OCEAN ACIDIFICATION IN CORAL REEFS : THE ROLE OF MICROBIAL COMMUNITY TO THE DISSOLUTION OF CALCIUM CARBONATE UNDER ELEVATED pCO2

THESIS

OCEAN ACIDIFICATION IN CORAL REEFS

THE ROLE OF MICROBIAL COMMUNITY TO THE DISSOLUTION OF CALCIUM CARBONATE UNDER ELEVATED pCO_2

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静岡大学　博士論文

OCEAN ACIDIFICATION IN CORAL REEFS

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サンゴ礁の海洋酸性化

高濃度二酸化炭素下における炭酸カルシウムの溶解に関する微生物活動の役割

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環境・エネルギーシステム専攻

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Md. Nazrul ISLAM

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List of Abbreviations

A_T	Total Alkalinity
BCDP	Bio-Chemical Dissolution Processes
DAPI	4',6-diamidino-2-phenylindole
DO	Dissolved Oxygen
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EPOCA	The European Project on OCean Acidification
GPP	Gross Primary Production
h	Height (length)
h	Hour
IPCC	Intergovernmental Panel on Climate Change
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology
NPP	Net Primary Production
NR	Natural Rubbles (with associated epilithic and endolithic communities)
NR+G	Natural Rubbles with addition of Glucose
NR+M	Natural Rubbles with addition of coral Mucus
POM	Particulate Organic Matter
r	Correlation coefficient
r	Radius
Rd	Dark respiration
SD	Standard Deviation
TOC	Total Organic Carbon
TR	Treated Rubble (keeping only their endolithic communities)
t-Test	Student's paired *t*-Test
UV	Ultra Violet
Vs.	Versus
WCSk	White Coral Skeleton
ΔCO_2	Net respiration [CO_2 final – CO_2 initial]
Ω	Solubility/Saturation state
Ω_{arg}	Saturation state (aragonite)
Ω_{cal}	Saturation state (calcite)

General Abstract

Ocean acidification has emerged as one of the biggest threats to coral reefs across the world. The effects of ocean acidification on calcifying organisms which are very abundant in coral reef are poorly understood. To determine the effects of ocean acidification on the dissolution of calcium carbonate, coral rubble with associated microbial community were incubated under natural and elevated pCO_2. The main objective of these incubations was to understand the role of associated microbial community for the dissolution of calcium carbonate in coral rubble under ocean acidification scenario. Incubations were conducted at Sesoko reef, Okinawa, Japan in May and September of 2011 and 2012. Short incubations (24 hours under natural illumination) and long incubations (4 days under natural illumination and dark condition) were carried out using white coral skeleton (control), natural rubble (with associated epilithic and endolithic communities), treated rubble (with removed epilithic community) and natural rubble with addition of organic matter (glucose or coral mucus) under different levels of pCO_2 (ambient, 520, 720 and 1120 ppm).

During short experiment under natural illumination, net primary production, net respiration and dissolution rates of treated coral rubble (only endolithic communities) were slightly lower than the natural rubble, indicating that the metabolism of endolithic community was much higher when compared to the epilithic community at all pCO_2 levels. In short incubation, treated rubble (with only endolithic communities) dissolution rates varied from 85-91%. In long experiment under natural illumination, carbonate dissolution rates varied from 23.7 ± 0.1 (ambient) to 50.5 ± 1.5 (1120 ppm) µmol $m^{-2}d^{-1}$ at night time and 3.2 ± 1.1 (ambient) to 5.8 ± 1.2 (1120 ppm) µmol $m^{-2}d^{-1}$ at day time. However, in long experiment under complete dark condition when photosynthesis was inhibited, carbonate dissolution increased to 81.6 ± 0.2 µmol $m^{-2}d^{-1}$ at high pCO_2. When compared natural rubble with white coral skeleton (control), around 80% of carbonate dissolution was due to the contribution of biological processes by the respiration of associated microbial communities. Conversely, very small amount of dissolution was observed from the control even

under high pCO_2 condition. These suggest that biological processes and the time scale play a significant role in determining calcification and dissolution. Dissolution of calcium carbonate occurred even aragonite saturation state (Ω_{arg}) remained higher than 1 and this effect was more noticeable with increasing pCO_2 levels.

Addition of bioavailable organic matter significantly enhanced bacterial abundance (*t*-test; p=0.01) and net respiration (*t*-test; p=0.0001) and these increased with increasing pCO_2 levels ($p<0.05$). With the increase in respiration, dissolution rates also increased. Heterotrophic microbial communities produced more CO_2 and promoted further carbonate dissolution. This pattern was also reflected at high pCO_2 incubations. In the organic matter addition incubation bottles, bacterial abundance increased by 3 to 4 orders of magnitude and the dissolution rates increased by 2.5 times to 10 times more than in control (white skeleton). The results show that inputs of organic matter in the reefs will enhance metabolic activities of microbial communities associated with coral rubble which ultimately increase dissolution of calcium carbonate. These suggest that availability of organic matter accelerates carbonate dissolution with enhancing microbial abundance and their physiological activities. Therefore, bioavailable organic matter has a potentially important role for calcium carbonate dissolution.

Elevated CO_2 influenced carbonate ($CaCO_3$) dissolution in white coral skeleton (WCSk; control) but the magnitude of dissolution rates was very small. On the other hand natural rubble (NR) showed higher dissolution rates than the control both at natural illumination and dark condition. This suggests that CO_2 produced by the microbial community respiration intensified dissolution in these experiments. Therefore, in coral reefs ecosystem, calcium carbonate ($CaCO_3$) dissolution is not only governed by the physico-chemical processes but also biological processes in determining calcification and dissolution. These concepts were clearly demonstrated in the “Bio-Chemical Dissolution Processes (BCDP)” model.

Outline of Thesis

The thesis contains 5 chapters arranged as follows:

Chapter 1 presents *General Introduction* with the relevant background information of Ocean acidification and their chemical and biological impacts on some calcifying organisms. It discusses the importance of coral rubble in the total coral reef ecosystem and its associated microbial communities. Additionally, this chapter summarizes the objectives of research and hypothesis.

Chapter 2 presents research *Methodology and Measurements*. It discuss about study site and samples collection, experimental design and details procedure of incubations experiments, several laboratory measurements and analysis.

Chapter 3 describes coral rubble associated microbial communities (epilithic and endolithic) with their daily metabolism and contribution to the dissolution of calcium carbonate under different levels of pCO_2 (ambient, 520, 720 and 1120 ppm). It compares physico-chemical vs. biological effects on carbonate dissolution under dark condition, where photosynthesis is inhibited; and also under natural day-night system.

Chapters 4 discuss how the availability of organic matter may accelerate carbonate dissolution by influencing bacterial activity under elevated pCO_2. This pattern is shown by a series of experiments under elevated pCO_2 and the addition of organic matter (glucose or coral mucus).

Chapter 5 concludes with remarks, summary of key findings, and presents a novel view of carbonate dissolution in coral reefs ecosystem the "Bio-Chemical Dissolution Processes (BCDP)".

Chapter 1

General Introduction

1.1 Background of research

1.1.1 Ocean Acidification

The acidification of the oceans is the ongoing decrease in pH of the oceans, caused by the uptake of anthropogenic CO_2 from the atmosphere (Caldeira and Wickett 2003). The last century has seen a rapidly growing global population and much more intensive use of resources, leading to greatly increased emissions of gases, such as carbon dioxide and methane, from the burning of fossil fuels (oil, gas and coal), and from agriculture, cement production and deforestation. Coal is now the largest fossil-fuel source of CO_2 emissions. Over 90% of the growth in coal emissions results from increased coal use in China and India. Global emissions per capita reached 1.3 tonnes of carbon and the developed countries still lead. However, evidence from the geological record is consistent with the physics that shows that adding large amounts of carbon dioxide to the atmosphere warms the world and may lead to higher sea levels and flooding of low-lying coasts; greatly changed patterns of rainfall; increased acidity of the oceans; and decreased oxygen levels in seawater.

Since the beginning of the industrial revolution, about 30–40% of the carbon dioxide (approximately 79 million tons per day) released into the atmosphere by human activities has been absorbed by the oceans (Feely et al. 2004; Sabine et al. 2004; IPCC 2007). The concentration of carbon dioxide (CO_2) in the atmosphere is 396 ppm (NOAA 2013; Fig. 1.1) and increased 2.1 ppm per year during past decade (2003-2012). However, consequence of increase in atmospheric CO_2, the world's ocean pH has dropped about 0.001 to 0.002 pH units per year over the past several decades (West 2011). This sounds like a tiny drop, but pH is measured on a logarithmic scale, so small changes in pH can indicate significant physical effects. The average ocean pH has shifted from a pre-industrial value of 8.2 to a current value of 8.1 (Raven et al. 2005) and is expected to decrease a further 0.3-0.4 pH units (Orr

et al. 2005), resulting in an ocean that is three times more acidic. Predictions by the Intergovernmental Panel on Climate Change (IPCC) suggest ocean-surface pH may drop to 7.8 by 2100, resulting in a marine environment more acidic than at any time in the past 12 million years (Fig. 1.2).

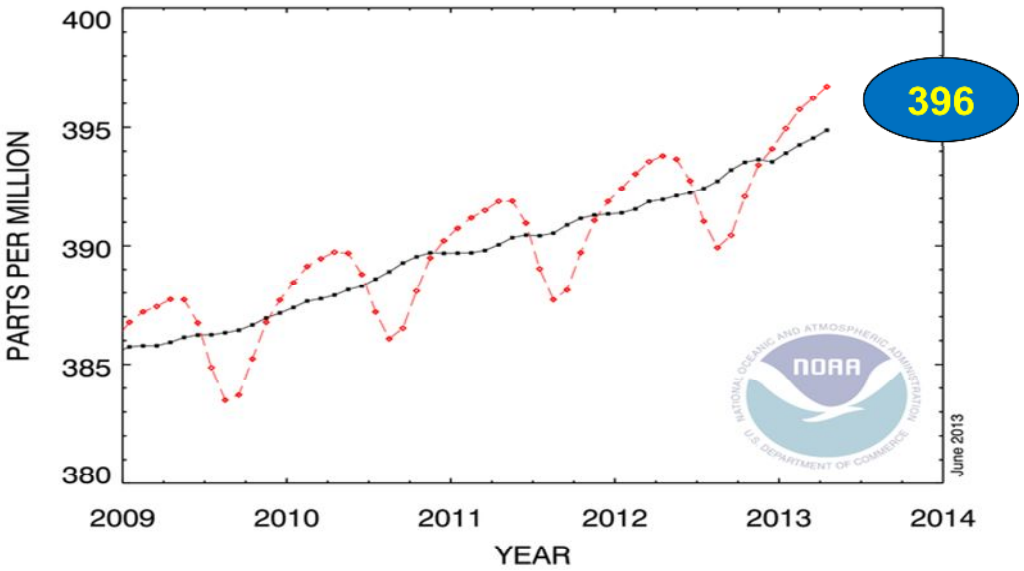

Fig. 1.1 Monthly mean carbon dioxide (CO_2) and trends in the atmosphere during past decade at Mauna Loa Observatory, Hawaii; NOAA 2013 [http://www.esrl.noaa.gov/gmd/ccgg/trends/]

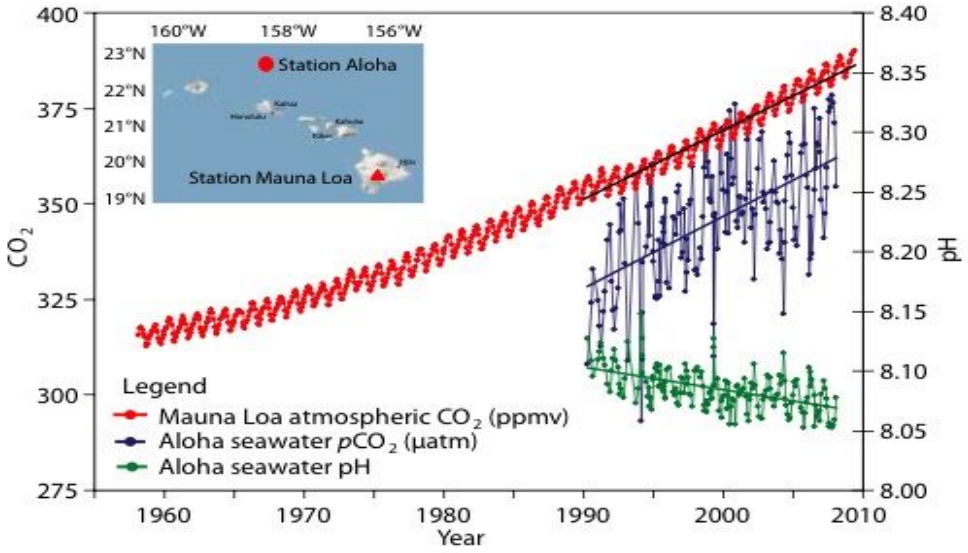

Fig. 1.2 Correlation between rising levels of carbon dioxide (CO_2) in the atmosphere and ocean; and shows pH of the ocean decreasing with accumulation of CO_2 [http://www.pmel.noaa.gov/co2/]

The ocean plays an important role in global carbon cycle, absorbing about half of the CO_2 emission, by anthropogenic activities. However, the hydrolysis of CO_2 is leading to a decrease in ocean pH. The atmospheric CO_2 dissolving into seawater is the formation of carbonic acid (H_2CO_3), which releases hydrogen ions (H^+) and decreases pH, a process referred to as ocean acidification (Caldeira and Wickett 2003). Excess hydrogen ions react with carbonate ions (CO_3^{2-}) to form bicarbonate ions (HCO_3^-), result in reducing carbonate ions (CO_3^{2-}) concentration and leads to dissolution of calcium carbonate ($CaCO_3$) (Fig. 1.3).

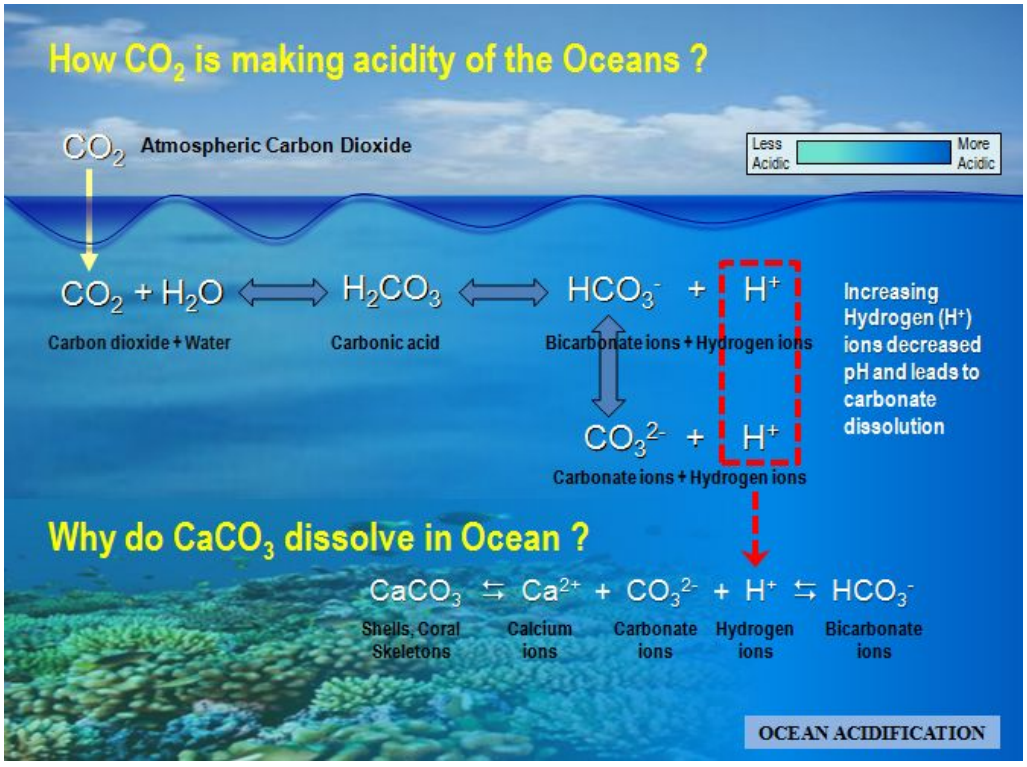

Fig. 1.3 Ocean acidification promoted carbonate dissolution

The seawater-mediated interaction of carbon dioxide (CO_2) and calcium carbonate ($CaCO_3$) illustrates carbonate dissolution by the following reaction.

$$CO_2 + H_2O + CaCO_3 \leftrightarrows Ca^{2+} + 2HCO_3^- \quad \text{[Dissolution]} \quad [1]$$

The saturation state of seawater with respect to aragonite can be defined as:

$$\Omega = [Ca^{2+}] [CO_3^{2-}] / K'_{sp} \quad \text{[Saturation state]} \quad [2]$$

Where K'_{sp} is the stoichiometric solubility product of aragonite derived from a function of salinity and temperature (Mucci 1983). Ω is largely determined by CO_3^{2-}, because Ca^{2+} is near conservative in seawater (Kleypas et al. 1999). Tropical surface seawaters are supersaturated (Ω > 1) with respect to all mineral phases, but the degree of saturation state varies from 5 to 6 for calcite (Ω_{cal}) and from 3 to 4 for aragonite (Ω_{arg}) (Kleypas et al. 1999). The decreased of saturation state could result in reduced calcification rates and promoted to calcium carbonate ($CaCO_3$) dissolution.

1.1.2 Biological impacts

Ocean acidification induces fundamental changes in seawater chemistry that could have dramatic effect and possible consequences on biological ecosystems as well as marine life (Doney et al. 2009a; Feely et al. 2009). Photosynthetic algae and seagrasses in the ocean may benefit from higher CO_2 conditions, as they require CO_2 to live just like plants. However, many studies have shown that a more acidic environment has a devastating impacts on some calcifying organisms, such as corals (Gattuso et al. 1998; Langdon 2000), oysters (Kurihara et al. 2007), sea urchins (Kurihara and Shirayama 2004), foraminiferans (Ricketts 2009) and also photosynthetic plankton coccolithophorids (Riebesell et al. 2000; Zondervan et al. 2001; Rost and Riebesell 2004; Kleypas et al. 2006; Yates and Halley 2006; Casareto et al. 2009) that have shells or plates of calcium carbonate ($CaCO_3$).

The formation of shells of $CaCO_3$ by calcification is a widespread phenomenon among the marine organisms. Although it is not always clear what function this calcification has, it seems integral to their biology; so any decrease in calcification, as a result of increased CO_2, is therefore likely to have significant consequences such as the weakening of coral skeletons and reef structures generally (Raven et al. 2005). Doney et al. (2009b) mentioned that, many calcifying species exhibit reduced calcification and growth rates in laboratory experiments under high

CO_2 conditions. Increasing ocean acidification has been shown to significantly reduce the ability of reef-building corals to produce their skeletons. A more acidic ocean could wipe out species, disrupt the food web and affect the overall structure of marine ecosystems.

