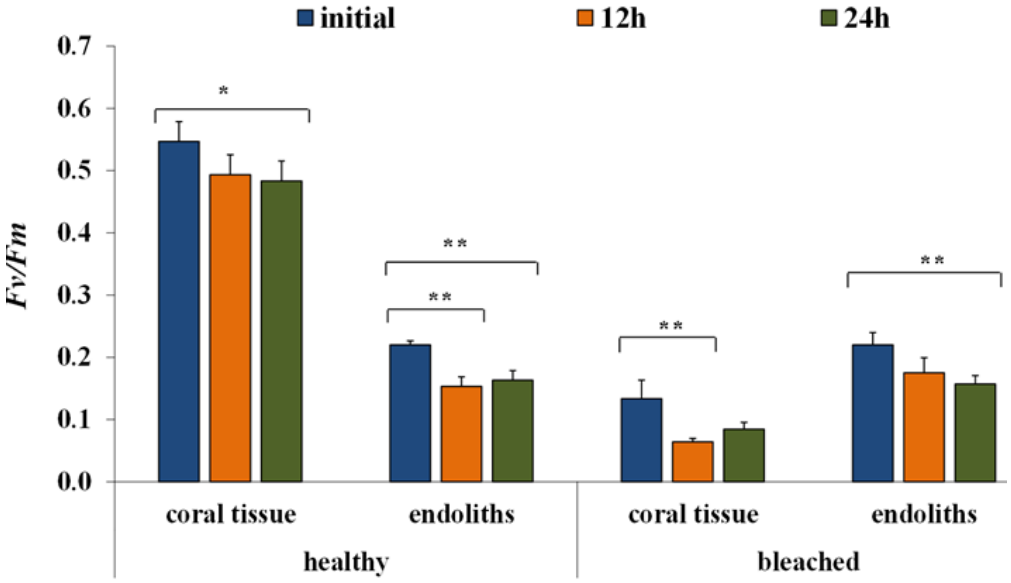


Figure 3.2 Light-microscope photographs of the endolithic community from the green layer in the skeleton of *Porites lutea*. *Ostreobium quekettii* ((*a*) main branches, (*b*) ultimate branches (arrow) and (c) swelling similar to sporangia), (*d*) holdfast of dasycladalean alga, (*e*) *Leptolyngbya terebrans*. (*f*) Fungi: hyaline hypha (head arrow), red hypha with septum (arrows).

Addition incubation



Injection incubation

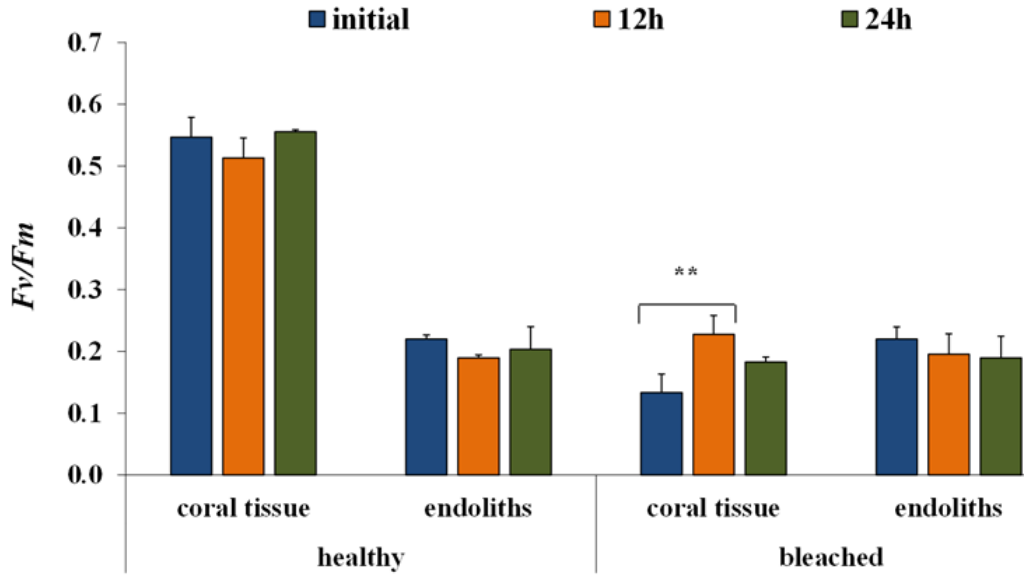


Figure 3.3 Maximum quantum yield (*Fv/Fm*, average ± standard error) of the coral tissues and endolithic algae in healthy and bleached corals (*n=3*) measured during Addition incubation (upper panel) and Injection incubation (lower panel). Three-way ANOVA and post hoc Turkey's test were used to determine significant differences among the conditions (healthy or bleached), layers, and times. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$.