The European Project on OCean Acidification (EPOCA) has mentioned that, the magnitude of ocean acidification can be predicted but the impacts of the acidification on marine organisms and their ecosystems are much less predictable. If pH levels drop enough, the shells will literally dissolve. Delicate corals may face an even greater risk than shellfish because they require very high levels of carbonate to build their skeletons. Consequently, the results of the decrease in carbonate ions might be catastrophic for calcifying organisms which play an important role in the food chain and form diverse habitats helping the maintenance of biodiversity. Recent research has shown that ocean acidification could compromise the successful fertilization, larval settlement and survivorship of Elkhorn coral, an endangered species. This research suggests that ocean acidification could severely impact the ability of coral reefs to recover from disturbance. However, effects of ocean acidification on organisms and ecosystems are still poorly understood. Ocean acidification research is still in its infancy and more studies are required to answer the numerous questions related to its biological and biogeochemical consequences.

1.2 Importance of coral rubble in coral reef ecosystems

Coral rubble are colonized by epilithic and endolithic algal communities and by other heterotrophic organisms including bacteria, foraminifera, nematodes, copepods, crustaceans etc. It's covered about 50% of reef lagoon bottom (Nakano and Nakai 2008) and they maintain the shape of coastal reef environment. Coral rubble derived from dead corals. The fragment of coral is one important source of sediment on reefs lagoon. Coral rubble is one of the most typical bottom habitats and particularly important as settling ground for new coral colonies (Mortensen et al. 1995; Takada et al. 2007). The dead coral framework can trap sands and mud for helping to build the reef structure and it is also an important habitat for other many tiny organisms. Rubble provides a unique niche for the growth of endolithic microbial

succession and established a chloraphyta-dominated endolithic community (Charpy et al. 2012), and the endolithic communities contribute as much primary production as the epilithic communities (Tribollet et al. 2006). At first glance, the rubble field seems an unproductive areas in the reefs (Fig. 1.4); however, recent studies suggested that coral rubble show high primary productivity and highest ability of nitrogen fixation (Berman-Frank et al. 2003; Casareto et al. 2008). Therefore, the biological activities of coral rubble associated microbial communities may play an important role in calcification and dissolution.

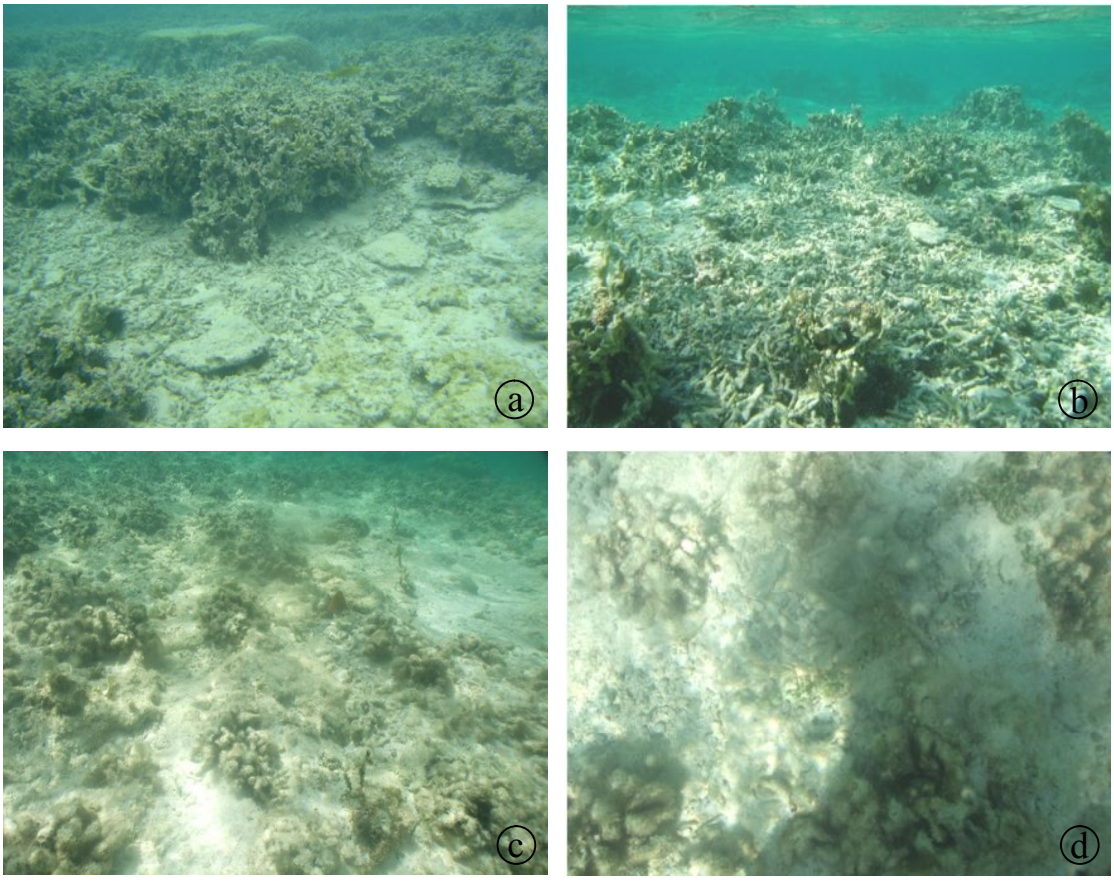

Fig. 1.4 Different view (a, b, c & d) of coral rubble field in shallow reef lagoon

1.3 Objectives of research

In the present study, objective of research was to determine the effects of Ocean acidification vs. role of microbial community on the dissolution of calcium carbonate by examining the response of coral rubble associated community (Autotrophs and Heterotrophs including bacteria) under elevated pCO_2. I wanted to know the role of epilithic and endolithic community on the dissolution of calcium carbonate and what changes in dissolution rate may occur when photosynthesis is inhibited. Moreover I wanted to determine the effect of bioavailable organic matter on the carbonate dissolution by influencing bacterial activity under different levels of pCO_2. The aspects included for the present studies are pointed below:

- to know the contribution of microbial communities associated with coral rubble (epilithic and endolithic) on the dissolution of calcium carbonate under high pCO_2;
- to determine the contribution of physico-chemical vs. biological processes for the dissolution of carbonate;
- to determine the effect of bioavailable organic matters on the dissolution of calcium carbonate;
- to evaluate the diurnal variation and the level of organic matter addition on the dissolution of calcium carbonate under different level of pCO_2.

1.4 Research hypothesis

Due to increase in atmospheric CO_2, the ocean pH has dropped and that could have dramatic effect on some calcifying organisms. Contrary, photosynthetic micro-macro algae in the ocean may benefit from higher CO_2 conditions, as they require CO_2 to enhance growth through primary production and organism abundance. However, increasing CO_2 enhanced primary production as a resulting in more CO_2 by physiological activities (respiration) which makes more corrosive of the ocean and promoted carbonate dissolution. The hypothesis of calcium carbonate ($CaCO_3$) dissolution under high pCO_2 condition is shown in schematic diagram (Fig. 1.5).

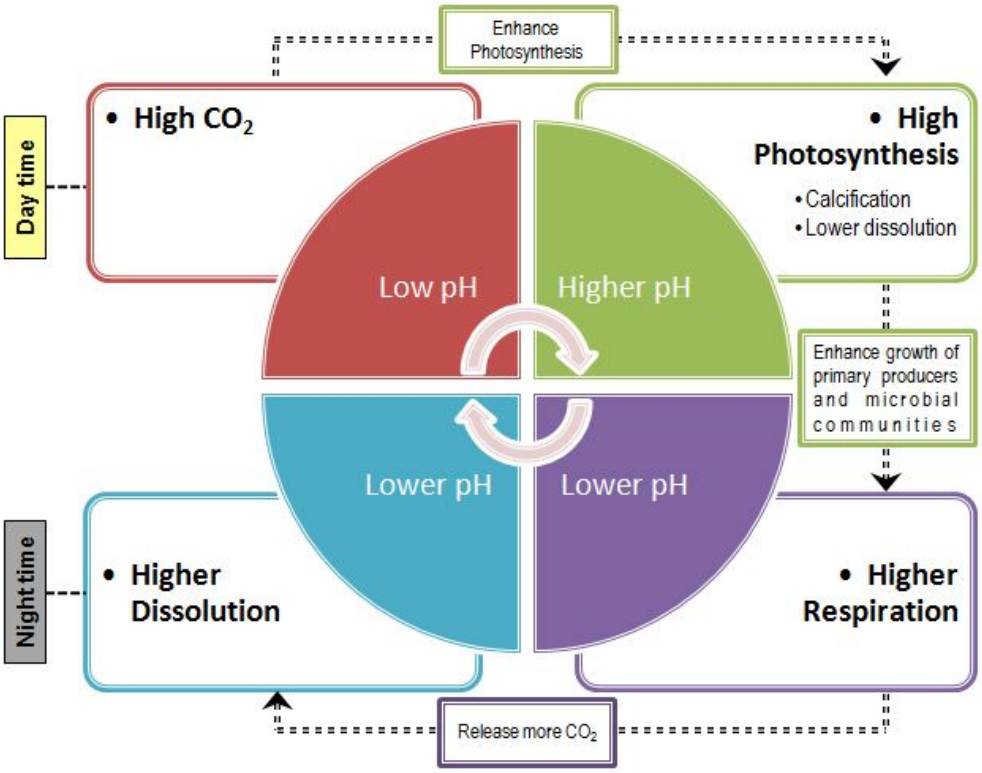

Fig. 1.5 Schematic diagram of calcium carbonate ($CaCO_3$) dissolution hypothesis under high pCO_2 condition

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Chapter 2

Methodology and Measurements

2.1 Study area and collection of samples

The study area is located in a shallow fringing coral reef at Sesoko Island, Ryukyu Archipelago, Okinawa, Japan between 26° 38′ N and 127° 51′ E (Fig. 2.1). This reef is in a post bleaching phase after important bleaching events in 1994 and 1996 (Nakano 2004). The reef is composed of less than 10% of living corals, 50% of coral rubbles, 30% of sandy bottom, and the rest covered by macroalgae and turf algae (Nakano and Nakai 2008).

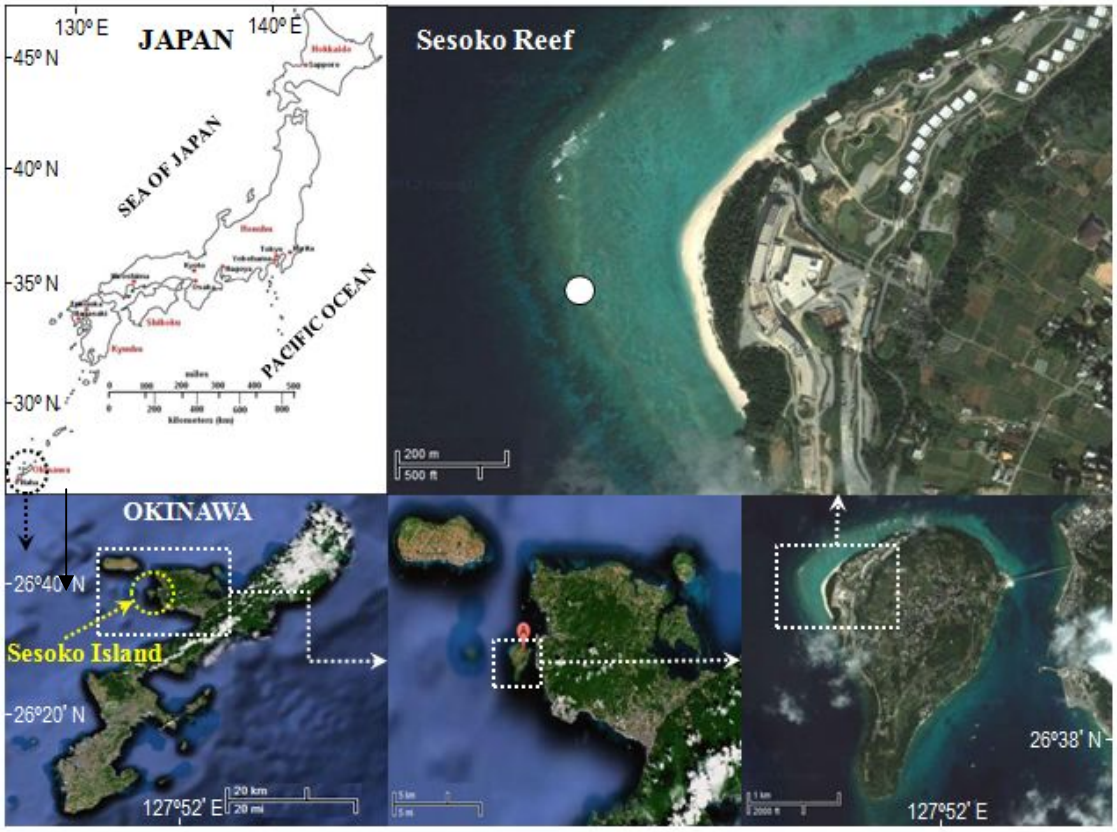

Fig. 2.1 Map showing the study area and the location of sample collection (○) at Sesoko Island, Okinawa, Japan

Coral rubble samples were collected in the lagoon at about 1~2 m depth in plastic bags (Zip Lock) together with surrounding seawater (Fig. 2.2~2.7), and immediately transported to the laboratory. Seawater was collected by using 10 L Nalgene bottle. After collection, seawater was filtered using a cartridge filter (0.2 μm isopore membrane filter) and dispensed into 1L Nalgene bottles for incubation. All the bottles were washed using neutral Extran (MA02; MERCK) detergent and rinsed with Milli-Q water before use.

Fig. 2.2 Sesoko island, Okinawa, Japan

Fig. 2.3 Sesoko beach, Okinawa, Japan

Fig. 2.4 Sample collection from the shallow lagoon

Fig. 2.5 Sample collection from the deep lagoon

Fig. 2.6 Rubble bed in reef lagoon

Fig. 2.7 Similar size rubble selection

2.2 Experimental design

Four incubation experiments (Incubation i, ii, iii and iv) were carried out in four different ways and purposes (Table 2.1): i) short time incubation (24 hours) in natural illumination and under ambient and high pCO_2 (1120 ppm) conditions using treated rubble (TR: keeping only their endolithic communities) and natural rubbles (NR: with associated epilithic and endolithic communities) to assess the role of epilithic and endolithic communities; ii) short time incubation (24 hours) in natural illumination and under ambient and high pCO_2 (1120 ppm) conditions using NR, NR with addition of organic matter glucose (NR+G) and coral mucus (NR+M) as source of organic matter to assess the role of bioavailable organic maters on the carbonate dissolution by influencing bacterial activity; iii) long time incubation (4 days) under dark condition using NR and NR with addition of different level of (1µM, 2µM, 4µM) organic matter glucose (G) under ambient, 520, 720 and 1120 ppm pCO_2 to test the contribution of physico-chemical vs. biological factors for the dissolution of calcium carbonate, and iv) long time incubation (4 days) under natural illumination using NR and NR with addition of organic matter glucose (G) to see the diurnal variation and the level of organic matter addition on the dissolution of calcium carbonate. As control, white coral skeleton (WCSk) was incubated in the same conditions as mentioned previously for all incubation types.

Table 2.1 Experimental design for short and long incubations

Incubation	Duration	Condition	pCO_2			
			Ambient	520 ppm	720 ppm	1120 ppm
i. Short	24h	Natural illumination	WCSk	-	-	WCSk
			TR	-	-	TR
			NR	-	-	NR
ii. Short	24h	Natural illumination	NR	-	-	NR
			NR+G	-	-	NR+G
			NR+M	-	-	NR+M
iii. Long	4 day's	Dark	WCSk	WCSk	WCSk	WCSk
			NR	NR	NR	NR
			NR+G	NR+G	NR+G	NR+G
iv. Long	4 day's	Natural illumination	WCSk	WCSk	WCSk	WCSk
			NR	NR	NR	NR
			NR+G1	NR+G1	NR+G1	NR+G1
			NR+G2	NR+G2	NR+G2	NR+G2
			NR+G4	NR+G4	NR+G4	NR+G4

WCSk: White coral skeleton; TR: Treated rubble keeping only their endolithic communities; NR: Natural rubbles with associated epilithic and endolithic communities; NR+G: Natural rubbles with addition of organic matter (Glucose); NR+M: Natural rubbles with addition of organic matter (10% Coral mucus); G1, G2 and G4: Level of organic matter addition (1µM, 2µM and 4µM of glucose); Natural illumination: Out door

2.3 Preparation of incubation experiments

Incubation experiments were carried out in the facilities of the Tropical Biosphere Research Center of the University of Ryukyus, Sesoko Island, Okinawa, Japan. Two small branches of undisturbed coral rubbles (NR = Natural Rubbles with associated epilithic and endolithic communities; Fig. 2.8) of similar sizes were placed into 1L Nalgene bottles which were filled with filtered seawater (0.2µm) collected simultaneously at the same sampling point. Other branches of similar size were previously brushed to remove the epilithic community (TR = Treated Rubble keeping only their endolithic communities; Fig. 2.8) and place in another incubation bottles which were also filled with filtered seawater (Fig. 2.10). The different levels of pCO_2 in the incubation bottles were adjusted by injecting CO_2 saturated seawater into the bottles at different volumes until pH values equivalent to the desired pCO_2 levels were obtained (Casareto et al. 2009) (Table 2.2). CO_2 saturated seawater was prepared by bubbling pure CO_2 gas into natural seawater until pH was stable (Fig. 2.10). Glucose stock solution was prepared as 1.8 g l^{-1}, and 100µl, 200µl, 400µl and 1000µl of this solution were added to the incubation bottles (1L) to make final concentration of 1µM, 2µM, 4µM and 10µM. In case of coral mucus, 10% mucus solution was added to the incubation bottles. Temperature and light intensity were monitored during all incubation period using *in situ* sensor (Fig. 2.9).

Table 2.2 Equivalent values of pCO_2 (ppm) at different pH levels

pH	pCO_2 (ppm)
8.500	160
8.400	218
8.300	295
8.200	**396**
8.103	520
8.100	525
8.000	690
7.984	720
7.900	900
7.816	1120
7.800	1168

Values at 28ºC temperature, 35‰ salinity, alkalinity 2255 µmol l^{-1}

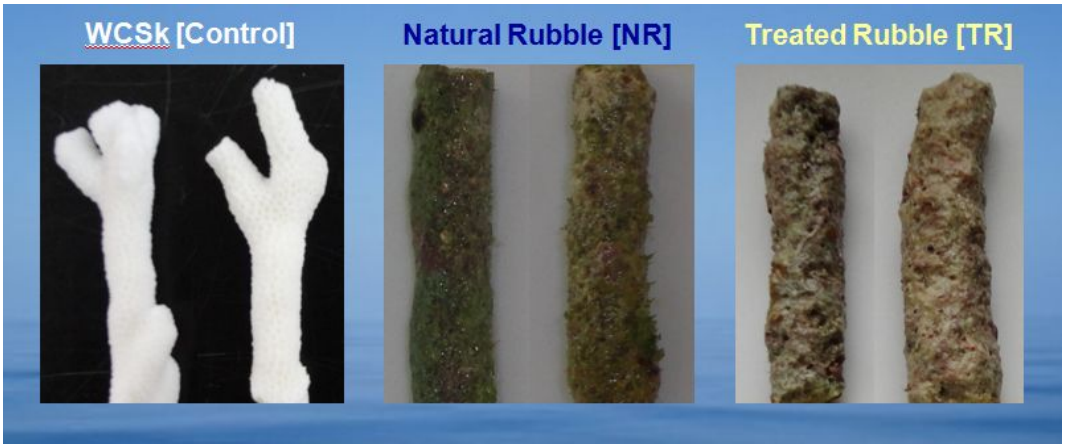

Fig. 2.8 Experimental rubble

[**WCSk:** White coral skeleton, without any attachment of living organisms; **NR:** Natural rubbles with associated epilithic and endolithic communities; **TR:** Treated rubble keeping only their endolithic communities.]