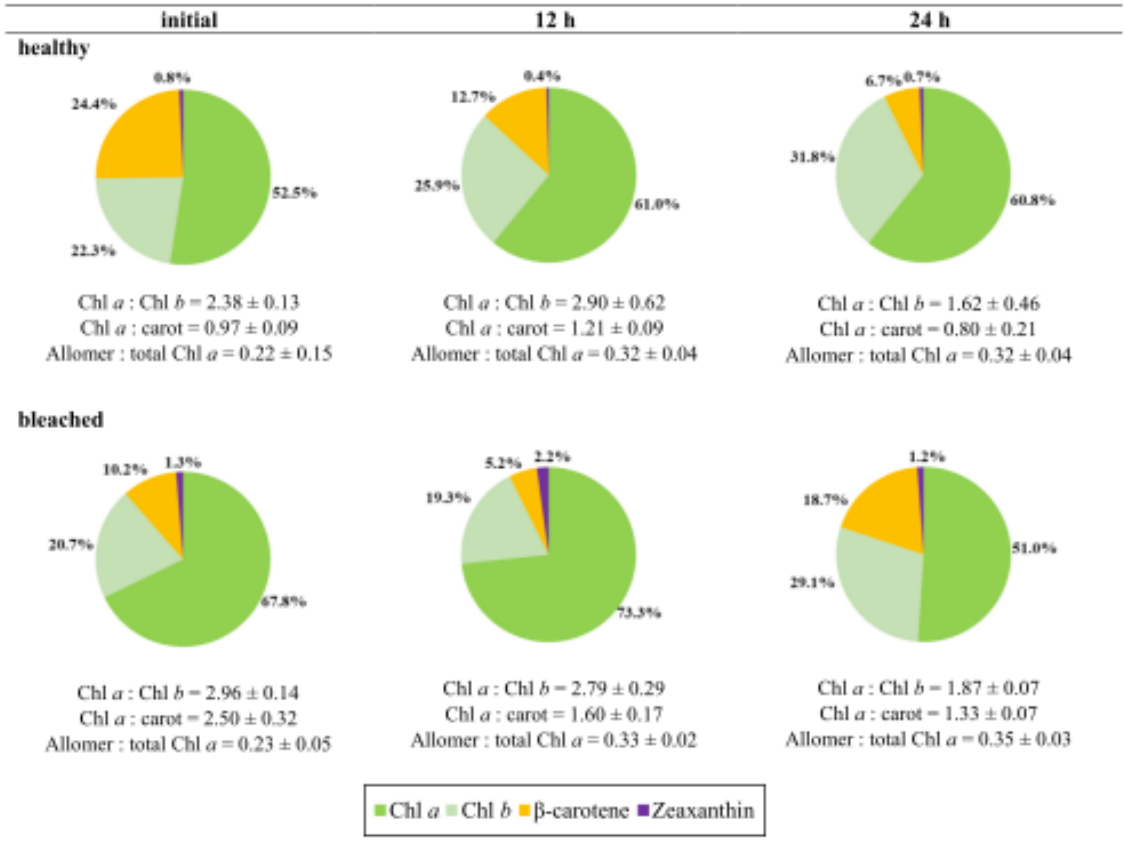


Figure 3.4 Percentage composition of chlorophylls and carotenoids in the endolithic algae of healthy and bleached corals at initial measurement, 12 h (light period), and 24 h (light and dark periods).

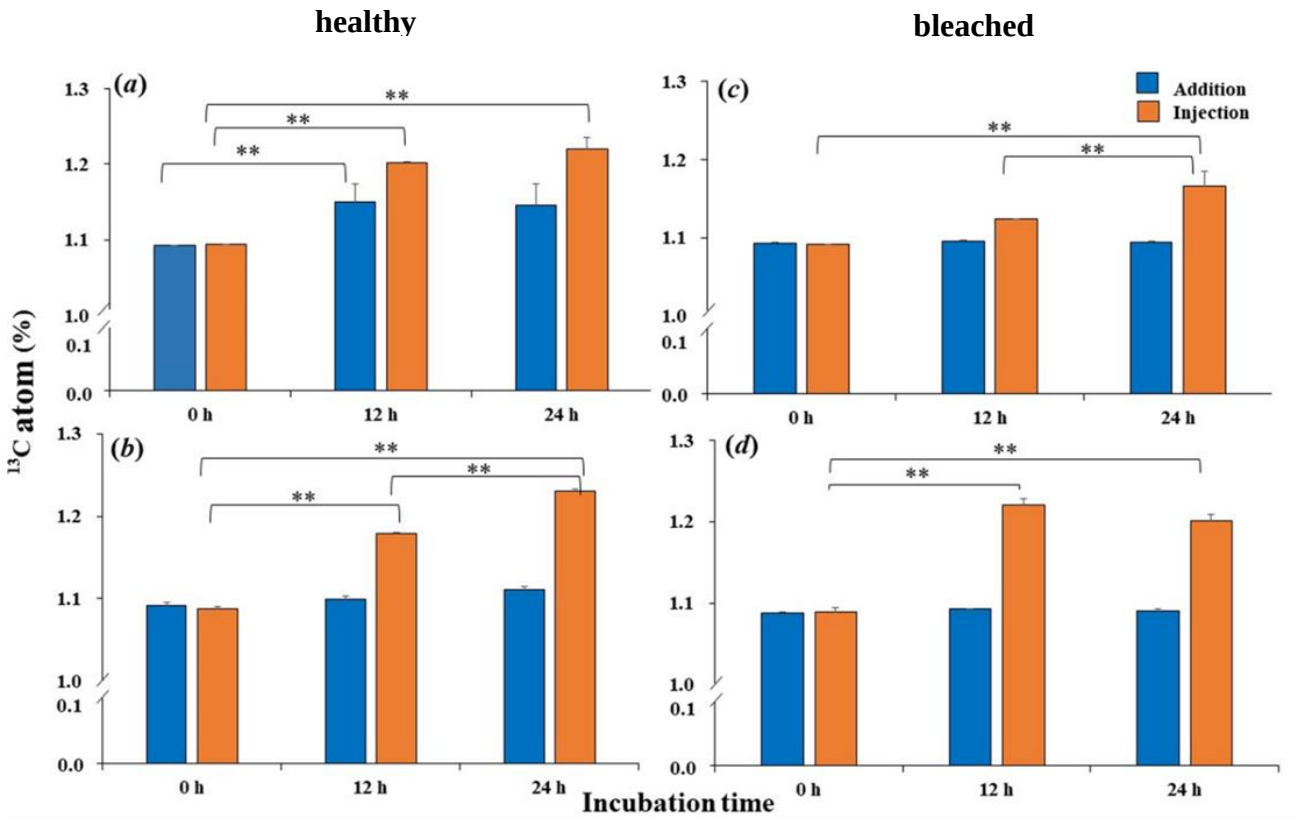


Figure 3.5 ^{13}C atom (%) recorded in *Porites lutea* (*n=3*) at initial measurement, 12 h (light period), and 24 h (light and dark periods). Left panel for healthy corals: (*a*) coral tissue and (*b*) endolithic algae. Right panel for bleached corals: (*c*) coral tissue and (*d*) endolithic algae. Two-way ANOVA and post hoc Turkey's test were used to determine significant differences among the different incubations and times. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Table 3.1 Pigment concentrations in the coral tissues and endolithic algae of *Porites lutea* before incubation, (μg cm^{-2}: mean ± standard error; *n=3*). One-way ANOVA and post hoc Turkey's test were used to determine significant differences between the different conditions (healthy or bleached). * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