Fig. 2.9 Preparation for incubation experiments

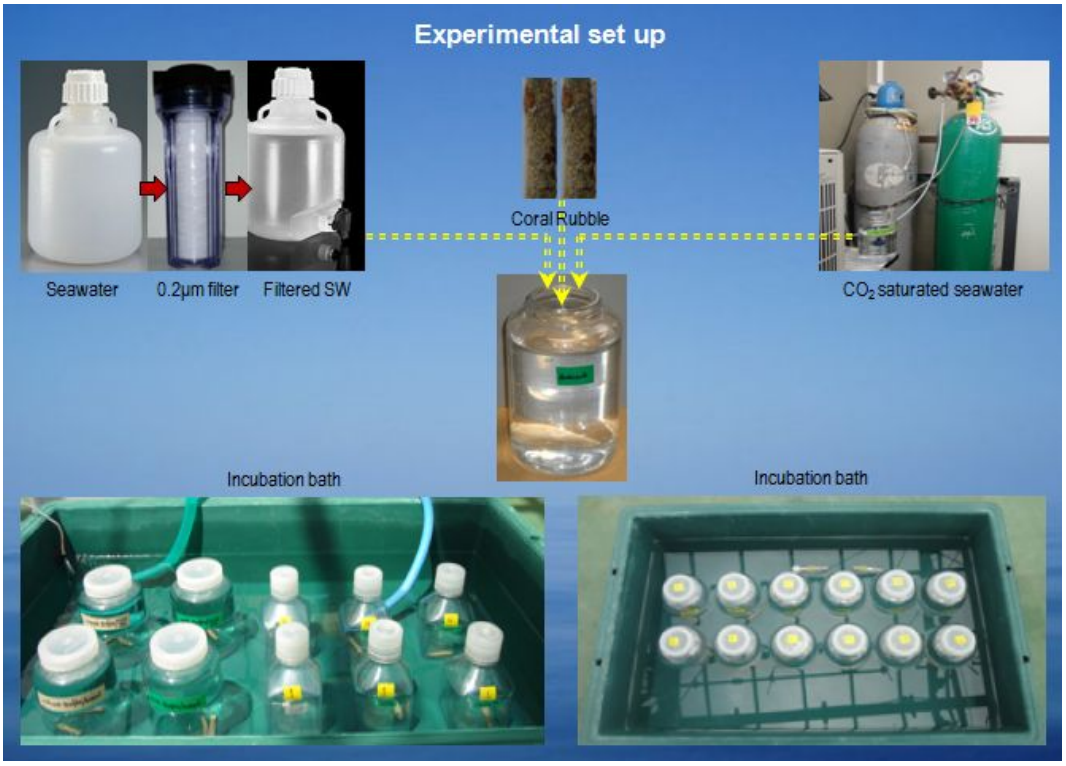

Fig. 2.10 Methods of experimental set up

2.4 Laboratory measurements

Measurement of short incubation experiment was done 24 times per day (1h interval) and 4 times per day (6h interval) for long incubation experiments.

2.4.1 Physico-chemical parameters

Natural illumination incubations were conducted in natural temperature at 25ºC to 33ºC and long incubation (dark) were conducted in a temperature-controlled water bath at 20°C. Temperature and light intensity were monitored all through the incubations using *in situ* sensor (MDS-MkV/T and MDS-MkV/L, Alec electronics).

pH and DO were measured using a pH meter ORION 4 STAR calibrated with NIST (NBS) scaled buffer solutions (Mettler pH 9.228 and 6.880 buffers at 20°C). Precision of pH measurement was ±0.005 pH unit (1σ, n = 10). Alkalinity (A_T) was measured by the total alkalinity titrator (KIMOTO ATT-05). Reproducibility of the A_T measurement was ± 2 µmol kg^{-1} (1σ; n = 10). Salinity was measured using Refractometer (ATAGO S/Mill-E).

Samples for analysis of dissolved organic matter (DOM) were collected using specially prepared acid washed cleaned pre-combusted (550°C) amber glass vials with Teflon lined screw cap. Before analysis of samples for dissolved organic carbon (DOC), a filtration procedure was done to eliminate particulate materials contain from the sample. Filtration was done under gravity using a clean acid washed pre-combusted (550°C) 100 ml glass syringe fixed with acid washed cleaned polypropylene filter holder equipped with 25mm GF/F micro glass fiber filter paper combusted at 550°C. At all times prior to filtration syringe was washed with Milli Q grade water and after with small amount of sample. During filtration about 10ml of sample was discarded to wash filters to avoid any contaminations. Finally, the concentration of dissolved organic carbon (DOC) was measured using total organic carbon analyzer (TOC-5000A; Shimadzu, Japan) and calculated following the formula: $x = (y - c)/m$, where c is Y intercept and m is slope or gradient.

2.4.2 Biological parameters

The length and diameter of coral rubble were measured considering a cylinder as most approximate geometrical shape using Slide Caliper and surface area was calculated following the equation: $A = 2\pi r^2 + 2\pi rh$; where h is length (height) of the rubble.

Rubble Chlorophyll measurement were performed by spectrophotometry (Bio Tek; Synergy HT). Rubbles were crushed into small pieces and then homogenized with cold 95% (v/v) methanol in a mortar for pigment extraction. Extracts were then filtered through a syringe filter (0.2 µm, Millex-LG, Millipore) to remove debris. The concentration of chlorophyll was determined by measuring the absorbance at 663 nm and 645 nm. Chlorophyll concentration was calculated using the extinction coefficients given by Arnon (1949) and Porra (2002) following formula:

Chl *a* (µg/ml) = $12.7 \times A_{663} - 2.69 \times A_{645}$

Chl *b* (µg/ml) = $22.9 \times A_{645} - 4.68 \times A_{663}$

Chl *a* + *b* (µg/ml) = $20.2 \times A_{645} + 8.02 \times A_{663}$

Finally, for calculation of coral rubble's chlorophyll were calculated following formula:

Chl *a* ($\mu g\ cm^{-2}$) = (12.7 x A_{663} - 2.69 x A_{645}) × Extraction volume ÷ Surface area

Chl *b* ($\mu g\ cm^{-2}$) = (22.9 x A_{645} - 4.68 x A_{663}) × Extraction volume ÷ Surface area

Total Chlorophyll ($\mu g\ cm^{-2}$) = (20.2 × A_{645} + 8.02 × A_{663}) × Extraction volume ÷ Surface area

2.5 Calculation and analysis

2.5.1 Microbial analysis

Epilithic communities of the coral rubble were observed microscopically after brushing of rubble and suspended in artificial seawater (0.2µm filtered). For assessing diversity of endolithic communities, rubble was treated with 10% HCl to remove carbonates. Every sample was fixed by glutaraldehide at 2% final concentration. Associated organisms were then examined microscopically and identified (Nikon; ECLIPSE/80i). Heterotrophic bacteria were collected on 0.2 µm black polycarbonate filters by filtering 10 to 15 ml aliquots that were previously stained with DAPI (4',6-diamidino-2-phenylindole) (1µg ml^{-1} final concentration). Abundance was assessed by counting bacteria cells under an epifluorescence microscope (Nikon; ECLIPSE/E600) Eclipse), using a UV-filter.

2.5.2 Statistical analysis

The mean values, standard deviation (SD), correlation coefficient (r) and regression were analyzed by using statistical software. Difference between dark dissolution and natural dark-light dissolution mean values were compared using Student's paired t-Test (two sample assuming equal variances), to analyze the variances with 0.05 level of significance.

2.5.3 Bio-chemical analysis

For the short time incubations, we assessed primary production and respiration of the rubble associated communities. Net primary production (NPP) (11h) and dark respiration (Rd) (13h) were calculated from changes in dissolve oxygen concentration.

From these data we calculated total respiration (R) over 24h and gross primary production (GPP) as follows: [GPP = NPP + R]. Contributions of epilithic and endolithic communities were estimated by comparing NPP and R from treated and natural ruble (TR and NR). All calculations of metabolic parameters were converted to carbon (C) using "Redfield-Richards Ratio" empirical formula (Libes, 1992).

Different levels of pCO_2 (ambient, 520, 720 and 1120 ppm) were calculated using pH and A_T according to the carbonate equilibrium in seawater described by Millero (1979) and Fujimura *et al.* (2001). Carbonate dissolution rates (µmol $l^{-1}d^{-1}$) were analyzed using the alkalinity anomaly technique (Smith, 1973; Gattuso *et al.*, 1997; Fujimura *et al.*, 2001) following the equation: Dissolution rate = $\Delta A_T/2$, where ΔA_T is the variation of A_T ($A_{T\ final} - A_{T\ initial}$) during the incubation period at the start and end of each incubation. The alkalinity anomaly method actually yields an estimate of the net value of the $CaCO_3$ precipitation/dissolution balance (Smith, 1978; Bensoussan and Gattuso, 2007).

The carbonate system parameters in seawater and saturation state of calcium carbonate (Ω_{arg}) were calculated with the program CO2SYS (Lewis and Wallace, 1998) using the apparent equilibrium constants K'_0 from Weiss (1974), K'_1 and K'_2 from Mehrbach *et al.* (1973) as described by Dickson and Millero (1987) and the HSO_4^- constant according to Dickson (1993). Saturation degree of calcium carbonate (Ω_{arg}) was calculated from pH, alkalinity, salinity and temperature data sets. The degree of saturation is defined as: $\Omega_{arg} = [Ca^{2+}]\ [CO_3^{2-}] / K'_{sp}$, where K'_{sp} is the stoichiometric solubility product of aragonite derived from a function of salinity and temperature (Mucci, 1983).

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Chapter 3

Contribution of coral rubble associated microbial community to the dissolution of calcium carbonate under high pCO_2

Abstract: The contribution of coral rubble associated microbial community to the dissolution of calcium carbonate at different pCO_2 levels were investigated using incubation experiments at Sesoko Island, Okinawa, Japan. Short incubation (24h under natural illumination) and long incubations (4 days under natural illumination and dark condition) were carried out using white coral skeleton (control), natural rubble, treated rubble (removed epilithic community) and natural rubble with added organic matter (glucose) under different levels of pCO_2 (ambient, 520, 720 and 1120 ppm). Net primary production, respiration and dissolution rates of the endolithic communities were much higher than the epilithic communities at all pCO_2 levels. During the long experiment under natural illumination, carbonate dissolution rates varied from 23.7 ± 0.1 (ambient) to 50.5 ± 1.5 (1120 ppm) μmol $m^{-2}d^{-1}$ at night time and 3.2 ± 1.1 (ambient) to 5.8 ± 1.2 (1120 ppm) μmol $m^{-2}d^{-1}$ at day time. However, in long experiment under complete dark condition dissolution increased to 81.6 ± 0.2 μmol $m^{-2}d^{-1}$ at high pCO_2. With addition of glucose as source of organic matter, growth of bacteria was enhanced and therefore respiration rate increased. Heterotrophic microbial communities produced more CO_2 and promoted carbonate dissolution**.** This was reflected by further decrease in aragonite saturation state (Ω_{arg}) and increase in pCO_2 levels. The results show that inputs of organic matter in the reefs enhance metabolic activities of microbial communities associated with coral rubble which ultimately increase dissolution of calcium carbonate. These suggest the importance of biological processes (photosynthesis vs. respiration) in determining calcification and dissolution.

Keywords: Ocean acidification, Coral rubble, Epilithic and endolithic communities, Heterotrophic bacteria, $CaCO_3$, Bio-Chemical Dissolution Processes (BCDP)

3.1 Introduction

Coral reef is one of the most diverse ecosystems in the world. Many calcareous organisms such as corals, mollusks and echinoderms are present in this ecosystem. Coral rubble derived from dead corals, often cover wide areas in shallow lagoons of coral reefs. At Sesoko reef about 50% of reef lagoon bottom is covered by coral rubble (Nakano and Nakai 2008) and they maintain the shape of coastal reef environment. At first glance, the rubble field seems an unproductive areas in the reefs; however, recent studies suggested that coral rubble show high primary productivity and highest ability of nitrogen fixation (Berman-Frank et al. 2003; Casareto et al. 2008). Casareto et al. (2008) reported that in coral rubble at Sesoko Island, Okinawa, the primary production ranged from 176 ~ 367 nmol C μg Chl-*a*$^{-1}$ day^{-1} which is comparable to that of living corals (Casareto unpublished data), and the N_2 fixation from 0.77 ~ 7.2 nmol N μg Chl-*a*$^{-1}$ day^{-1}. Coral rubble is colonized by epilithic and endolithic microbial communities, and the endolithic communities contribute as much primary production as the epilithic communities (Tribollet et al. 2006a). Micro-algae growing onto (epilithic) and into (endolithic or boring algae) the rubble show high diversity of Cyanophytes (Casareto et al. 2008; Charpy et al. 2010, 2012) which enable them to fix N_2. Epilithic and endolithic microbial communities of coral rubble associated with heterotrophic bacteria are defined as "microbial community". The biological activities of microbial communities may play important role in calcification or dissolution of rubble (Ferran 2006).

Coral reefs are one of the first ecosystems to be recognized as vulnerable to ocean acidification (Kleypas and Yates 2009). The concentration of carbon dioxide (CO_2) in earth atmosphere is approximately 396 ppm (NOAA 2013) and rose by 2.0 ppm yr^{-1} during 2000–2010, with a corresponding decrease in pH of 0.0015 yr^{-1} (Midorikawa et al. 2010). The atmospheric carbon dioxide (CO_2) levels will exceed 1000 ppm by 2100 if emissions are not reduced considerably (The Royal Society 2005). The acidification of the oceans could affect the calcification and dissolution of calcium carbonate ($CaCO_3$) present in corals (Gattuso et al. 1998; Langdon 2000) and other calcifying organisms, such as sea urchins (Kurihara and Shirayama 2004), oysters (Kurihara et al. 2007), foraminifers (Ricketts 2009) and calcareous plankton

coccolithophorids (Riebesell et al. 2000; Zondervan et al. 2001; Rost and Riebesell 2004; Kleypas et al. 2006; Yates and Halley 2006). It is also thought that calcification rate in scleractinian corals and calcifying macro-algae decreases and their dissolution rate increases under the high pCO_2 (Kleypas and Langdon 2006). If calcification in corals declines, then reef-building capacity also declines (Kleypas et al. 1999). It is thought that ocean acidification affects not only the living organisms, but also the reefs they build. Several researches indicate that calcification rate of many organisms decreases with decreasing $CaCO_3$ saturation state (Ω) resulting in increase in carbonate dissolution in corals and carbonate sediments (Gattuso et al. 1999; Kleypas et al. 1999; Yamamoto et al. 2012).

Until now many studies on ocean acidification have focused on the physico-chemical aspects of calcification and dissolution working mainly in open ocean environments (Kleypas et al. 1999; Orr et al. 2005; Midorikawa et al. 2010). Only a few studies focused on the importance of biological processes on the calcification of calcareous organisms (Casareto et al. 2009; McCulloch et al. 2012). Casareto et al. (2009) reported that biological processes are more important than physico-chemical reactions in determining calcification and dissolution in coccolithophorids. In the present study I investigate the role of microbial community in the dissolution of coral rubble by examining the response of coral rubble and associated community to elevated pCO_2 and abundance of bacteria. I aimed to know the role of epilithic and endolithic community on the dissolution of calcium carbonate and what changes in dissolution rate may occur when photosynthesis is inhibited. I also aimed at assessing the contribution in dissolution rate by heterotrophic bacterial community with addition of organic matter under different levels of pCO_2.

3.2 Materials and Methods

3.2.1 Study site and collection of samples

The study site is located in a shallow fringing coral reef at Sesoko Island, Ryukyu Archipelago, Okinawa, Japan between 26° 38′ N and 127° 51′ E (Fig. 3.1). This reef is in a post bleaching phase after important bleaching events in 1994 and 1996 (Nakano 2004). The reef is composed of less than 10% of living corals, 50% of coral rubbles, 30% of sandy bottom, and the rest covered by macroalgae and turf algae (Nakano and Nakai 2008). Coral rubble were collected in the lagoon at about 1~2 m depth in plastic bags (Zip Lock) together with surrounding seawater, and immediately transported to the laboratory. Seawater was collected by using 10 L Nalgene bottle. After collection, seawater was filtered using a cartridge filter (isopore membrane filter) of 0.2 μm pore size and dispensed into 1L Nalgene bottles for incubation. All the bottles were washed using neutral Extran (MA02; MERCK) detergent and rinsed with Milli-Q water before use.