pigments	healthy	bleached
coral tissue		
Chlorophyll *a*	13.59 ± 0.97	2.11 ± 0.25 **
Chlorophyll c_2	0.26 ± 0.05	0.38 ± 0.08
cPPB-*a*E	0.51 ± 0.13	0.75 ± 0.12
Peridinin	5.93 ± 0.39	2.18 ± 0.10 **
Diadinoxanthin	2.35 ± 0.04	1.86 ± 0.09 **
β-carotene	0.25 ± 0.04	0.25 ± 0.04
[1] Zeaxanthin	0.98 ± 0.24	1.08 ± 0.15
[1] Lutein	0.40 ± 0.15	0.74 ± 0.39
[2] Chlorophyll *a* : Carotenoids	1.61 ± 0.13	0.49 ± 0.06 **
Allomer : total Chl *a*	0.30 ± 0.06	0.35 ± 0.02
endolithic algae		
Chlorophyll *a* spp.	2.96 ± 0.13	2.27 ± 0.05
Chlorophyll *b* spp.	1.26 ± 0.04	0.69 ± 0.04
Zeaxanthin	0.04 ± 0.00	0.04 ± 0.00
β-carotene	1.38 ± 0.00	0.34 ± 0.02 **
Chlorophyll *a* : Chlorophyll *b*	2.38 ± 0.13	2.96 ± 0.14
Chlorophyll *a* : Carotenoids	0.97 ± 0.09	2.50 ± 0.32 **
Allomer : total Chl *a*	0.22 ± 0.15	0.23 ± 0.05

Unit: μg cm^{-2}

1 Pigment of associated algae (other than zooxanthellae) with coral tissue

2 Carotenoids found in zooxanthellae

Table 3.2 Primary production and N_2 fixation in the coral tissues and endolithic algae of healthy and bleached corals (*n = 3*) after 12 h (light period) and 24 h (light and dark periods) measured during Addition and Injection incubations.

Incubation	**condition**	**Layer**	**primary production (µg C cm^{-2} $time^{-1}$)**		**N_2 fixation (ng N cm^{-2} $time^{-1}$)**	
			12 h	**24 h**	**12 h**	**24 h**
Addition	healthy	tissue	39.88±0.6	35.23±2.2	29.61±9.3	13.41±3.1
		endoliths	0.23±0.0	0.54±0.0	2.12±0.1	1.77±0.5
	bleached	tissue	0.64±0.0	2.51±0.0	7.66±0.7	12.38±2.0
		endoliths	0.16±0.0	0.11±0.0	1.59±0.2	1.32±0.1
Injection	healthy	tissue	69.67±4.6	65.79±0.3		
		endoliths	2.18±0.7	6.24±0.7		
	bleached	tissue	8.73±0.0	22.94±1.3		
		endoliths	3.45±0.0	3.16±0.1		

Table 3.3 Pigment concentration (µg cm $^{-2}$) in the coral tissues and in the endolithic algae measured during incubations at initial, 12 h (light period), and 24 h (light and dark periods).

Pigment	healthy			bleached		
	initial	**12 h**	**24 h**	**initial**	**12 h**	**24 h**
coral tissue						
Chlorophyll *a*	13.59 ± 0.97	11.01 ± 0.24	13.47 ± 0.24	2.11 ± 0.25	0.55 ± 0.14	0.87 ± 0.05
Chlorophyll c_2	0.26 ± 0.05	0.15 ± 0.01	0.14 ± 0.01	0.38 ± 0.08	0.08 ± 0.02	0.07 ± 0.00
cPPB-*a*E	0.51 ± 0.13	0.67 ± 0.18	0.44 ± 0.10	0.75 ± 0.12	1.17 ± 0.24	0.66 ± 0.02
Peridinin	5.93 ± 0.39	3.85 ± 0.09	5.34 ± 0.45	2.18 ± 0.10	0.77 ± 0.06	1.02 ± 0.06
Diadinoxanthin	2.35 ± 0.04	1.11 ± 0.13	0.65 ± 0.03	1.86 ± 0.09	0.55 ± 0.02	0.57 ± 0.03
β-carotene	0.25 ± 0.04	0.19 ± 0.04	0.34 ± 0.07	0.25 ± 0.04	0.15 ± 0.06	0.28 ± 0.07
[1] Zeaxanthin	0.98 ± 0.24	0.96 ± 0.20	0.58 ± 0.14	1.08 ± 0.15	0.14 ± 0.06	0.30 ± 0.12
[1] Lutein	0.40 ± 0.15	0.58 ± 0.27	0.28 ± 0.11	0.74 ± 0.39	0.06 ± 0.02	0.23 ± 0.11
[2] Chlorophyll *a*: diatoxanthin	4.44 ± 1.04	1.75 ± 0.45	1.85 ± 0.79	2.60 ± 0.68	1.11 ± 0.18	1.18 ± 0.49
[3] Chlorophyll *a*: Carotenoids	1.61 ± 0.13	2.15 ± 0.08	2.19 ± 0.17	0.49 ± 0.06	0.36 ± 0.09	0.46 ± 0.02
Allomer: total Chl *a*	0.30 ± 0.06	0.42 ± 0.07	0.36 ± 0.10	0.35 ± 0.02	0.53 ± 0.08	0.43 ± 0.04
endolithic algae						
Chlorophyll *a* spp.	2.96 ± 0.13	2.73 ± 0.16	3.04 ± 0.05	2.27 ± 0.05	1.12 ± 0.23	1.62 ± 0.04
Chlorophyll *b* spp.	1.26 ± 0.04	1.16 ± 0.20	1.59 ± 0.18	0.69 ± 0.04	0.30 ± 0.06	0.92 ± 0.04
Zeaxanthin	0.04 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.01	0.04 ± 0.00
β-carotene	1.38 ± 0.00	0.57 ± 0.18	0.33 ± 0.22	0.34 ± 0.02	0.08 ± 0.03	0.63 ± 0.06
[2] Chlorophyll *a*: Siphonein	4.39 ± 1.02	4.68 ± 1.52	7.65 ± 2.62	4.26 ± 1.05	7.20 ± 2.71	3.00 ± 0.37
[2] Chlorophyll *a*: Neoxanthin	8.34 ± 3.57	12.27 ± 2.25	7.90 ± 4.80	14.64 ± 2.62	9.77 ± 3.33	7.94 ± 2.03
Chlorophyll *a*: Chlorophyll *b*	2.38 ± 0.13	2.90 ± 0.62	1.62 ± 0.46	2.96 ± 0.14	2.79 ± 0.29	1.87 ± 0.07
Chlorophyll *a*: Carotenoids	0.97 ± 0.09	1.21 ± 0.09	0.80 ± 0.21	2.50 ± 0.32	1.60 ± 0.17	1.33 ± 0.07
Allomer: total Chl *a*	0.22 ± 0.15	0.33 ± 0.04	0.35 ± 0.03	0.23 ± 0.05	0.33 ± 0.02	0.35 ± 0.03