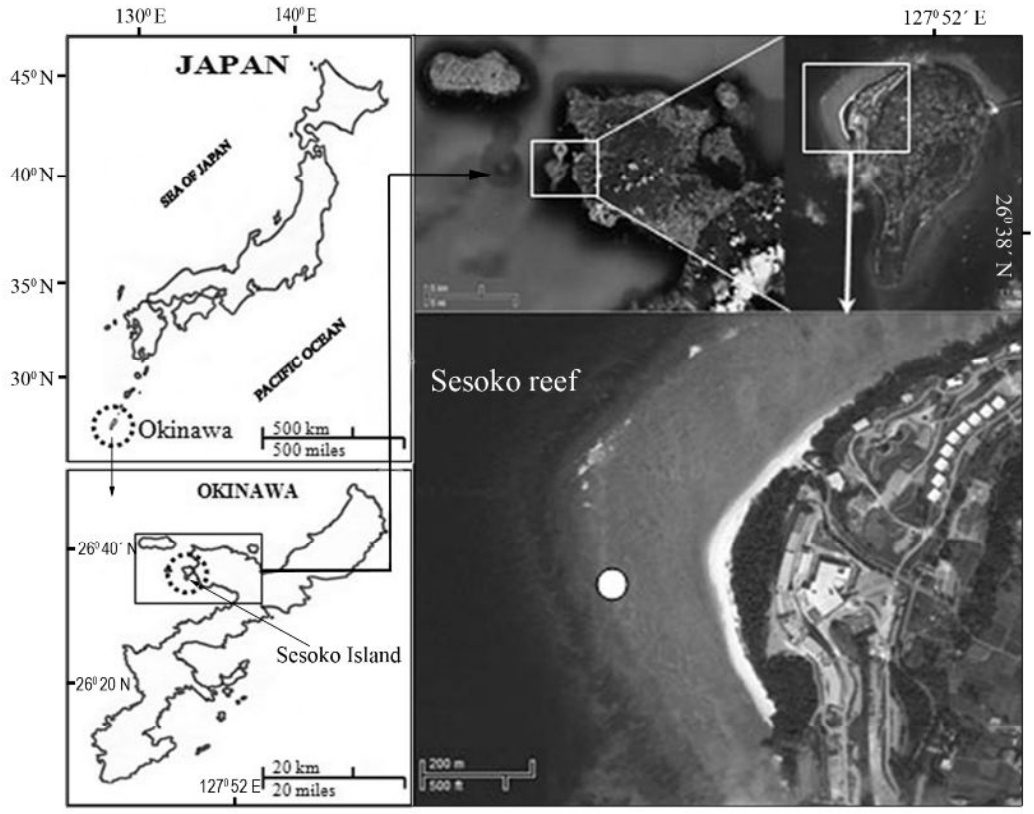

Fig. 3.1 Map showing the study area and the location of sample collection (○) at Sesoko Island, Okinawa, Japan

3.2.2 Preparation of incubation experiments

Incubation experiments were carried out in the facilities of the Tropical Biosphere Research Center of the University of Ryukyus, Sesoko Island, Okinawa, Japan. Two small branches of undisturbed coral rubbles (NR = Natural Rubbles with associated epilithic and endolithic communities) of similar sizes were placed into 1L Nalgene bottles which were filled with filtered seawater collected simultaneously at the same sampling point. Other two branches of similar size were previously gentle brushed using tooth brush and water pik to remove the epilithic community (TR = Treated Rubble keeping only their endolithic communities) and placed in another incubation bottles which were also filled with filtered seawater. Three (03) replicates were used for each incubation experiment. Incubations were carried out in three different ways (Table 3.1): i) short time incubation (24 hours) in natural illumination and under ambient and high pCO_2 (1120 ppm) condition to assess the role of epilithic and endolithic communities; ii) long time incubations (4 days) under dark conditions using NR and NR + 4 µM glucose as source of organic matter under ambient, 520, 720 and 1120 ppm pCO_2 to test the contribution of physico-chemical vs. biological factors for the dissolution of calcium carbonate, and iii) long time incubations (4 days) under natural illumination using NR and NR + 4 µM glucose to see the diurnal variation in the dissolution of calcium carbonate. As control, white coral skeleton was incubated in the same conditions as mentioned above. The different levels of pCO_2 in the incubation bottles were adjusted by injecting CO_2 saturated seawater into the bottles at different volumes until pH values equivalent to the desired pCO_2 levels were obtained (Casareto et al. 2009). CO_2 saturated seawater was prepared by bubbling pure CO_2 gas into natural seawater until pH was stable. Glucose stock solution was prepared as 1.8 g l^{-1} and 400 µl of this solution were added to the incubation bottles (1L) to make final concentration of 4µM. All incubations were conducted in a temperature-controlled water bath at 20°C. Light intensity was monitored during the experiments using *in situ* sensor (MDS-MkV/L, Alec electronics).

Table 3.1 Experimental design for short and long incubations

Incubation	Duration	Condition	pCO_2			
			Ambient	520 ppm	720 ppm	1120 ppm
Short	24h	Natural illumination	WCSk	-	-	WCSk
			NR	-	-	NR
			TR	-	-	TR
Long	4 days	Dark	WCSk	WCSk	WCSk	WCSk
			NR	NR	NR	NR
			NR + 4µM G	NR + 4µM G	NR + 4µM G	NR + 4µM G
Long	4 days	Natural illumination	WCSk	WCSk	WCSk	WCSk
			NR	NR	NR	NR
			NR + 4µM G	NR + 4µM G	NR + 4µM G	NR + 4µM G

WCSk: White coral skeleton; NR: Natural rubbles with associated epilithic and endolithic communities; TR: Treated rubble keeping only their endolithic communities; µM G: micro mole glucose; Natural illumination: out door

3.2.3 Laboratory measurements

Temperature, pH and DO were measured using a pH meter with three (3) sensors ORION 4 STAR calibrated with NIST (NBS) scaled buffer solutions (Mettler pH 9.228 and 6.880 buffers at 20°C). Alkalinity (A_T) was measured by the total alkalinity titrator (KIMOTO ATT-05). Reproducibility of the A_T measurement was ± 2 µmol kg^{-1} (1σ; n = 10). Measurement of short incubation experiment was done 24 times per day (1h interval) and 4 times per day (6h interval) for long incubation experiments. The length and diameter of coral rubble were measured using Slide Caliper and rubble chlorophyll measurement was performed by spectrophotometry (Bio Tek; Synergy HT).

3.2.4 Microbial analysis

Epilithic communities of the coral rubble was observed microscopically after brushing of rubble and suspended in seawater. After removing epilithic communities from the coral rubble, a portion (around 10 mm in length and 7 mm in diameter) of rubble was cut off and then decalcified using 10% HCl solution. After decalcification, a mass (colony) of endolithic algae was exposed and observed under microscope. Every sample was fixed by glutaraldehyde (1% final concentration) and kept cool at

4ºC. Associated organisms were then examined microscopically and identified (Nikon; ECLIPSE/80i). Heterotrophic bacteria were collected on 0.2 μm black polycarbonate filters by filtering 10 to 15 ml aliquots that were previously stained with DAPI (4',6-diamidino-2-phenylindole) at 1 μg ml^{-1} concentration. Abundance was assessed by counting bacteria cells under an epifluorescence microscope (Nikon; ECLIPSE/E600), using a UV-filter.

3.2.5 Statistical analysis

The mean values, standard deviation (SD), correlation coefficient (r) and regression were analyzed by using statistical software. Difference between dark dissolution and natural dark-light dissolution mean values were compared using Student's paired t-Test (two sample assuming equal variances), to analyze the variances with 0.05 level of significance.

3.2.6 Calculation and analysis

For the short time incubations, we assessed primary production and respiration of the rubble associated communities. Net primary production (NPP) (11h) and dark respiration (Rd) (13h) were calculated from changes in dissolve oxygen concentration. From these data we calculated total respiration (R) over 24h and gross primary production (GPP) as follows: [GPP = NPP + R]. Contributions of epilithic and endolithic communities were estimated by comparing NPP and R from treated and natural ruble (TR and NR). All calculations of metabolic parameters were converted to carbon (C) using "Redfield-Richards Ratio" empirical formula (Libes 1992).

Coral rubble surface area was calculated following the equation: $A = 2\pi r^2 + 2\pi rh$; where π (pi) is a mathematical constant that is the ratio of a rubble circle's circumference to its diameter and is approximately equal to 3.141, r is radius, h is length (height), πr^2 is the area of the top or bottom and $2\pi rh$ is the area of the side. Different levels of pCO_2 (ambient, 520, 720 and 1120 ppm) was calculated using pH and A_T according to the carbonate equilibrium in seawater described by Millero (1979) and Fujimura et al. (2001). Carbonate dissolution rates (μmol $l^{-1}d^{-1}$) were analyzed using the alkalinity anomaly technique (Smith 1973; Gattuso et al. 1997;

Fujimura et al. 2001) following the equation: Dissolution rate = $\Delta A_T/2$, where ΔA_T is the variation of A_T ($A_{T\ final} - A_{T\ initial}$) during the incubation period at the start and end of each incubation. The alkalinity anomaly method actually yields an estimate of the net value of the $CaCO_3$ precipitation/dissolution balance (Smith 1978; Bensoussan and Gattuso 2007).

The carbonate system parameters in seawater and saturation state of aragonite (Ω_{arg}) were calculated with the program CO2SYS (Lewis and Wallace 1998) using the apparent equilibrium constants K'_0 from Weiss (1974), K'_1 and K'_2 from Mehrbach et al. (1973) as described by Dickson and Millero (1987) and the HSO_4^- constant according to Dickson (1993). Saturation degree of aragonite (Ω_{arg}) was calculated from pH, alkalinity, salinity and temperature data sets. The degree of saturation is defined as: $\Omega_{arg} = [Ca^{2+}]\ [CO_3^{2-}] / K'_{sp}$, where K'_{sp} is the stoichiometric solubility product of aragonite derived from a function of salinity and temperature (Mucci 1983).

3.3 Results

3.3.1 Daily variation of physico-chemical parameters

Natural illumination incubations were conducted in natural temperature at 25ºC to 33ºC (Fig. 3.2). The maximum light intensity was 678 µmol $m^{-2}s^{-1}$ and the average was 362 µmol $m^{-2}s^{-1}$ (Fig. 3.3). During 24h short incubation, pH and dissolved oxygen (DO) (Fig. 3.4 and 3.5) were higher at day time and lower at night time, whereas alkalinity (Fig. 3.6) were higher at night time and lower at the day time. Over the 24h observation pH, DO and alkalinity were varied 7.8 ~ 8.5; 6.01 ~ 8.45 mg l^{-1} and 2245 ~ 2299 µmol l^{-1} respectively. In case of 4 days long incubation experiment, the total alkalinity was very little change at day time but significantly increased 100 ~ 250 µmol l^{-1} at night time. Average salinity of sample seawater was 35‰.

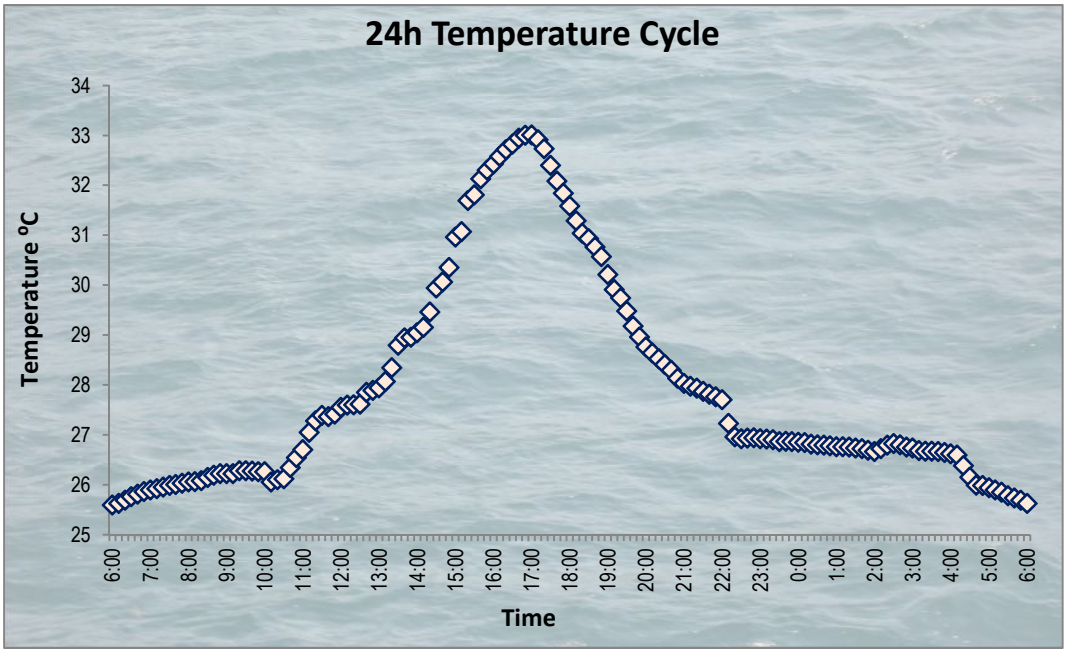

Fig. 3.2 Over 24h temperature measurement from the natural illumination experiment

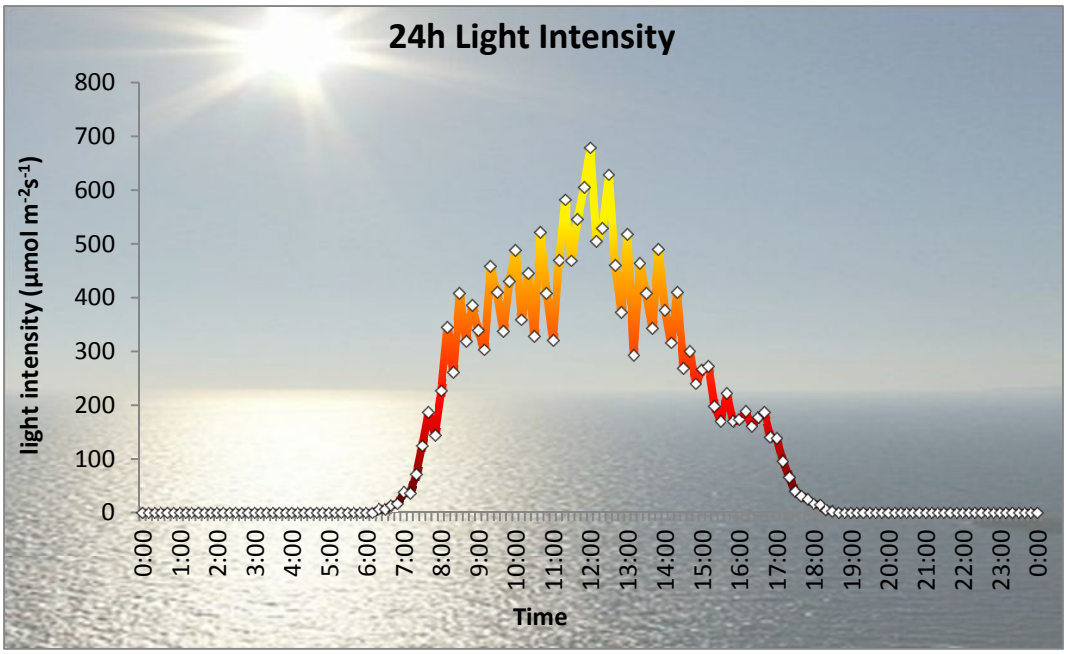

Fig. 3.3 Over 24h light intensity measurement from the natural illumination experiment

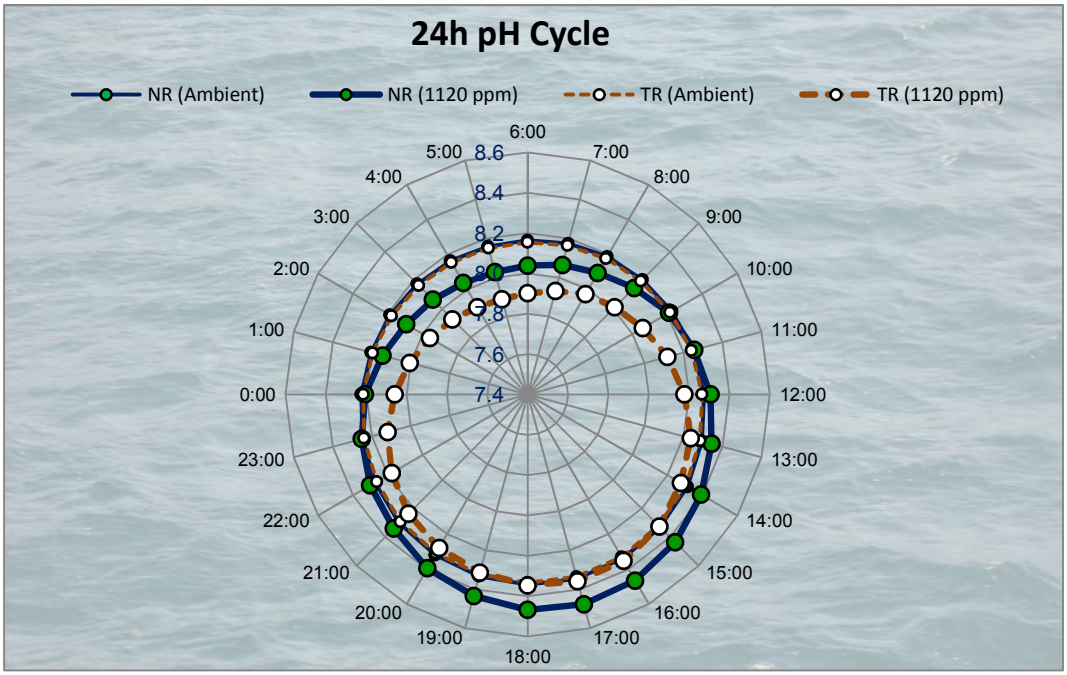

Fig. 3.4 Over 24h pH measurement from the natural illumination experiment

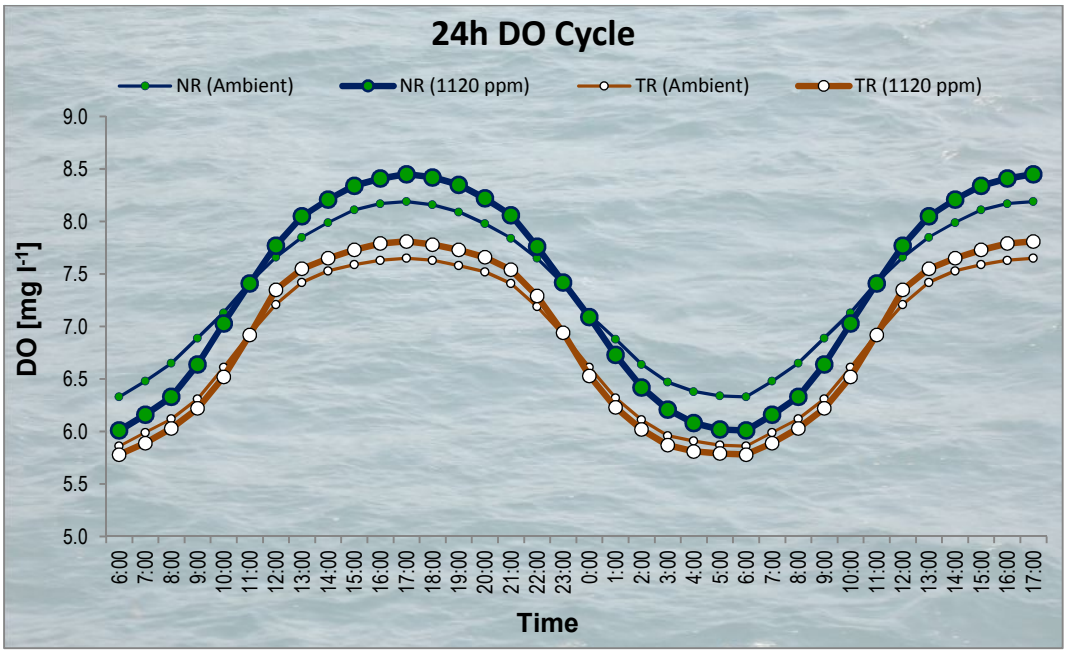

Fig. 3.5 Over 24h DO measurement from the natural illumination experiment

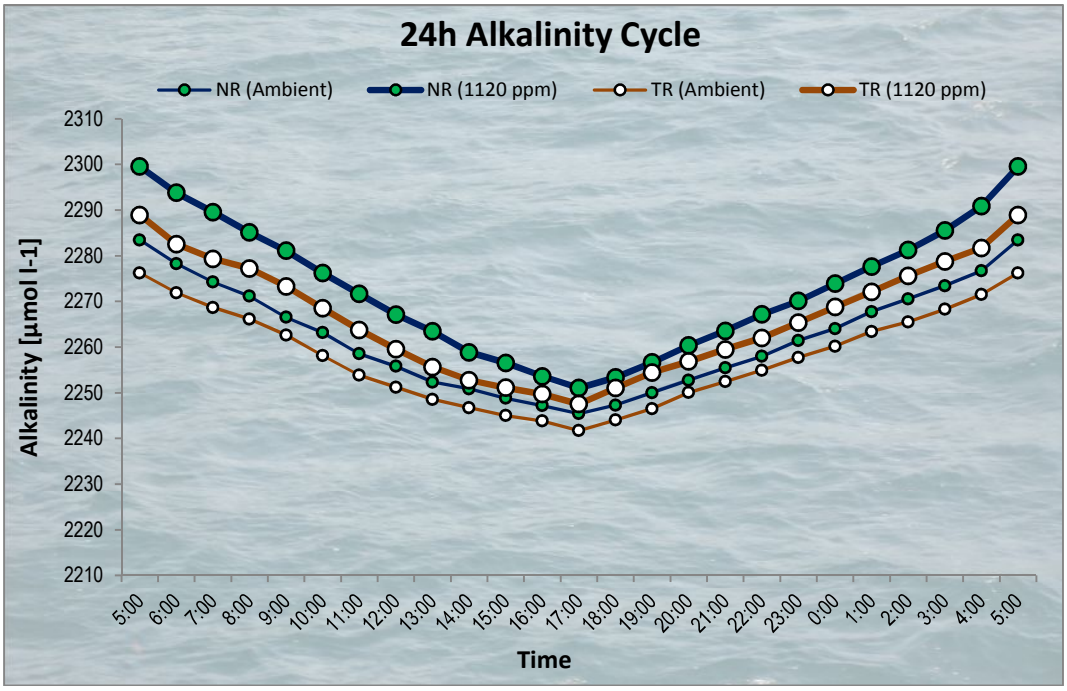

Fig. 3.6 Over 24h alkalinity measurement from the natural illumination experiment

3.3.2 Associated communities in coral rubble

Coral rubble was colonized by epilithic and endolithic communities. Epilithic community was composed of several algal taxa including Chlorophyta, Cyanophyta, Phaeophyta, Rhodophyta and Bacillariophyta. Endolithic community was composed mainly by Chlorophytes and Cyanophytes. Other organisms of Heterotrophs as foraminifera, nematodes, copepods and other crustaceans were found associated to the surface of coral rubble (Fig. 3.7 ~ 3.9; Table 3.2). Rubble size was standardized by surface area. The average rubble length, diameter and surface area were 5.51 ± 0.02 cm, 1.00 ± 0.01 cm, and 18.84 ± 0.18 cm^2 respectively.

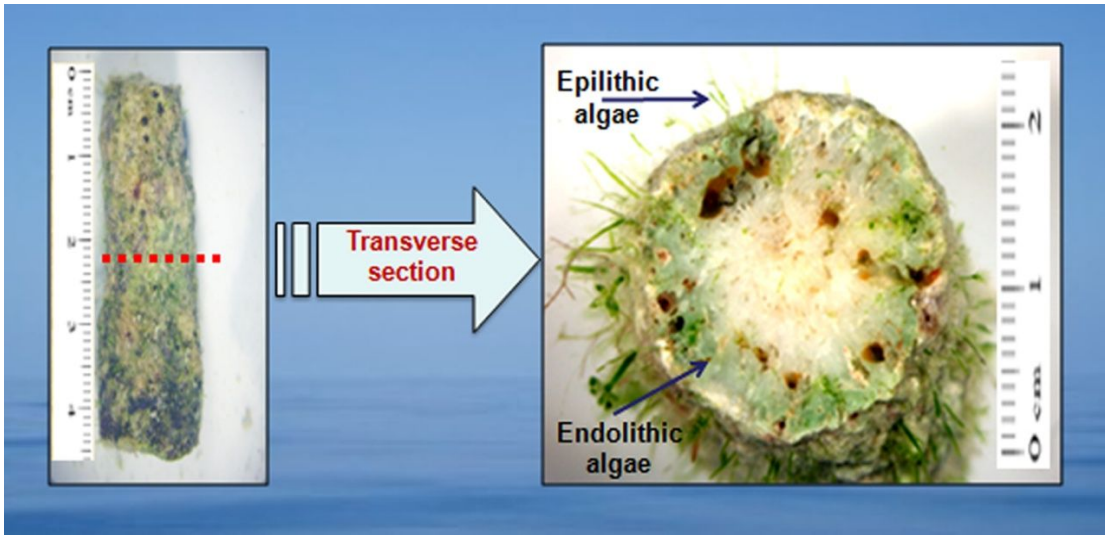

Fig. 3.7 Photograph of natural coral rubble (left) and a transverse section of it (right) showing the endolithic (green band) and epilithic algae (Islam et al. 2012)

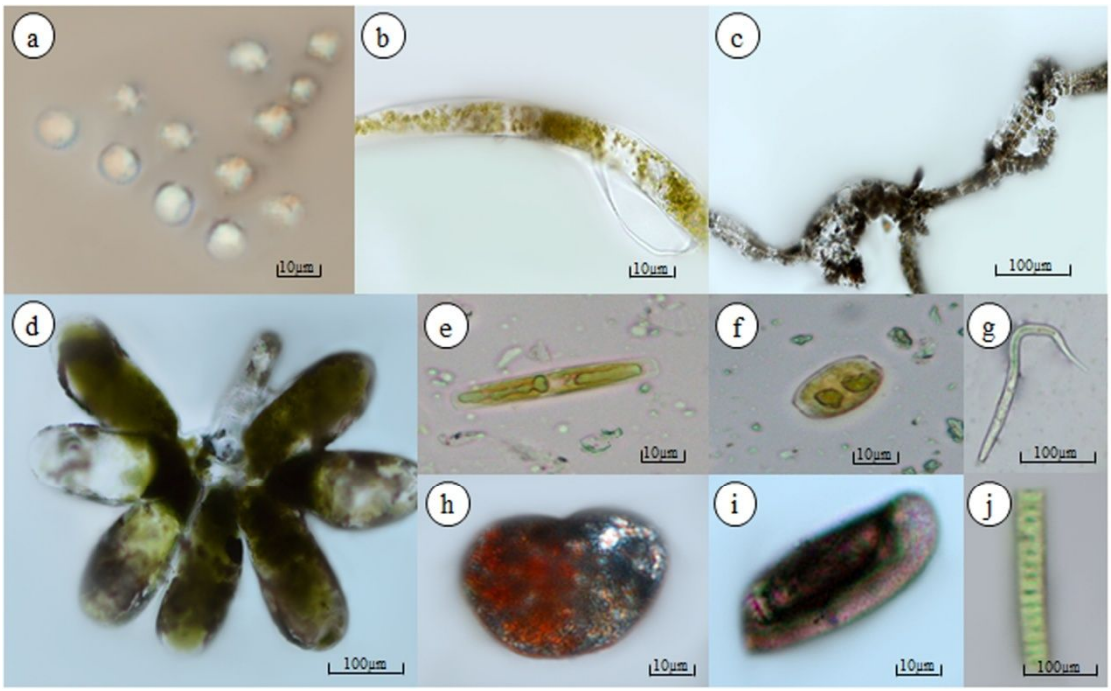

Fig. 3.8 Photographs of epilithic microbial communities (a) coccoid green algae; (b) green algae; (c) red calcareous algae; (d) algal colony; (e, f) diatoms; (g) nematode; (h, i) foraminifers; (j) cyanobacteria (Islam et al. 2012)

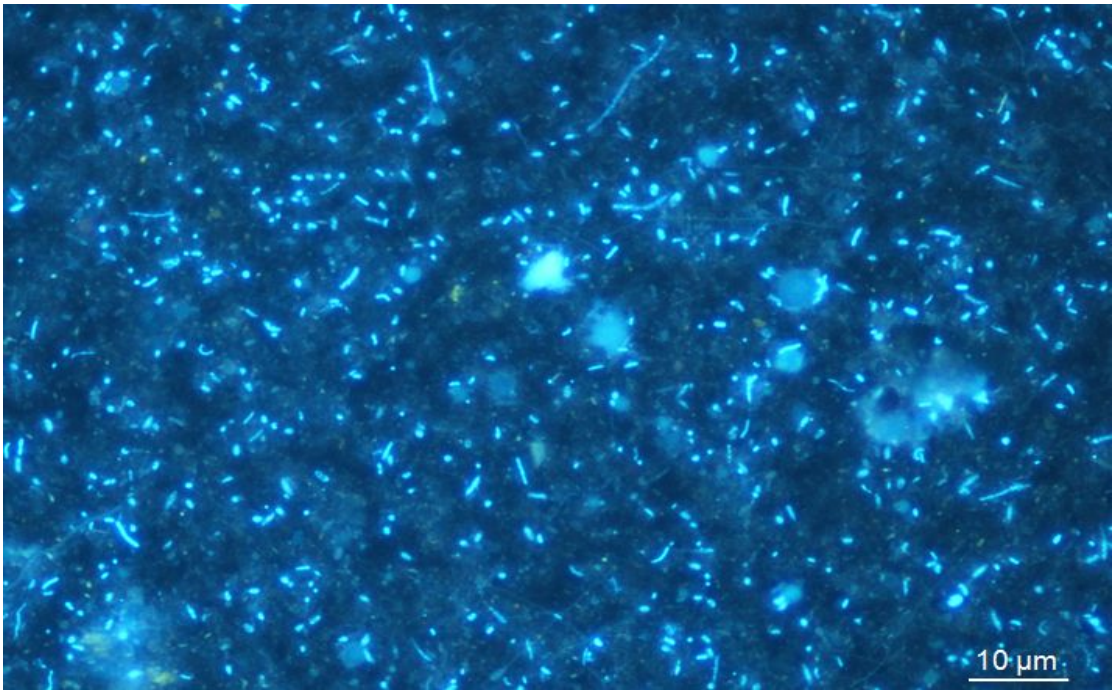

Fig. 3.9 Heterotrophic bacterial communities in coral rubble (Islam et al. 2012)

3.3.3 Daily metabolism of rubble associated community and their contribution to carbonate dissolution

The net primary production (NPP) and respiration rates (R) of natural rubble (NR) were 1373 ± 2.8 $\mu gCm^{-2}d^{-1}$ and 2536 ± 2.4 $\mu gCm^{-2}d^{-1}$ in ambient condition and 1803 ± 1.5 $\mu gCm^{-2}d^{-1}$ and 3328 ± 0.0 $\mu gCm^{-2}d^{-1}$ in high pCO_2 (1120 ppm) condition respectively. In case of treated rubble (TR), NPP and R were 1321 ± 1.4 and 2442 ± 2.8 $\mu gCm^{-2}d^{-1}$ in ambient condition and 1499 ± 1.5 and 2768 ± 0.0 $\mu gCm^{-2}d^{-1}$ in high pCO_2 (1120 ppm) condition respectively (Table 3.3). Respiration increased with primary production in high pCO_2 levels ($r = +1$; $p < 0.0005$). P/R ratios of natural rubble and treated rubble at both ambient and high pCO_2 (1120 ppm) conditions were similar (~1.54). Rates of gross primary production (GPP), net primary production (NPP), total respiration (R) and dark respiration rates (Rd) of the endolithic communities were higher than those of the epilithic communities.

Table 3.2 A species list of epilithic and endolithic communities of coral rubble at Sesoko Island, Okinawa, Japan

Epilithic Communities			
Autotrophs (Primary Producers)		**Heterotrophs**	
Species name	Abundance	Species name	Abundance
Chlorophyta (green algae)		**Protozoa (Sarcomastigophora)**	
Cladophora sp.	C	**Foraminifera**	A
Parvocaulis clavata	C	**Ciliophora (Ciliates)**	
Cyanophyta (blue-green algae)		*Undella* sp.	C
Hyella (cf.) *caespitosa* (spherical part)	C	*Dadayiella ganymedes*	C
Hydrocoleum homoeotrichum	C	**Nematoda**	
Lyngbya majuscula	C	*Acantholaimus* sp.	A
Oscillatoria sp.	F	*Halalaimus* sp.	C
Phormidium sp.	F	**Arthopoda**	
Calothrix sp.	F	**Crustacea (Copepods)**	
Phaeophyta (brown algae)		*Oithona nana*	A
Sphacelaria sp.	F	*Oithona simplex*	A
Rhodophyta (red algae)		*Microsetella norvegica*	C
Chroodactylon ornatum	F		
Polysiphonia sp.	F		
Bacillariophyta (diatom)			
Navicula sp.	A		
Nitzchia sp.	A		
Pleurosygma sp.	A		
Endolithic Communities			
Chlorophyta (green algae)			
Ostreobium sp.	A		
Holdfasts of *Parvocaulis* spp.	A		
Gomontia polyrhiza	F		
Cyanophyta (blue-green algae)			
Hyella (cf.) *caespitosa*	A		
Plectonema (cf.) *norvegium*	A		
Mastigocoleus testarum	A		
Scytonema sp.	A		
Schzothrix sp.	C		

F = Few; C = Common; A = Abundant

In addition, older coral rubble was contained more chlorophyll concentration than the young rubble (Fig. 3.10). Therefore, older rubble shows higher primary productivity. The endolithic communities were one of the major primary producers (83~96%) in coral rubbles indicating the importance of endolithic community over the epilithic community for the total rubble metabolism.

Dissolution rates were also high in natural rubble than in treated rubble at all pCO_2 levels. Therefore, a strong positive correlation between rubble community respiration and dissolution rates ($r = +0.98$; $p = 0.014$) was found. Endolithic communities contributed 85~91% of dissolution in coral rubble. No dissolution in the control (white coral skeleton) was found at both ambient and high pCO_2 (1120 ppm) conditions.

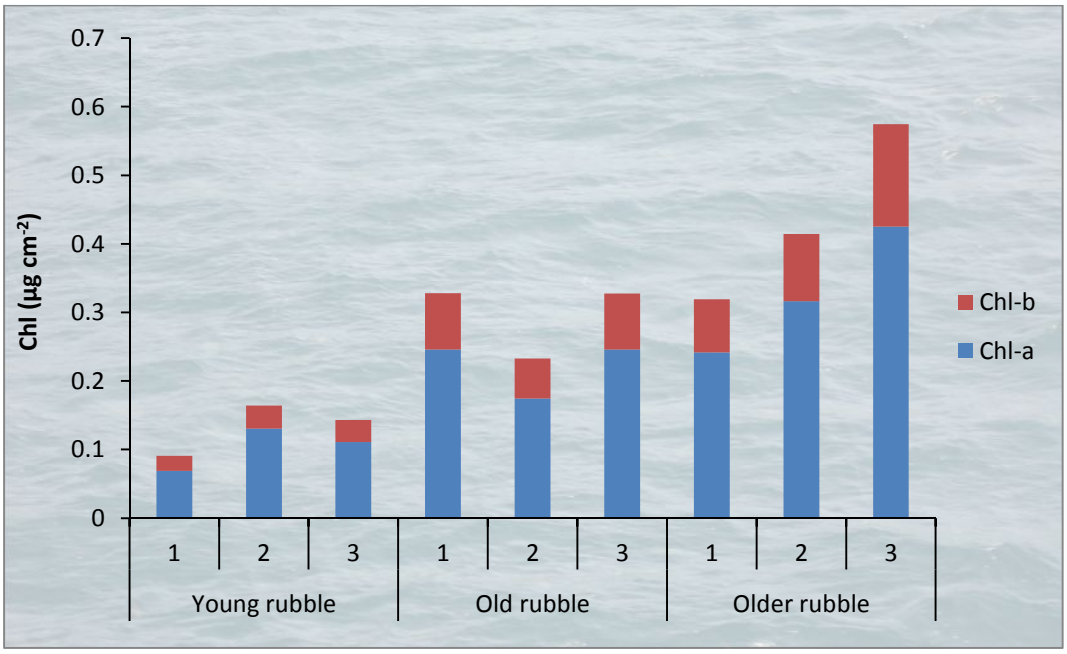

Fig. 3.10 Measurement of chlorophyll in different groups of coral rubble

Table 3.3 Summary result of 24h natural illumination short incubation experiment

Treatments	GPP [$\mu gCm^{-2}d^{-1}$]	NPP [$\mu gCm^{-2}d^{-1}$]	R [$\mu gCm^{-2}d^{-1}$]	Rd [$\mu gCm^{-2}d^{-1}$]	P/R	DR [$\mu mol\ m^{-2}d^{-1}$]
Ambient condition						
TR (endo)	3763 ± 0.2	1321 ± 1.4	2442 ± 2.8	1263 ± 1.6	1.54	90.8 ± 0.0
NR (epi + endo)	3909 ± 0.3	1373 ± 2.8	2536 ± 2.4	1293 ± 1.2	1.54	100.0 ± 0.1
Contribution of TR	96.25%	96.21%	96.27%	97.71%		90.84%
High pCO_2 (1120 ppm) condition						
TR (endo)	4269 ± 1.4	1499 ± 1.5	2768 ± 0.0	1433 ± 2.0	1.54	108.9 ± 0.1
NR (epi + endo)	5129 ± 1.4	1803 ± 1.5	3328 ± 0.0	1721 ± 1.1	1.54	127.6 ± 0.0
Contribution of TR	83.23%	83.09%	83.17%	83.26%		85.32%

NR: Natural rubbles with associated epilithic and endolithic communities; TR: Treated rubble keeping only their endolithic communities; GPP: Gross primary production rates; NPP: Net primary production rates; R: Total respiration; Rd: Dark respiration rates; P/R: ratio of gross primary production and respiration; DR: $CaCO_3$ dissolution rates; Mean ±SD (n = 3)

3.3.4 Physico-chemical and biological effects on carbonate dissolution

During long experiment (4 days) under dark condition, respiration increased with increasing pCO_2 levels from 62 ± 0.2 to 128 ± 1.5 ppm (NR). Consistent with increase in respiration, dissolution rates also increased from 38.4 ± 2.4 to 81.6 ± 0.2 $\mu mol\ m^{-2}d^{-1}$ in ambient and high pCO_2 condition respectively. On the other hand, with addition of glucose (4µM), bacterial abundance, respiration rates as well as dissolution rates were further increased (Table 3.4). Glucose addition had a significant effect on bacterial abundance ($r = +0.96$; $p = 0.03$); and strong positive correlation between bacterial abundance and respiration ($r = +0.99$; $p = 0.008$) as well as carbonate dissolution ($r = +0.99$; $p = 0.003$) was found. The aragonite saturation state (Ω_{arg}) decreased from 2.5 to 1.2 and strong negative correlation between saturation state and dissolution was observed ($r = –0.99$; $p = 0.01$) (Fig. 3.11).