Unit: µg cm^{-2}

1 Pigment of associated algae (other than zooxanthellae) with coral tissue

2 Ratio calculated from peak area of HPLC elution profiles

3 Carotenoids found in zooxanthellae

Table 3.4 POC and PON (mg cm $^{-2}$ ± standard error) in the healthy and bleached *Porites lutea* (coral tissues and endolithic community) during the two incubations at initial, 12 h (light period), and 24 h (light and dark periods).

Incubation	Condition	Layer	POC			PON			POC: PON		
			initial	12 h	24 h	initial	12 h	24 h	initial	12 h	24 h
Addition	healthy	tissue	5.81±0.85	8.13±6.84	8.20±3.29	0.71±0.07	0.81±0.41	0.62±0.05	8.11±0.43	9.07±1.74	13.01±2.77
		endoliths	0.80±0.28	0.46±0.05	0.35±0.11	0.06±0.00	0.03±0.00	0.03±0.01	13.87±3.99	13.93±1.30	10.39±0.90
	bleached	tissue	4.84±0.55	2.26±0.06	3.66±0.54	0.49±0.00	0.25±0.01	0.37±0.09	9.79±1.17	9.16±0.30	10.11±0.87
		endoliths	0.37±0.07	0.41±0.11	0.32±0.01	0.05±0.01	0.04±0.01	0.03±0.01	7.24±0.42	10.96±0.60	9.70±1.60
Injection	healthy	tissue	5.30±0.06	6.61±0.21	4.56±1.31	0.76±0.01	0.89±0.08	0.58±0.14	6.98±0.02	7.46±0.51	7.81±0.38
		endoliths	1.01±0.04	0.27±0.11	0.39±0.02	0.14±0.01	0.03±0.01	0.05±0.01	7.19±0.16	8.60±0.66	8.51±0.32
	bleached	tissue	4.72±0.10	2.97±0.63	4.63±1.72	0.71±0.02	0.24±0.02	0.53±0.16	6.68±0.06	12.15±1.16	8.55±0.43
		endoliths	0.59±0.00	0.25±0.03	0.29±0.01	0.01±0.01	0.03±0.01	0.04±0.01	7.40±0.49	7.54±0.36	7.33±0.36

Table 3.5 ^{13}C atom (%) recorded in healthy and bleached *Porites lutea* (coral tissue and endolithic community) during two incubations at initial, 12 h (light period), and 24 h (light and dark periods)

incubation	**condition**	**replicates**	**^{13}C atom (%)**		
			initial	**12 h**	**24 h**
Addition	healthy	tissue 1	1.0921	1.1319	1.1656
		tissue 2	1.0918	1.1668	1.1262
		tissue 3	1.0916	1.1556	1.1317
		endoliths 1	1.0898	1.1015	1.1082
		endoliths 2	1.0943	1.0966	1.1138
		endoliths 3	1.0928	1.0899	1.0939
	bleached	tissue 1	1.0939	1.0959	1.0951
		tissue 2	1.0921	1.0944	1.0939
		tissue 3	1.0943	1.0971	1.1186
		endoliths 1	1.0871	1.0930	1.0921
		endoliths 2	1.0884	1.0916	1.0896
		endoliths 3	1.0871	1.0894	1.0836
Injection	healthy	tissue 1	1.0945	1.2188	1.2311
		tissue 2	1.0945	1.1841	1.2091
		tissue 3	1.0898	1.1644	1.2454
		endoliths 1	1.0895	1.1821	1.2291
		endoliths 2	1.0867	1.1770	1.2321
		endoliths 3	1.0921	1.1422	1.2696
	bleached	tissue 1	1.0917	1.1231	1.1535
		tissue 2	1.0919	1.1241	1.1812
		tissue 3	1.0881	1.0937	1.1636
		endoliths 1	1.0867	1.2262	1.1959
		endoliths 2	1.0924	1.2138	1.1969
		endoliths 3	1.0884	1.2025	1.1822

Chapter 4

Response to oxidative stress: H_2O_2 scavenging activity in coral tissue and endolithic community under healthy and bleached conditions of *Porites lutea*

4.1 Abstract

This chapter was dedicated to exploring the responses of coral host and endolithic community to oxidative stress, H_2O_2 scavenging activity was measured in the coral tissue and the endolithic community under healthy and bleached *P. lutea*. Coral nubbins were incubated under two conditions of temperature (27 °C as normal temperature or 34 °C), two conditions of illumination (360 as normal and 680 µmol photons m^{-2} s^{-1}), and the combination of them during 6h. *Fv/Fm* of healthy coral tissue decreased from initial after exposure to light and high (34 °C) temperature stresses. However, the *Fv/Fm* in bleached coral tissue increased along or remained the same in all treatments. Endolithic algae showed the same pattern in both coral conditions with a decrease in *Fv/Fm* under high irradiance and the combined treatment. Corals showed a significant increase in H_2O_2 scavenging activity during light and thermal stresses, however scavenging activity was low under combined stresses. On the other hand, endolithic algae showed increase in scavenging activity at all treatments. These results show the capacity of rapid response of endolithic algae to environmental stresses, however in coral tissues, the stress levels in combined treatment overpassed the coral capacity to fight against oxidative stress.