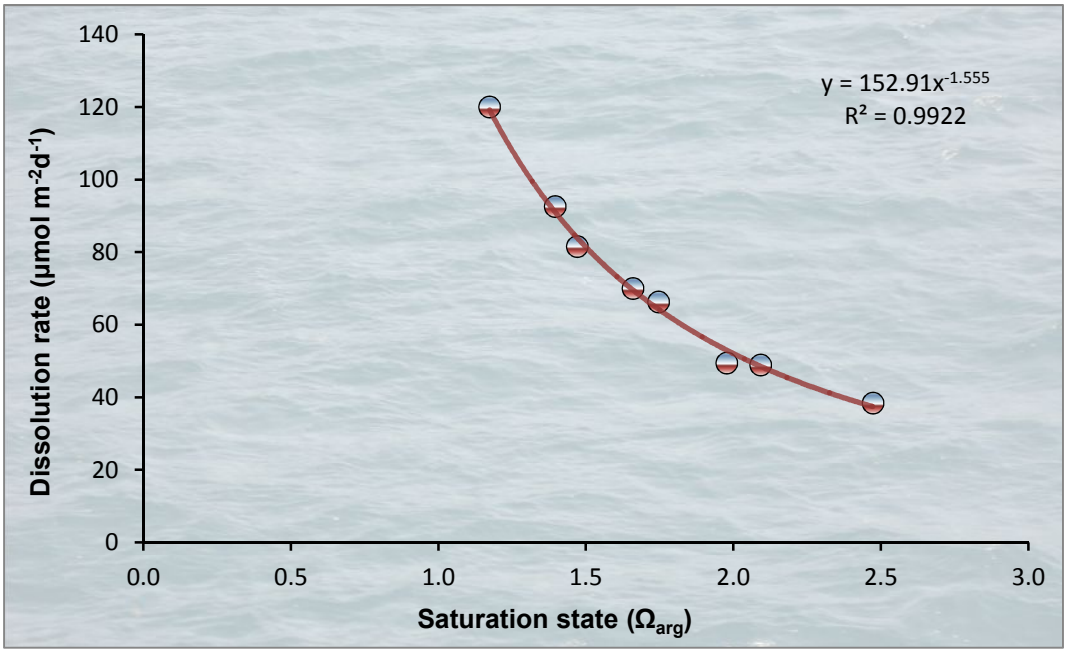

Fig. 3.11 Relationship between aragonite saturation state (Ω_{arg}) and carbonate dissolution rates

Table 3.4 Summary results of 4 days long incubation under dark condition

Treatments	Net Respiration [ΔCO_2 ppm]	Bacterial Abundance [Cells $\times 10^6$ ml^{-1}]	Dissolution rate [µmol $m^{-2}d^{-1}$]	Saturation state [Ω_{arg}]
Natural Rubble (NR)				
ambient	62 ± 0.2	4.3 ± 3.5	38.4 ± 2.4	2.5
520 ppm	76 ± 0.5	4.9 ± 2.0	48.9 ± 1.7	2.1
720 ppm	102 ± 0.1	5.5 ± 3.5	66.3 ± 0.7	1.7
1120 ppm	128 ± 1.5	6.1 ± 2.5	81.6 ± 0.2	1.5
NR with addition of glucose (4µM)				
ambient	243 ± 0.5	6.6 ± 5.5	49.5 ± 0.2	2.0
520 ppm	217 ± 1.1	7.1 ± 7.0	70.0 ± 0.2	1.7
720 ppm	281 ± 1.3	8.1 ± 3.5	92.6 ± 0.1	1.4
1120 ppm	323 ± 1.7	9.8 ± 4.5	120.0 ± 0.1	1.2

NR: Natural rubbles with associated epilithic and endolithic communities; Net respiration: [ΔCO_2 = CO_2 final – CO_2 initial]; Mean ±SD (n = 3)

3.3.5 Carbonate dissolution under natural system

Under natural illumination, the bacterial abundance and carbon dioxide production by respiration increased significantly with increasing pCO_2 levels ($p < 0.05$). Glucose addition further enhanced bacterial abundance and increased respiration rate (Table 3.5). The dissolution rates of carbonate increased with increasing pCO_2 levels ($r = 0.98$; $p = 0.015$) and with the addition of glucose (Table 3.5). Diurnal variation of carbonate dissolution was 23.7 ± 0.1 to 85.8 ± 1.2 µmol $m^{-2}d^{-1}$ at night time and 3.2 ± 1.1 to 8.4 ± 1.5 µmol $m^{-2}d^{-1}$ at day time (Fig. 3.12). The natural illumination experiment shows very small dissolution during day time and very high dissolution during night time (10 times higher than day time) (Table 3.5).

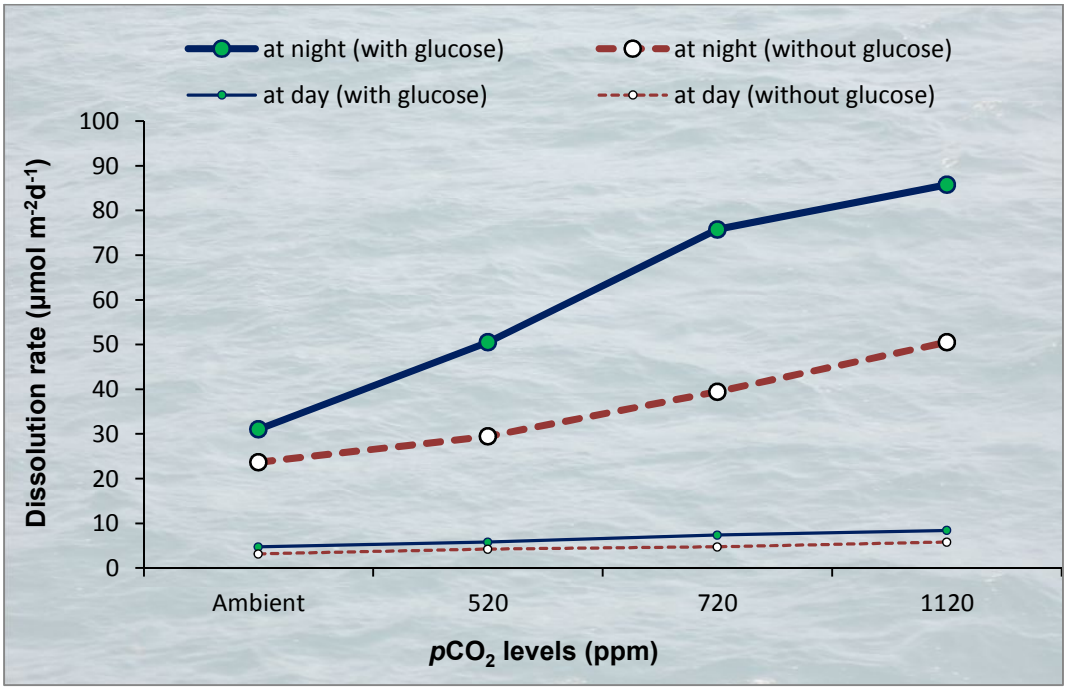

Fig. 3.12 Carbonate dissolution rates during day and night under different pCO_2 levels and addition of glucose

Table 3.5 Summary results of 4 days long incubation under natural illumination

Treatments	Bacterial Abundance [Cells $\times 10^6$ ml^{-1}]	Net Respiration [ΔCO_2 ppm]		Dissolution rate [µmol $m^{-2}d^{-1}$]		Saturation state [Ω_{arg}]	
		at night	at day	at night	at day	at night	at day
Natural Rubble (NR)							
ambient	4.5 ± 4.0	70 ± 0.1	9 ± 0.3	23.7 ± 0.1	3.2 ± 1.1	3.0	4.7
520 ppm	5.3 ± 1.5	119 ± 0.2	12 ± 0.8	29.5 ± 0.2	4.2 ± 1.3	2.5	4.6
720 ppm	6.2 ± 3.5	180 ± 0.5	16 ± 1.1	39.5 ± 1.1	4.7 ± 1.6	2.0	4.6
1120 ppm	7.2 ± 3.0	252 ± 0.9	19 ± 1.0	50.5 ± 1.5	5.8 ± 1.2	1.6	4.5
NR with addition of glucose (4µM)							
ambient	7.3 ± 6.0	109 ± 0.8	11 ± 0.6	31.1 ± 1.3	4.7 ± 0.7	2.8	4.6
520 ppm	9.4 ± 4.0	164 ± 0.9	16 ± 1.3	50.5 ± 0.5	5.8 ± 0.4	2.3	4.5
720 ppm	12 ± 6.5	234 ± 2.1	22 ± 1.1	75.8 ± 1.4	7.4 ± 1.3	1.9	4.1
1120 ppm	14 ± 7.0	351 ± 2.7	34 ± 2.1	85.8 ± 1.2	8.4 ± 1.5	1.5	4.0

NR: Natural rubbles with associated epilithic and endolithic communities; Values are given for midnight (00:00) and noon (12:00) measurements as night and day respectively; Net respiration: [ΔCO_2 = CO_2 final – CO_2 initial]; Mean ±SD (n = 3)

3.3.6 Carbonate balance

During 24h short incubation under natural illumination, the calcification and dissolution rates were 86 to113 µmol $m^{-2}d^{-1}$ and -100 to -128 µmol $m^{-2}d^{-1}$ under ambient and high pCO_2 condition respectively (Fig. 3.13).

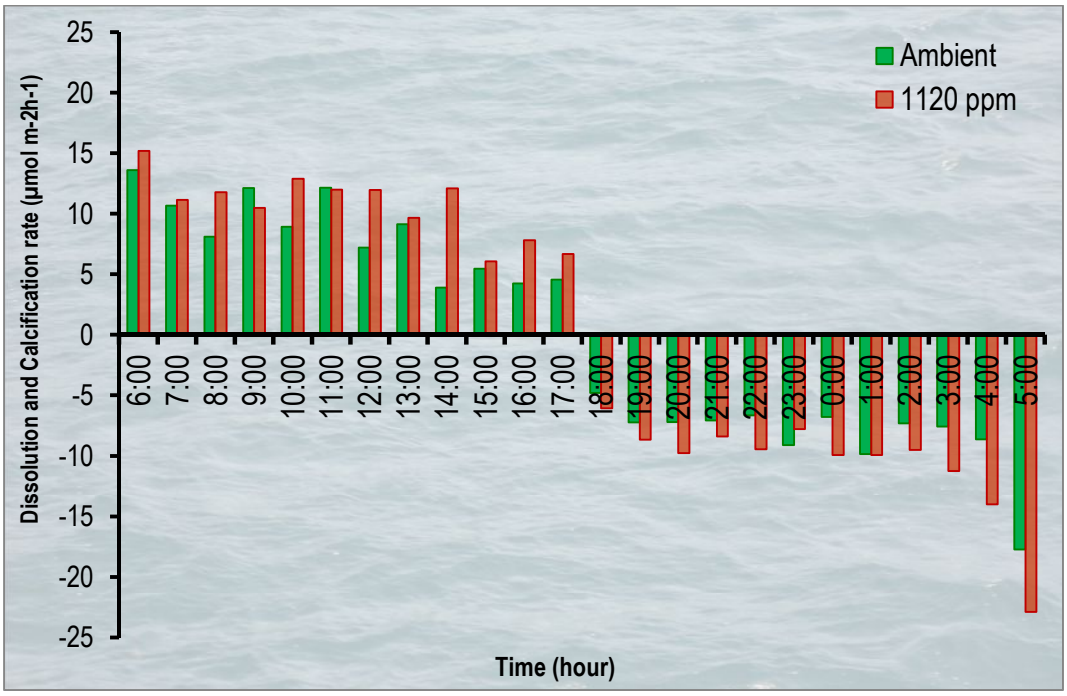

Fig. 3.13 Calcification and dissolution rates during short incubation

On the other hand, during 4 day's long incubation experiment under natural illumination; very small amount of dissolution were observed at day time (10 times lower than night time), and the average calcification and dissolution rates were 55 µmol $m^{-2}d^{-1}$ and -53 µmol $m^{-2}d^{-1}$ (Fig. 3.14). In the dark experiment when photosynthesis was inhibited, dissolution of calcium carbonate increased with increasing time scale and pCO_2 concentration level (Fig. 3.15).

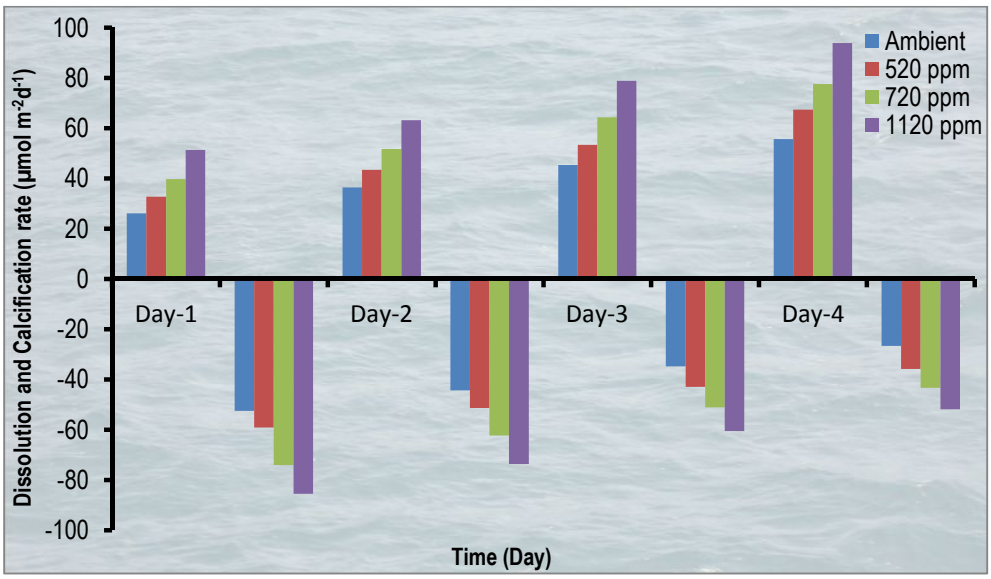

Fig. 3.14 Calcification and dissolution rates during long incubation

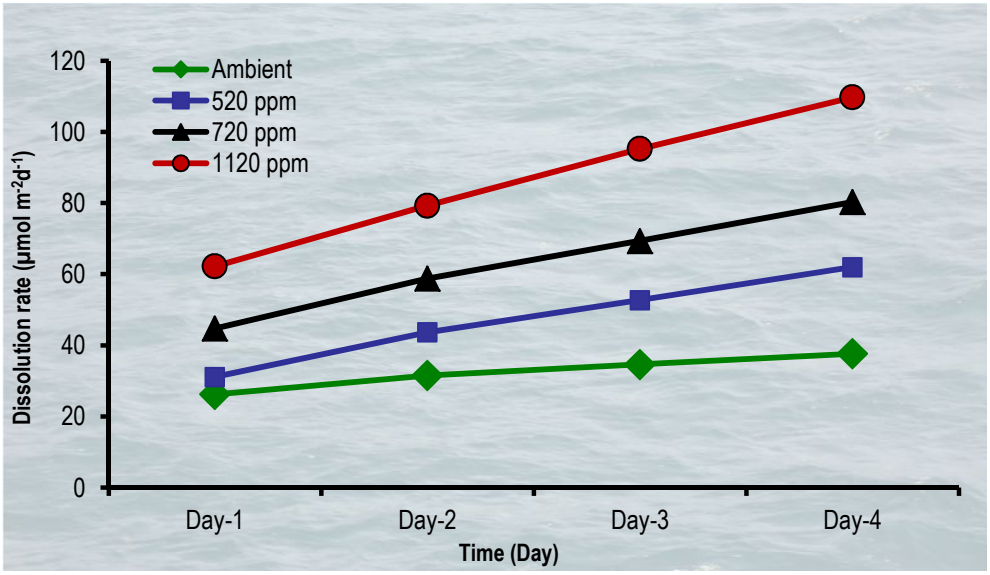

Fig. 3.15 Carbonate dissolution rates under dark incubation

3.4 Discussion

3.4.1 Rubble associated community and their contribution to carbonate dissolution

Assessment of rubble associated community (epilithic and endolithic) metabolism such as photosynthesis, respiration, calcification, and dissolution, has made it possible to characterize reef health. Endolithic phototrophs are one of the major primary producers (56~81% or 191±25 mmol C $m^{-2}d^{-1}$) in dead corals and coral rubbles (Wanders 1977; Tribollet et al. 2006b). Casareto et al. (2008) indicated that heterocysts possessing cyanobacteria are the main components of endolithic algae that contributed with 356~366 nmol C µg Chl-$a^{-1}d^{-1}$ as net primary production in coral rubble at Sesoko Island, Okinawa, Japan. In the natural illumination experiment, endolithic communities contributed 83~96% of net primary production. Significant effect of pCO_2 on net primary production and respiration of associated communities in coral rubble was found. Net primary production of epilithic communities was also enhanced and its contribution to NPP increased from 4% to 17%. The results showed that respiration was higher than net primary production over 24 h periods. Consistent with the increase of respiration, dissolution also increased with pCO_2. Rate of dissolution was higher for endolithic community (85~91%) than the epilithic. Previous studies also found that endolithic community produced large amount of CO_2 during respiration contributing in carbonate dissolution (Tudhope and Risk 1985; Tribollet et al. 2002; Tribollet and Golubic 2005; Ferran 2006). The results showed that for each micromole of CO_2 released from respiration of coral rubble community, 0.17 micromole of $CaCO_3$ was dissolved (Table 3.3). On the other hand, low magnitude of dissolution rate from the epilithic communities might be due to a rapid exchange of CO_2 in the surrounding seawater.

3.4.2 Physico-chemical vs. biological effects on carbonate dissolution

Ocean acidification as a result of rise in CO_2 is increasing the pCO_2 levels of the ocean. It has been shown that carbonate dissolution increases at 800 ~ 1000 ppm levels of pCO_2 (Halley et al. 2005). In this experiment, increase in the dissolution of calcium carbonate with increasing pCO_2 levels. It has been shown that dissolution of

calcium carbonate of calcareous organisms and sediments occurs due to reduction in CO_3^{2-} saturation state by ocean acidification (Gattuso et al. 1999; Kleypas et al. 1999; Barnes and Cuff 2000; Halley and Yates 2000; Andersson et al. 2003). Previous studies suggested that carbonate dissolution occurs when saturation state with respect to carbonate minerals is less than one (Gattuso et al. 1998; Langdon et al. 2000; Leclercq et al. 2002; Yamamoto et al. 2012). In coral reefs, large variation in CO_2 concentration and Ω occur due to their high diversity and productivity. It has been shown that the value of $\Omega_{arg} < 1$ (undersaturated) promotes dissolution (Gattuso et al. 1999; Kleypas et al. 1999). However others have shown that in coral reefs dissolution occurred even when Ω_{arg} remained at supersaturated state. The degree of super saturation (Ω) ranges from 3 ~ 4 for aragonite (Gattuso et al. 1999; Kleypas et al. 1999) in coral reefs. Previous studies reported that calcium carbonate dissolution occurred when Ω_{arg} ranged from 4.38 to 2.84 (Yates and Halley 2006); 3.06 to 1.83 (Ohde and van Woesik 1999) and 3.7 to 1.3 (Yamamoto et al. 2012). In this experiment also dissolution of calcium carbonate occurred when the saturation state (Ω_{arg}) ranged from 3.0 to 1.2. Therefore, Ω does not seem to be an appropriate indicator for calcification and dissolution in coral reef ecosystems.