Keywords: *Porites lutea*, bleached coral, oxidative stress, total protein concentration

4.2 Introduction

Environmental and anthropogenic stresses are the main cause of coral bleaching by promoting the disruption of the symbiotic relationship. Many studies had tried to elucidate the mechanism by which high illumination and elevated temperature stresses affect the coral physiology compared to other causative factors of bleaching. Oxidative stress is one of the most important trigers of bleaching. Elevated temperature and high illumination may promote the formation of reactive oxygen species (ROS) in both, coral host and the zooxanthellae (Lesser, 2006; Lesser and Farrell, 2004). Damage of cell membranes and DNA molecule due to the formation of ROS is the cause of zooxanthellae looses resulting in bleaching. Hydrogen peroxide (H_2O_2) belong to a group of ROS, it is the most stable of the reactive oxygen species and easily diffuses across biological membranes. Typically,the host and their organisms possess defense mechanisms to prevent oxidative stress by the utilization of various antioxidant enzymes: superoxide dismutase (SOD), catalase and glutathione peroxidase and non-enzymatic mechanism: ascorbic acid, glutathione, α-tocopheral (vitamin E), carotenoids and mycosporine-like amino acids (MAAs) (Lesser, 2006). Previous studies found that the endolithic community provides photo-protection for the coral host (Schlichter et al., 1997; S Yamazaki et al., 2008), particularly during bleaching events. However, the formation of H_2O_2 and scavenging of H_2O_2 in endolithic community were almost not reported until now. Thus, this chapter was dedicated for investigating the responses of coral tissue and endolithic community under health and bleached conditions affected by strong solar irradiance and high temperature. Photosynthetic performance, and H_2O_2 scavenging were measured before and after incubations under stressful conditions.

4.3 Materials and Methods

4.3.1 Coral sampling

Fragments of healthy colonies of *Porites lutea* were collected with permission from Okinawa Prefectural Government, No. 28 – 6 during May 2017. Sampling was done during low tide (depth 0.5 - 1 m). Coral samples were transported to the laboratory and kept in an aquarium with running natural seawater. Multi-sensor Hydrolab MS5 Multi-parameter Sonde (OTT Hydromet, Kempten, 2, Germany) were used to measurement the environmental conditions at the time of sampling. The temperature and salinity of seawater were 27.5 °C and 34 psu, respectively.

4.3.2 Incubation design

Coral colonies were divided into two parts: healthy coral fragments were acclimatized for three days under the ambient conditions with running seawater and the remaining coral fragments were induced to bleach at 33 ºC of seawater temperature. Four sets of condition were selected: high temperature and light intensity (34 ºC and 680 µmol photons m^{-2} s^{-1}; combined treatment), ambient temperature and high light intensity (27 ºC and 680 µmol photons m^{-2} s^{-1}; light treatment), high temperature and ambient light intensity (34 ºC and 360 µmol photons m^{-2} s^{-1}; thermal treatment) and ambient temperature and light intensity (27 ºC and 360 µmol photons m^{-2} s^{-1}; normal condition) as a control. Short-term incubation experiments were performed in which corals were exposed to experimental conditions for 6.

4.3.3 The maximum quantum yield of photosystem II (*Fv/Fm*)

Chlorophyll *a* fluorescence was measured at the start and after 6 h stress period using a pulse-amplitude-modulated (PAM) fluorometer (JUNIOR PAM, Walz, Germany) according to the

method of Schreiber et al. (1998). The maximum quantum yield was calculated as *Fv/Fm*, where *Fv = Fm – Fo*. *Fo* is the initial fluorescence after dark adaptation and *Fm* is the maximum fluorescence after dark adaptation (Krause and Weis, 1991). Coral samples were kept for 15–30 min in the dark condition for adaptation before the fluorescence measurements were made. The fluorescence data were measured from 3 to 6 different points in coral tissues and endolithic algae, from which averages and standard errors were calculated.

4.3.4 Measurement of H_2O_2 and total protein concentration

At the end of each experiment a set of corals samples were prepared for analysis of H_2O_2 scavenging activity and total protein concentration. Coral tissues and endolithic algae were removed from the surface using whetstone drilling. Extracted materials from coral pieces were dissolved in 1ml of 50 mM Tris-HCl buffer, pH 7.5. Samples solution were kept in 1.5 ml microtubes and centrifuged at 10,000 g for 3 min. The supernatant was snap frozen in liquid nitrogen and preserved at -20 °C until analysis. Process measurement was done following Palmer et al., (2009). 40 µl of supernatant was placed into 96 well microtiter plate and absorbance was detected every 81s for 30 mins. The H_2O_2 standard curve of serial dilution (0, 6.25, 12.5, 25 and 50 mM) was used to calculate H_2O_2 concentration, which was normalized to protein concentration units.

For the total protein concentration measurements, triplicate 25 µl aliquots of supernatant were placed into 96 well microtiter plate. The samples were quantified using the Pierce™ BCA Protein Assay kit (Thermo scientific, Rockford, USA). The absorbance was measured at 562 nm using Multi-Detection Microplate Reader (Bio-tek Synergy™ HT, USA)

4.3.5 Statistical analysis

ANOVA test was performed to determine the differences among experiment conditions at different times. Post hoc Tukey test was used to assess pairwise differences when ANOVA revealed statistically significant effects. The software MINITAB ver. 14 was used for all statistical analysis.

4.4 Results

4.4.1 The maximum quantum yield of photosystem II (*Fv/Fm*)

The maximum quantum yield of photosystem II (*Fv/Fm*) of the healthy coral tissue significantly decreased ($P < 0.01$) from initial values for temperature and light stresses but it did not change in case of combined stresses (figure 4.1). Whereas the *Fv/Fm* values in bleached coral tissue increased and were statistically significant ($P < 0.01$) for light and thermal stresses. Endolithic algae showed the same pattern in both coral conditions (healthy and bleached) where the *Fv/Fm* values significantly increased ($P < 0.01$) from initial values during normal and thermal stress, and decreased (but not statistically significant) in high illumination and combined treatments.

4.4.2 H_2O_2 scavenging activity

Increasing of H_2O_2 scavenging activity in coral tissue was observed under high light intensity and high temperature stress treatments at both, healthy and bleached conditions with higher values when the coral is healthy (figure 4.3). However, corals (healthy and bleached) under the combined stresses of high temperature and high illumination did not show increase in their H_2O_2 scavenging activity. In the endolithic community, statistically significantly

increased of H_2O_2 scavenging activity was observed at high temperature, high illumination and the combination of these stresses ($P < 0.01$) (figure 4.4).

4.5 Discussion

The results from short-term incubation (6 h stresses) under different temperatures and light intensities revealed that the maximum quantum yield of photosystem II (*Fv/Fm*) in tissue of healthy corals were decreased from initial value during all stress conditions (figure 4.1 and figure 4.2). However, the photosynthetic performance of bleached corals was not affected and slightly increased in all treatments. On the other hand, *Fv/Fm* of endolithic community decreased under high illumination and combined stresses. This pattern shows that endolithic community are more sensitive to higher illumination, especially when the seawater temperature is higher than normal.

H_2O_2 scavenging activity is an important parameter to evaluate the stress level in corals and their associated microbes. Coral tissues showed higher H_2O_2 scavenging activity under high illumination ($P < 0.01$) followed by thermal stress ($P < 0.01$), however the combination of these two stresses showed no significant responses. This may indicate that the stress levels overpassed the coral capacity to fight against oxidative stress. In all cases healthy corals showed higher capacity to fight oxidative stress, however bleached corals may be supported by the endolithic community, judging from the photosynthetic performance results. The endolithic community showed higher H_2O_2 scavenging activity under light stress followed by thermal stress and their combination. This shows the capacity of endoliths to fight against stresses. Increasing of H_2O_2 scavenging in endolithic algae were found under high

temperature followed by high light intensity and combined treatments with concomitant decreased of *Fv/Fm*. The highest scavenging was found in healthy coral. This is agreement with previous study (Fine et al., 2005) who showed that thermal stress of light stress alone cannot affect the photosynthetic performance of endoliths, but it was affected by their combination. These results show that high irradiance might affect the photosystem of endolithic algae rather than high temperature stress, but their recovery capacity suggests that they may provide photo protection for corals under high-light stress (Yamazaki et al., 2008). Previous studies (Halldal, 1968) showed that the endolithic algae can adapt to wide changes in irradiance, from shading to strong light levels. This ability of endolithic algae to acclimatize under wide light intensity variations shows the domination of endolithic algal blooms after mass coral bleaching (Fine et al., 2004; Fine and Loya, 2002). Increasing in the biomass of endolithic algae may result in an increased production of photo-assimilates, which are taken up by the coral tissue (Fine and Loya 2002; Sangsawang et al. 2017).

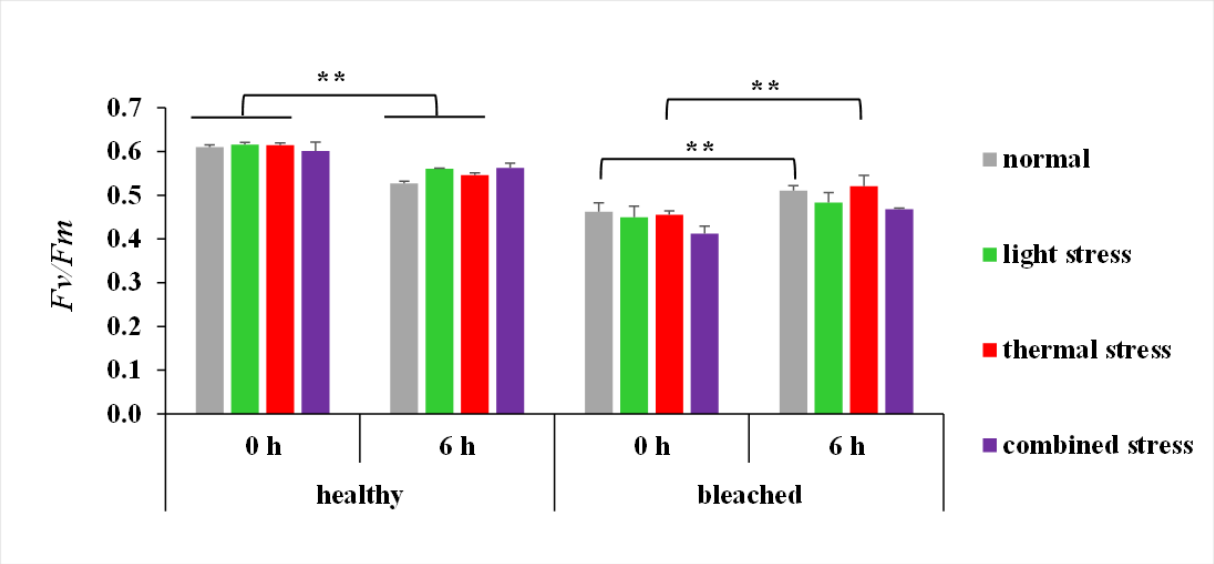


Figure 4.1 Maximum quantum yield of photosystem II (*Fv/Fm*) of coral tissue under healthy and bleached conditions at difference light and temperature.