In the dark experiment where photosynthesis was inhibited, dissolution of calcium carbonate increased with increasing pCO_2 levels. This suggests that CO_2 produced by respiration intensified dissolution in our experiment. It was found that dissolution increased with increase in abundance of bacteria. Moreover, when the abundance of bacteria was enhanced with the addition of glucose, the rate of dissolution was further increased (Table 3.4). This suggests that increasing the abundance of bacteria increased respiration and that resulted in dissolution of calcium carbonate. To estimate the contribution of bacteria for the total community respiration, compare data of night respiration from long incubation experiments at ambient pCO_2 conditions for NR vs. NR with addition of glucose. Addition of organic matter may not affect epilithic and endolithic algae respiration at natural pCO_2 and dark, however resulted in the growth of bacteria community. Increase of bacteria numbers with the addition of glucose might be correlated with the increase of respiration at dark period.

From this data set result concluded that an increase of 1 million bacteria per ml of seawater will increase night respiration in about 79 ppm of CO_2. Moulin et al. (1985) mentioned the bacterial respiration can induce rapid dissolution of carbonate in the sediments. Therefore, these results suggest that the availability of organic matter accelerates carbonate dissolution by increasing bacterial respiration.

3.4.3 Carbonate dissolution under natural system

Photosynthesis plays an important role in calcification (Schmalz and Swanson 1969; Yates and Halley 2006). Primary production by microalgae and cyanobacteria is enhanced under elevated pCO_2 (Riebesell et al. 1993; Wolf-Gladrow et al. 1998). Thomas et al. (2005) described that cyanobacteria can grow under elevated pCO_2, even up to 100% CO_2 in freshwater and other marine ecosystems. In this experiment primary production also increased with pCO_2 levels. This also reflects that increasing photosynthesis removes more CO_2. Therefore in the natural illumination experiment the dissolution rate were higher at night than on day. These results showed that the rates of dissolution were 23.7 to 85.8 μmol $m^{-2}d^{-1}$ at night time and 3.2 to 8.4 μmol $m^{-2}d^{-1}$ at day time, respectively. Diurnal variation of carbonate dissolution corresponding to day and night natural system was also found by Kinsey (1978) in One Tree Island Reef and by Barnes and Devereux (1984) in Rib Reef of the Great Barrier Reef in Australia. Important dissolution rates during dark period were also documented by previous studies (Leclercq et al. 2002; Yates and Halley 2006).

Further the dissolution rates were higher in dark incubation experiment where only respiration occurred compared to natural illumination experiment (*t*-test; $p = 0.004$) where both, photosynthesis and respiration occurred (Table 3.4 and 3.5). This suggests the importance of biological processes in determining calcification and dissolution. Similar to the dark experiment, addition of glucose enhanced heterotrophic bacterial abundance and their metabolic activities (respiration) enhanced dissolution. Therefore natural system may control dissolution and calcification through physiological activities (respiration and photosynthesis).

3.4.4 Carbonate balance

Acidification of the oceans by increasing anthropogenic CO_2 emissions will cause a decrease in biogenic calcification and an increase in carbonate dissolution (Yamamoto et al. 2012). The impact to coral reefs will depend in part upon the balance between calcification and dissolution. Due to nocturnal carbonate dissolution, negative carbonate balance was observed in short incubation. However, in the long incubation under natural illumination, dissolution rates gradually decreased and calcification rates increased with time scale and pCO_2 concentration level. Therefore, positive carbonate balance was observed in long incubation. High CO_2 enhanced growth of primary producers and microbial communities intensified calcification. These suggest that biological processes and the time scale play a significant role in determining calcification and dissolution.

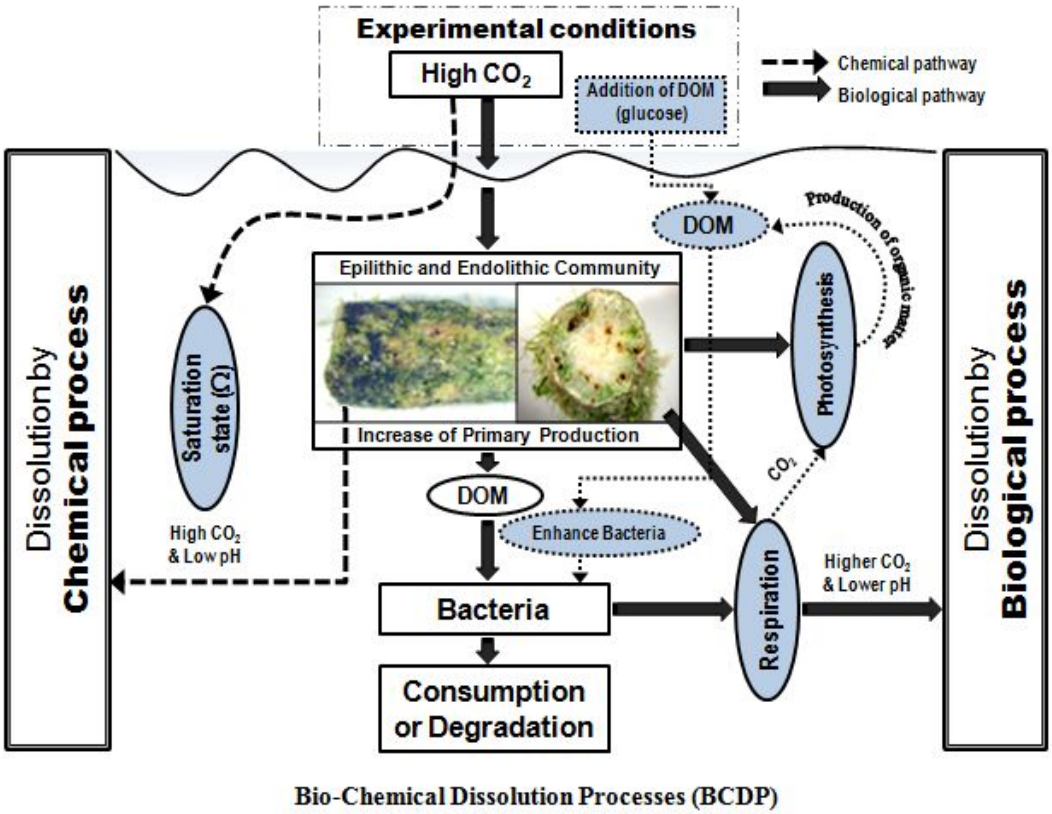

Fig. 3.16 Schematic diagram of "Bio-Chemical Dissolution Processes" (BCDP) showing the relative importance of biological vs. chemical processes to the dissolution of calcium carbonate. Dotted arrow indicates minor contribution for dissolution and solid arrow indicates major contribution for dissolution (Islam et al. 2012)

3.5 Conclusion

In conclusion, it seems that calcification and dissolution of calcium carbonate is not only governed by physico-chemical parameters but also by biological processes demonstrated by a "Bio-Chemical Dissolution Processes (BCDP)" (Fig. 3.16). In coral rubble the endolithic community seems to play a crucial role as primary producers and control dissolution of calcium carbonate. Furthermore, inputs of organic matter in the reefs also promote carbonate dissolution by enhancing heterotrophic microbial communities' activity (respiration).

3.6 References

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Chapter 4

The availability of organic matter accelerates carbonate dissolution by influencing bacterial activity under elevated pCO_2

Abstract: To determine the effects of organic matter and their availability accelerates carbonate dissolution by influencing bacterial activity under different level of pCO_2 was carried out using incubation experiments at Sesoko Island, Okinawa, Japan. Short incubation (24h under natural illumination) and long incubations (4 days under dark condition) were carried out using white coral skeleton (no attachment of living organism; control), natural rubble (with associated epilithic and endolithic communities) and natural rubble with addition of organic matter (glucose and coral mucus). Addition of bioavailable organic matter significantly enhanced bacterial abundance (*t*-test; p=0.01) and net respiration (*t*-test; p=0.0001) with increasing pCO_2 levels ($p<0.05$). Consistent with increase in respiration, dissolution rates also increased from 0.14 ± 0.0 to 0.65 ± 0.1 mmol $m^{-2}d^{-1}$. Under dark condition, where photosynthesis was inhibited, dissolution of calcium carbonate further increased with addition of different level of organic matter. In the organic matter addition incubation bottles, bacterial abundance and dissolution rates both increased 5~6 times more than the control (NR). On the other hand white coral skeleton without any attachment of living organism (control), very small amount (0.00~0.03±0.5 mmol $m^{-2}d^{-1}$) of dissolution were observed by the pressure of CO_2 only. This suggests that availability of organic matter accelerates carbonate dissolution with enhancing bacterial abundance and their physiological activities. Therefore, bioavailable organic matter has a potentially important role for calcium carbonate dissolution.

Keywords: Organic matter, Bacterial activity, Carbonate dissolution

4.1 Introduction

The organic matter in aquatic environment occurs in form of living organisms, organic detritus and dissolved substances. In case of coral reef ecosystems, habitually coral colonies release organic matter to the seawater as dissolved (DOM) and particulate organic matter (POM) (Tanaka et al. 2008). The coral-derived organic matter (DOC) is often collectively referred to as mucus, and that is rapidly utilized by bacteria for their enhanced growth and abundance. The coral mucus has been regarded as ecologically important, bacterial aggregation was found in coral mucus (Ducklow and Mitchell 1979) and coral exudates actually enhanced the growth of pico- and nanoplankton (Ferrier-Pagès et al. 2000). Some part of DOC in coral mucus was rapidly mineralized by bacteria into CO_2 in the reef sediment (Wild et al. 2004) and conversely, remain labile DOC contributes to long term C fixation as refractory organic matter (Ikeda et al. 2003; Tanaka et al. 2008).

Glucose, the most common monosaccharide (MCHO) in the seawater (Mopper et al. 1980), was used as the MCHO supplement because seawater concentrations remain consistently low (Burney et al. 1981; Johnson and Sieburth 1977), possibly indicating rapid uptake to a threshold level by the indigenous bacterial populations. Nevertheless, the marine environment is extremely oligotrophic (Morita 1988; Morita 1990; Munster 1993), which implies that microorganisms must face significant periods of shortage of carbon and energy (Kirchman 1990; Kjelleberg et al. 1987).

The purpose of the study is to investigate the role of bacteria to the dissolution of coral rubble by examining the response of coral rubble associated microbial community under elevated pCO_2. Moreover, to determine the effect of bioavailable organic matter accelerates carbonate dissolution by influencing bacterial activity under different levels of pCO_2.

4.2 Materials and methods

4.2.1 Study area and collection of samples

The study area is located in a shallow fringing coral reef at Sesoko Island, Ryukyu Archipelago, Okinawa, Japan between 26° 38′ N and 127° 51′ E (Fig. 2.1 in Chapter 2). The details description of study area has given in Chapter 2. Coral rubble samples was collected in the lagoon at about 1~2 m depth, and coral mucus was collected from *Acropora digitifera* species using air exposure method (Wild et al. 2004) (Fig. 4.1) into cleaned syringe and transferred into washed clean beaker. Seawater was collected by using 10 L Nalgene bottle. After collection, seawater was filtered using a cartridge filter (0.2 µm isopore membrane filter). The details sampling procedure has given in Chapter 2.

Fig. 4.1 Collection of coral mucus from *Acropora digitifera* species

4.2.2 Experimental design

Incubation experiment were carried out in natural illumination (24h short) and dark (4 day's long) under different level of pCO_2 conditions using natural rubble (NR: with associated epilithic and endolithic communities); natural rubble with addition of different level of glucose (NR+G) and natural rubble with coral mucus (NR+M) as source of organic matter to assess the role of organic maters on carbonate dissolution. As control, white coral skeleton (WCSk) was incubated in the same conditions as mentioned previously. The details experimental design has given in Table 2.1 in Chapter 2. Short (24h) and long (4 day's) time incubation experiments has been described in Fig. 4.2 and 4.3.

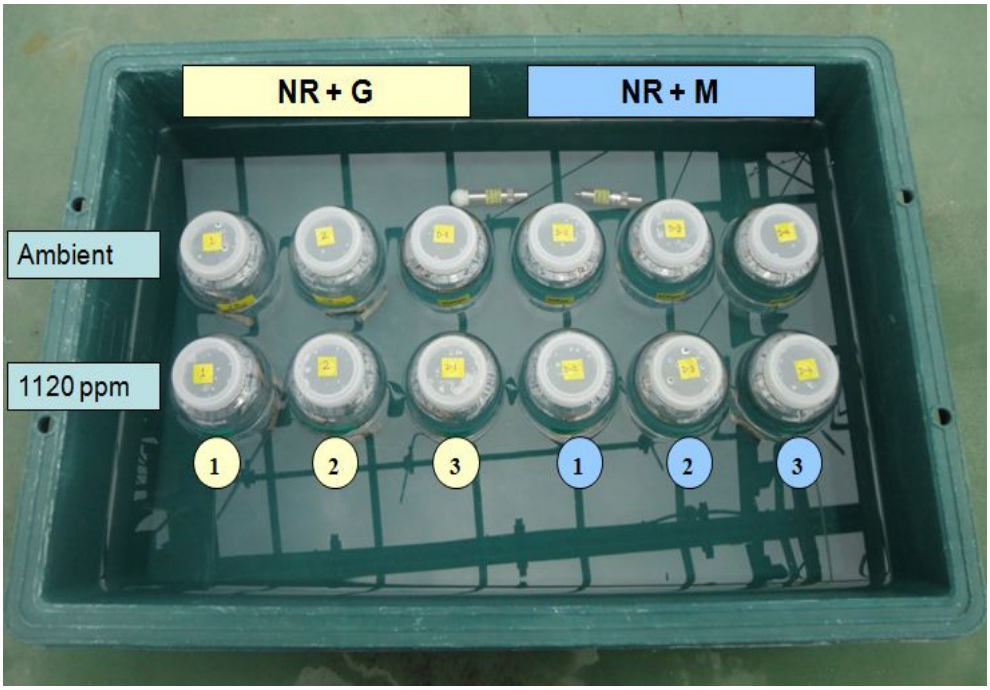

Fig. 4.2 Short incubation experiment (24h) under natural illumination system

Incubation were carried out in natural illumination condition (out door) under ambient and high pCO_2 (1120 ppm) using natural rubble with addition of organic matter glucose (NR+G) and coral mucus (NR+M) as source of organic matter to assess the role of bioavailable organic maters accelerates carbonate dissolution by influencing bacterial activity. [NR: Natural Rubble with associated epilithic and endolithic communities; NR+G: Natural rubble with addition of Glucose; NR+M: Natural rubble with addition coral Mucus]

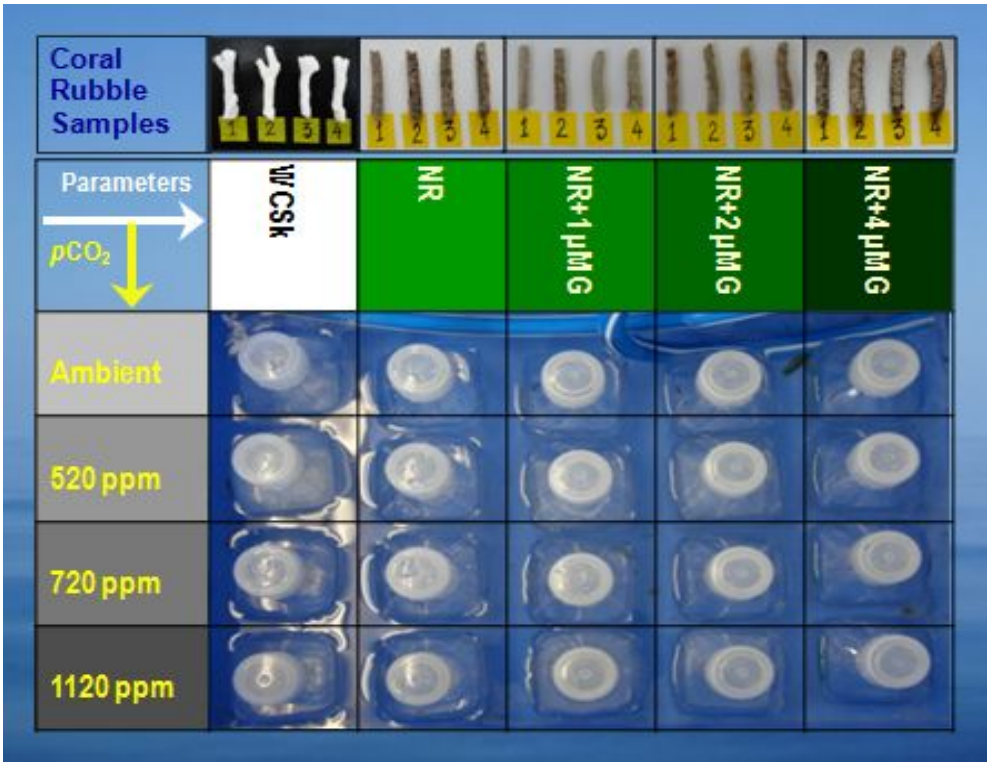

Fig. 4.3 Long incubation experiment (4 day's) under dark condition

incubation were carried out in dark condition using WCSk, NR and NR with addition of different level of (1µM, 2µM, 4µM) of glucose (G) as sources of organic matter under ambient, 520, 720 and 1120 ppm pCO_2 to test the contribution of physico-chemical vs. biological processes for the dissolution of calcium carbonate. [WCSk: White coral skeleton; NR: Natural Rubble with associated epilithic and endolithic communities]

4.2.3 Preparation of incubation experiments

The different levels of pCO_2 in the incubation bottles were adjusted by injecting CO_2 saturated seawater into the bottles until pH values equivalent to the desired pCO_2 levels were obtained. Glucose stock solution was prepared as 1.8 g l^{-1}, and 100µl, 200µl, 400µl and 1000µl of this solution were added to the incubation bottles (1L) to make final concentration of 1µM, 2µM, 4µM and 10µM. In case of coral mucus, 10% mucus solution was added to the incubation bottles. Incubations were conducted in natural temperature varied at 25°C to 33°C. Temperature and light intensity were monitored during the experiments using *in situ* sensor. The details procedure has been given in Chapter 2.

4.2.3 Laboratory measurement and analysis

pH, DO and Alkalinity (A_T) were measured 24 times per day (1h interval) for short incubation experiment and 4 times per day (6h interval) for long incubation experiment. Heterotrophic bacterial abundance was assessed by counting bacteria cells under an epifluorescence microscope. The details methods and procedure has been given in Chapter 2. Statistical data were analyzed and the variances with 0.05 level of significance. Labile DOC of each sample can be estimated from the difference between initial and final concentration of DOC. Net respiration was converted to carbon (C) using "Redfield-Richards Ratio" empirical formula (Libes 1992). Carbonate dissolution rates (µmol $l^{-1}d^{-1}$) were analyzed using the alkalinity anomaly technique (Smith 1973; Gattuso et al. 1997; Fujimura et al. 2001) and saturation state of calcium carbonate (Ω_{arg}) were calculated with the program CO2SYS (Lewis and Wallace 1998). The details calculation has described in Chapter 2.