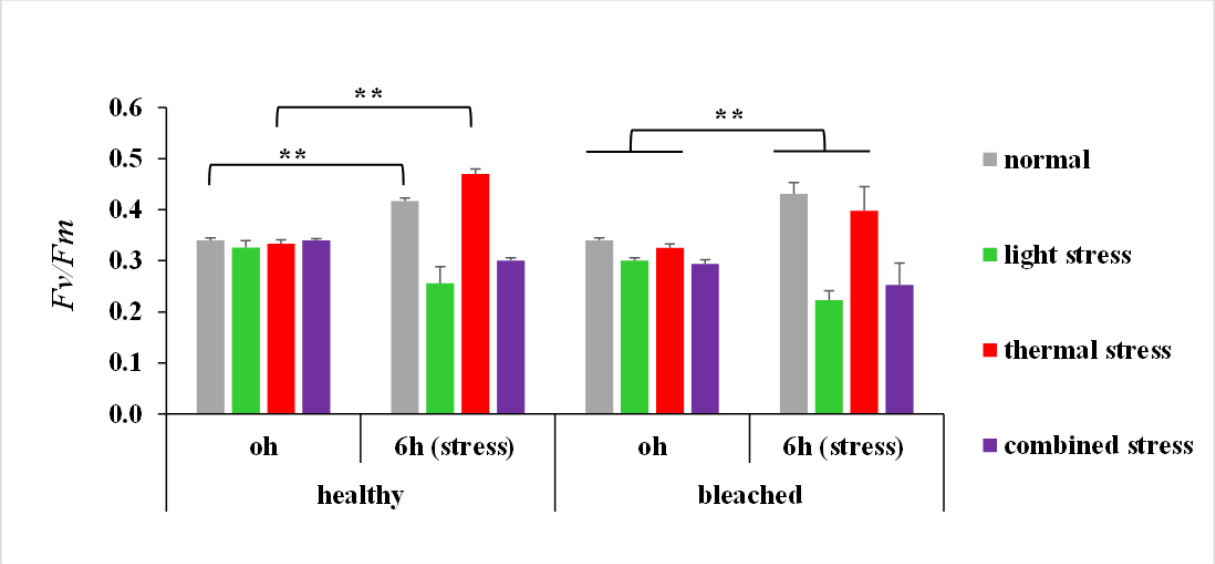


Figure 4.2 Maximum quantum yield of photosystem II (*Fv/Fm*) (mean ± standard error) of endolithic community under healthy and bleached conditions at difference light and temperature.

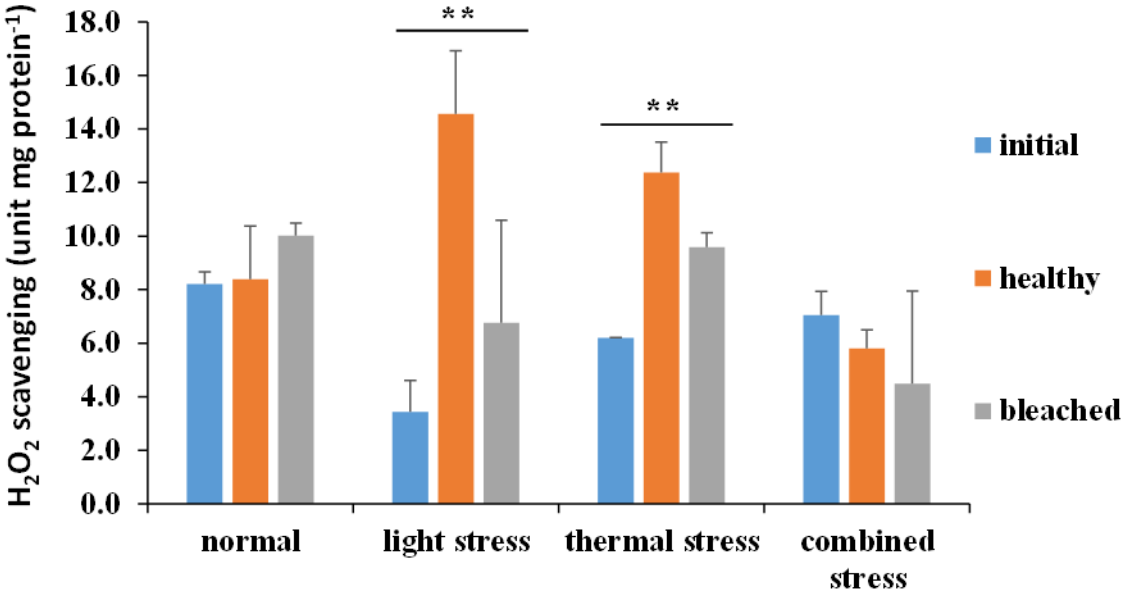


Figure 4.3 H_2O_2 scavenging activity (unit mg protein^{-1} ± standard error) in coral tissue under healthy and bleached conditions at difference stress (light, thermal and combined stress).

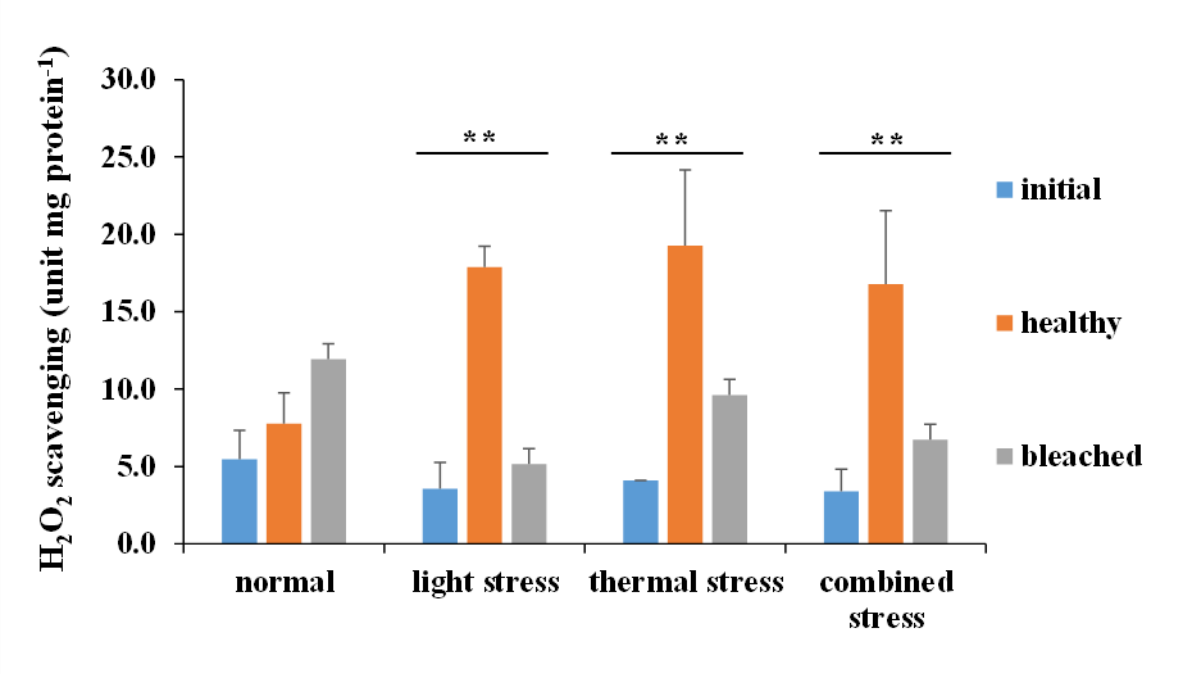


Figure 4.4 H_2O_2 scavenging activity (unit mg protein^{-1} ± standard error) in endolithic community under healthy and bleached conditions at difference stress (light, thermal and combined stress) compare to initial samples.

Chapter 5

General discussion

5.1 *Porites lutea* and endolithic community

In 1998, 2001 and 2007, corals living in shallow waters around Okinawa Island, Japan were damaged by bleaching. However, some coral species were found to be more tolerant to thermal stress and could recover after those bleaching events. The massive coral *P. lutea* is one of the dominant coral species commonly found in shallow intertidal pool of Sesoko, Okinawa (Cabaitan et al., 2012; Woesik et al., 2004). *P. lutea* is a tolerant coral species that can withstand even under stress of environmental changes (Loya et al., 2001). Besides the main energy from zooxanthellae, endolithic community may also provide the organic matter (Fine and Loya, 2002) and photoprotection (Yamazaki et al.,2008) to coral host.

In this study, the chlorophyte algae: *Ostreobium quekettii* and the cyanophyte: *Leptolyngbya terebrans* were found to dominate the endolithic community of *P. lutea*. *O. quekettii* is known for its capacity to harvest light under low light intensity (Kühl et al., 2008) because of its exclusive photosynthetic pigment composition as Chl *b* and far red absorbing Chl *a* antenna pigment (Halldal, 1968). Previous studies found that endolithic community can absorb red and far-red wavelengths, which reach levels of >2% of incident irradiance in contrast to <0.1% of PAR, as those wavelengths are not used by the zooxanthellae (Fork and Larkum, 1989; Koehne et al., 1999; Magnusson et al., 2007). Moreover, *L. terebrans* is known to fix nitrogen at low oxygen levels and not in anoxic

environment as most of other diazotrophs. These characteristics exemplify the high acclimation capacity of endolithic community for living in a shaded environment (under the coral tissue) and obtaining supplying their nitrogen requirements by their N_2 fixation capacity. Moreover, these capacities features are most appropriate to have an extended photo-productive period (longer than the 12 h that were set up in the present experiments) and fixing N_2 even during the illuminated hours.

The current study is the first to measure primary production and N_2 fixation rates in the endolithic community. Moreover, it provided a quantification of the translocated organic matter to the coral host. The study also showed that translocation is possible at both, normal as well as stressful (bleaching) conditions. Moreover, this study revealed the capacity of endoliths to be tolerant under high temperature stress and acclimatization under high illumination stress. H_2O_2 scavenging activity was also revealed and this showed the capacity of endoliths to provide photo-protection to the coral host. Based on the results of this study, the endolithic community plays a crucial role in supporting the coral holobiont under normal as well as stressful conditions.

5.2 Future research

The present study greatly provides understanding in the quantitative primary production and nitrogen fixation of the endolithic community and translocation of photo assimilates and nitrogen reach compound to coral host. This may demonstrates that the endolithic community forms a mutual metabolic network with the zooxanthellae and may explain why coral with high density of endolithic as the massive coral *Porites lutea* can be tolerance stress condition.

However, there are some hypotheses of the function of endolithic that required more research. Regarding the photo-protection by the production of antioxidants as mycosporine-like amino acids (MAAs) in the endolithic community needs further investigation.

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Appendices

A-I: Study area at Sesoko beach, Okinawa, Japan

Environment around Sesoko beach, during low tide

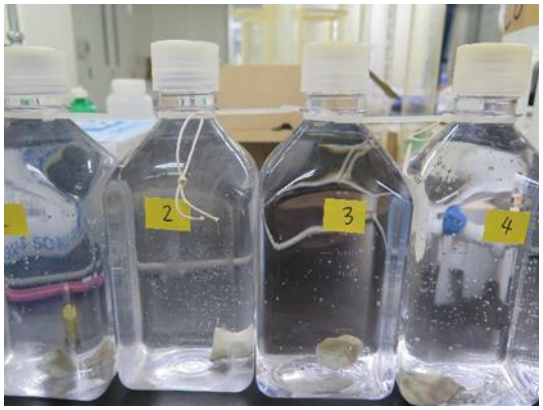
Exposure of *Porites lutea*

Porites lutea

Endolithic algae underneath coral tissue

A-II: Incubation set-up for chapter 2 and 3

Prepared samples for in sit u incubation



Incubation bottles with coral sample after added ^{13}C and ^{15}N

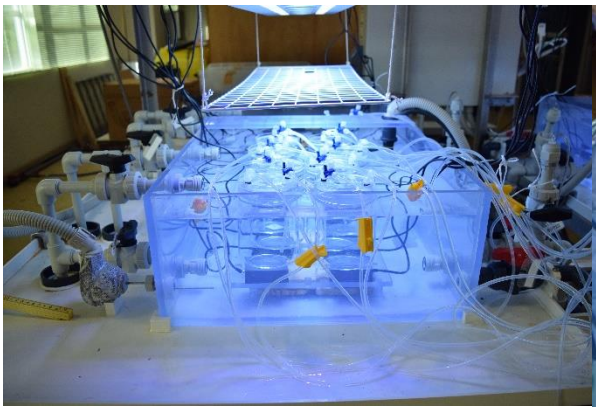
Bring to incubation place

During incubation

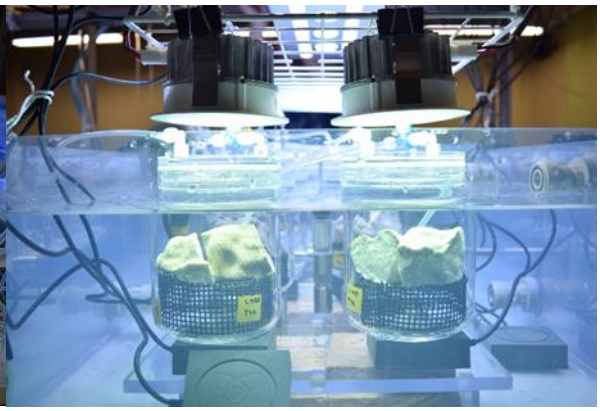
A-III: Incubation system for chapter 4

Healthy *P. lutea*

Bleached *P. lutea*



Incubation system



Light exposure period