4.3 Results

4.3.1 Addition of bioavailable organic matter

Bioavailable organic matter (glucose and coral mucus), was added to the incubation bottles to revitalize bacterial activity and their physiological activities accelerates carbonate dissolution. Addition of bioavailable organic matter enhanced bacterial abundance and dissolution rates were observed under natural rubble (NR), natural rubble with addition of glucose (NR+G) and with addition of coral mucus (NR+M) in ambient and high pCO_2 condition (Fig. 4.4 and Table 4.1). During 24h natural illumination experiment, the bacterial abundance (*t*-test; p=0.01) and net respiration (*t*-test; p=0.0001) increased significantly with addition of bioavailable organic matter and also with increasing pCO_2 levels ($p<0.05$). Consistent with increase in respiration, dissolution rates also increased (Table 4.1). In the organic matter addition incubation bottles, bacterial abundance and dissolution rates both increased 5~6 times more than the control (NR). The initial DOC value decreased exponentially to a constant level that represented refractory organic carbon. There were strong positive correlation between labile DOC and bacterial abundance (r= 0.99; p=0.0002) (Table 4.1).

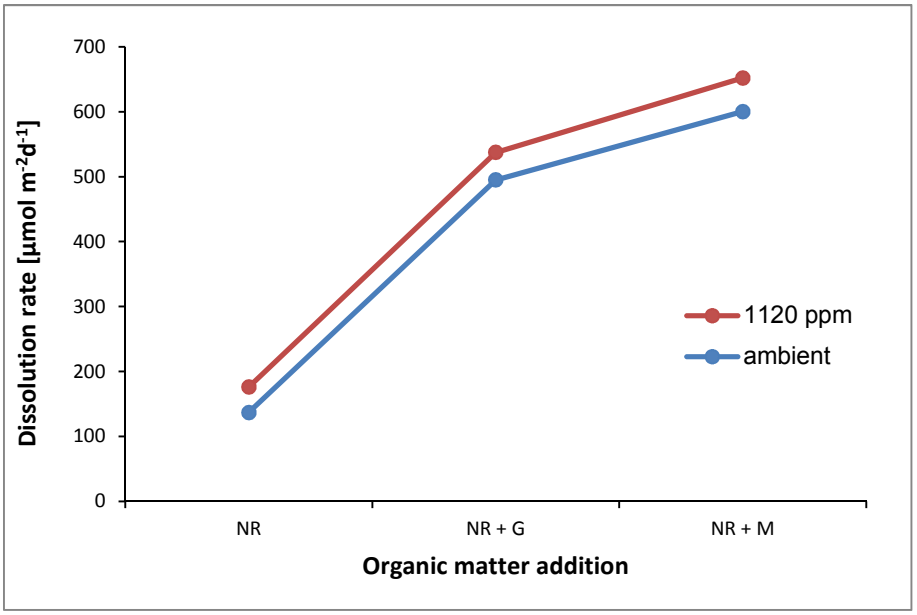

Fig. 4.4 Addition of organic matter increased carbonate dissolution

Table 4.1 Summary result of 24h natural illumination short incubation experiment

	Labile DOC (Initial – Final) [µmol C l^{-1}]	**Bacterial abundance** [Cells × 10^5 ml^{-1}]	**Net Respiration** [$mgCm^{-2}d^{-1}$]	**Dissolution rate** [mmol $m^{-2}d^{-1}$]	**Saturation state** [Ω_{arg}]
Ambient condition					
NR	29.10 ± 0.12	4.5 ± 0.01	316 ± 1.5	0.14 ± 0.0	2.87
NR + G	61.91 ± 0.32	16.4 ± 0.01	527 ± 0.8	0.50 ± 0.0	1.54
NR + M	74.01 ± 0.66	22.3 ± 0.02	612 ± 1.6	0.60 ± 0.1	1.22
High pCO_2 (1120 ppm) condition					
NR	31.83 ± 0.23	5.2 ± 0.02	354 ± 0.8	0.18 ± 0.1	2.42
NR + G	65.65 ± 0.46	18.2 ± 0.00	564 ± 1.0	0.54 ± 0.0	1.32
NR + M	80.38 ± 0.15	25.3 ± 0.01	683 ± 1.0	0.65 ± 0.1	1.13

NR: Natural rubbles with associated epilithic and endolithic communities; NR + G: Natural rubbles with addition of organic matter (Glucose); NR + M: Natural rubbles with addition of organic matter (10% Coral mucus); Natural illumination: Out door; Mean ± SD (n = 3)

4.3.1.1 Effect of glucose addition

After addition of glucose; bacterial abundance, net respiration rates as well as dissolution rates were increased. The bacterial abundance, net respiration and dissolution rates were 16.4±0.01×10^5 cells ml^{-1}, 527±0.8 $mgCm^{-2}d^{-1}$ and 0.50±0.0 mmol $m^{-2}d^{-1}$ in the ambient condition, whereas in high pCO_2 (1120 ppm) condition were 18.2±0.00×10^5 cells ml^{-1}, 564±1.0 $mgCm^{-2}d^{-1}$ and 0.54±0.0 mmol $m^{-2}d^{-1}$ respectively (Table 4.1). Glucose addition had a significant effect on bacterial abundance (*t*-test; p=0.01); and strong positive correlation between bacterial abundance vs. respiration (r=+0.99; p=0.0001) as well as carbonate dissolution (r=+0.99; p=0.0001) was found.

4.3.1.2 Effect of coral mucus addition

On the other hand, with addition of coral mucus (10%) as source of natural organic matter, bacterial abundance, net respiration rates as well as dissolution rates also increased higher than the glucose addition (Table 4.1). The bacterial abundance, net respiration and dissolution rates were 22.3±0.02×10^5 cells ml^{-1}, 612±1.6 $mgCm^{-2}d^{-1}$ and 0.60±0.1mmol $m^{-2}d^{-1}$ in the ambient condition, whereas in high pCO_2 (1120 ppm) condition were 25.3±0.01×10^5 cells ml^{-1}, 683±1.0 $mgCm^{-2}d^{-1}$ and 0.65 ± 0.1 mmol $m^{-2}d^{-1}$ respectively (Table 4.1). Addition of coral mucus also had a significant effect on bacterial abundance (*t*-test; p=0.01); and strong positive correlation between bacterial abundance vs. respiration (r=+0.99; p=0.0001) as well as carbonate dissolution (r=+0.99; p=0.0001) was found.

4.3.2 Level of organic matter addition

During 4 day's long incubation experiment under dark condition, where photosynthesis was inhibited, dissolution of calcium carbonate increased with increasing pCO_2 levels and different levels (1µM, 2µM and 4µM) of organic matter addition (glucose). After addition of different level of organic matter, the bacterial abundance and net respiration as well as dissolution rates were increased significantly ($p<0.05$). During incubation experiment of without addition of organic matter, the

bacterial abundance were 1.82±0.001×10^6 cells ml^{-1} of sample water and dissolution rate were 0.07±0.2 mmol $m^{-2}d^{-1}$ in the ambient condition, whereas in high pCO_2 (1120 ppm) condition were 2.39±0.001×10^6 cells ml^{-1} and 0.08±0.1 mmol $m^{-2}d^{-1}$ respectively. But in case of different levels of organic matter addition, the bacterial abundance were increased 8.24±0.002×10^6 ~ 14.62±0.002×10^6 cells ml^{-1} and dissolution rates were increased 0.15±0.2 ~ 0.32±0.2 mmol $m^{-2}d^{-1}$ with increasing both of glucose levels and pCO_2 levels ($p<0.05$) (Table 4.2). There were strong positive correlation between different level of organic matter addition and calcium carbonate dissolution (r=+0.99; p=0.0001).

Table 4.2 Summary results of 4 day's long incubation under dark condition with different level of organic matter addition

	Bacterial abundance [Cells ×10^3 ml^{-1}]	**Net Respiration** [ΔCO_2 ppm]	**Dissolution rate** [mmol $m^{-2}d^{-1}$]	**Saturation state** [Ω_{arg}]
WCSk				
Ambient	1.08 ± 0.014	03 ± 0.0	0.00 ± 0.0	1.53
520 ppm	1.17 ± 0.011	07 ± 0.0	0.01 ± 0.4	1.28
720 ppm	1.25 ± 0.015	12 ± 1.0	0.02 ± 0.4	1.06
1120 ppm	1.37 ± 0.010	16 ± 1.0	0.03 ± 0.5	0.88
NR	[Cells ×10^6 ml^{-1}]			
Ambient	1.82 ± 0.001	56 ± 1.5	0.07 ± 0.2	1.82
520 ppm	2.09 ± 0.002	80 ± 2.0	0.07 ± 0.1	1.53
720 ppm	2.19 ± 0.001	84 ± 1.0	0.08 ± 0.1	1.34
1120 ppm	2.39 ± 0.001	95 ± 1.0	0.08 ± 0.1	1.12
NR + G 1µM				
Ambient	8.24 ± 0.002	272 ± 2.0	0.15 ± 0.2	1.42
520 ppm	8.40 ± 0.001	284 ± 1.5	0.16 ± 0.4	1.28
720 ppm	8.60 ± 0.001	296 ± 2.5	0.17 ± 0.1	1.15
1120 ppm	8.94 ± 0.002	301 ± 1.0	0.17 ± 0.1	0.99
NR + G 2µM				
Ambient	9.00 ± 0.001	315 ± 1.0	0.17 ± 0.2	1.37
520 ppm	9.21 ± 0.002	326 ± 2.1	0.18 ± 0.1	1.24
720 ppm	9.33 ± 0.001	342 ± 2.6	0.19 ± 0.1	1.11
1120 ppm	9.62 ± 0.002	357 ± 1.5	0.20 ± 0.1	0.98
NR + G 4µM				
Ambient	11.95 ± 0.001	458 ± 3.3	0.28 ± 0.1	1.28
520 ppm	12.54 ± 0.001	472 ± 2.1	0.29 ± 0.1	1.17
720 ppm	13.57 ± 0.001	484 ± 2.5	0.30 ± 0.1	1.07
1120 ppm	14.62 ± 0.002	496 ± 2.5	0.32 ± 0.2	0.96

NR: Natural rubbles with associated epilithic and endolithic communities; NR + G: Natural rubbles with addition of different level of organic matter (1µM, 2µM & 4µM of Glucose); Net respiration: [ΔCO_2 = CO_2 final – CO_2 initial]; Mean ± SD (n = 3)

4.3.3 Carbonate dissolution vs. Saturation state

During 24h natural illumination experiment, with increasing carbonate dissolution rates (from 0.14±0.0 to 0.65±0.1 mmol $m^{-2}d^{-1}$), aragonite saturation state (Ω_{arg}) were decreased (from 2.87 to 1.13) in ambient and high pCO_2 condition respectively. Coral mucus addition had more significant effect than glucose addition for carbonate dissolution as well as CO_3^{2-} saturation state. In case of 4 day's long incubation experiment (under dark condition), carbonate dissolution rates were increased from 0.07±0.2 to 0.32±0.2 mmol $m^{-2}d^{-1}$ and aragonite saturation state (Ω_{arg}) were decreased from 1.82 to 0.96 under different level of organic matter (glucose) addition and different pCO_2 levels. There were strong negative correlation between carbonate dissolution rate and saturation state (r=–0.99; p=0.0002) (Fig. 4.5).

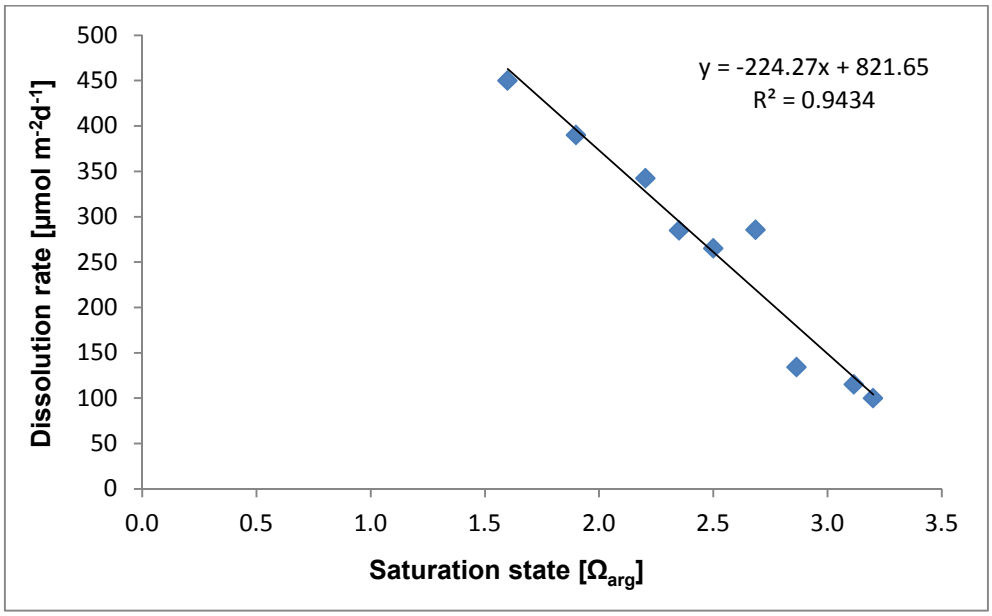

Fig. 4.5 Relationship between aragonite saturation state (Ω_{arg}) and carbonate dissolution rates

4.4 Discussion

4.4.1 Addition of bioavailable organic matter

Natural seawater normally contains 10^5 to 10^6 bacterial cells ml^{-1} (Van Es et al. 1982). The bacterial composition of natural seawater is regulated by such factors as grazing pressure (Simek, et al. 1992; Wright 1988), nutrient limitation or starvation (Kirchman 1990; Kjelleberg et al. 1993), and various other physicochemical stresses (e.g., temperature variation, oxidative stress, etc.). Addition of bioavailable organic matter (glucose and coral mucus) to the samples was influencing bacterial activity (Natori et al. 2006), growth and their abundance; and caused subsequent increase (5~6 times more than the control) of calcium carbonate dissolution in the dissolution experiments (Table 4.2). Activated bacteria produce CO_2 by the respiration and accelerated carbonate dissolution by addition of bioavailable organic matter. As a source organic matter, coral mucus addition effect were higher than the glucose effect for activation of bacteria and carbonate dissolution acceleration with increasing pCO_2 levels ($p<0.05$). Results suggest that the availability of organic matter accelerates carbonate dissolution in seawater by influencing bacterial activity.

4.4.2 Level of organic matter addition

Natural rubble (NR) is colonized by epilithic and endolithic communities with other organisms of Heterotrophs as foraminifera, nematodes and copepods (Islam et al. 2012). When photosynthesis is inhibited under dark condition, calcium carbonate dissolution was increased with increasing pCO_2 and addition of glucose levels. On the other hand without any attachment of living organism, very small amount ($0.00\sim0.03\pm0.5$ mmol $m^{-2}d^{-1}$) of dissolution were observed from white coral skeleton (control) by the pressure of CO_2 only. This suggests that dissolution increased with enhance bacterial abundance and their physiological activities, and availability of organic matter also accelerates carbonate dissolution. Different level of organic matter (glucose) addition regulates bacterial activity as well as dissolution rates. The effect of adding glucose (3 mM) was examined by Eguchi et al. (1996); bacterial cells grow instantaneously and increased cell density after glucose addition, probably because of rapid initial intracellular poly glucose accumulation (Preyer et al. 1993). Church et al.

(2000) found that biomass production of heterotrophic bacteria in the Southern Ocean was stimulated by addition of organic carbon. Organic carbon additions also consistently stimulated bacterial growth rates in the subarctic Pacific (Kirchman 1990) and the equatorial Pacific (Kirchman and Rich 1997). Moulin et al. (1985) mentioned the bacterial respiration of organic matter can induce rapid dissolution of a significant amount of carbonate in the sediments. However, during 4 day's long incubation experiment under dark condition, bacterial abundance 14 times higher than the normal seawater with addition of different level of organic matter (1µM, 2µM and 4µM glucose), and also found highly significant effect (r=+0.999; p=0.0005) on different level of organic matter addition. Consequently, results suggest that level of organic matter addition increased bacterial abundance and their physiological activity (especially respiration) regulates carbonate dissolution in seawater.

4.4.3 Carbonate dissolution vs. Saturation state

Calcium carbonate dissolution occurs due to reduction in CO_3^{2-} saturation state and the value of $\Omega_{arg} < 1$ promotes dissolution (Gattuso et al. 1999; Kleypas et al. 1999; Barnes and Cuff 2000; Halley and Yates 2000; Andersson et al. 2003). However, in coral reefs shows large variation in CO_2 concentration and Ω occur due to their high diversity and productivity. Therefore, below the super saturation threshold value 3~4 for aragonite (Gattuso et al. 1999; Kleypas et al. 1999) dissolution were observed. Previous studies reported that calcium carbonate dissolution occurred when Ω_{arg} ranged from 4.38 to 2.84 (Yates and Halley 2006); 3.06 to 1.83 (Ohde and van Woesik 1999); 3.7 to 1.3 (Yamamoto et al. 2012) and 3.0 to 1.2 (Islam et al. 2012). In this experiment dissolution of calcium carbonate occurred when the saturation state (Ω_{arg}) ranged from 2.87 to 1.13 in natural illumination experiment and from 1.82 to 0.96 in dark incubation experiment under different level of organic matter addition with increasing pCO_2 levels. Result showed strong negative correlation between carbonate dissolution rate and saturation state (r=–0.99; p=0.0002) (Fig. 4.2).

4.5 Conclusion

Bioavailable organic matter has a potentially important role for calcium carbonate dissolution. Levels of organic matter in the coral reefs also accelerate carbonate dissolution by enhancing growth of bacteria and their activity (respiration) under different levels of pCO_2.

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Chapter 5

Bio-Chemical Dissolution Processes (BCDP): A novel view of carbonate dissolution in coral reef ecosystems

Abstract: Calcium carbonate dissolution governed by "Bio-Chemical Dissolution Processes (BCDP)" in coral reef ecosystems. It was carried out using incubation experiments under different level of pCO_2 at Sesoko Island, Okinawa, Japan. Experiments were conducted using white coral skeleton (no attachment of living organism), natural rubble (with associated epilithic and endolithic communities) and natural rubble with addition of organic matter (glucose and coral mucus) under natural illumination and dark condition. Elevated CO_2 influenced carbonate ($CaCO_3$) dissolution in white coral skeleton (WCSk; control) but the magnitude of dissolution rates was very small. On the other hand natural rubble (NR) showed higher dissolution rates than the control in both of dark and natural condition. During dark incubation experiment when photosynthesis is inhibited, the respiration of coral rubble associated communities contributed around 80% of carbonate dissolution under elevated pCO_2. Additionally, CO_2 influenced carbonate dissolution in white coral skeleton (WCSk; control), but the magnitude of dissolution was very small. This suggests that CO_2 produced by the microbial community respiration intensified dissolution in our experiment. In case of organic matter (glucose and coral mucus) addition; bacterial abundance, respiration rates as well as dissolution rates were further increased. Therefore, in coral reef ecosystem, calcium carbonate ($CaCO_3$) dissolution is not only governed by chemical processes but also biological processes (photosynthesis and respiration) has induced and play a significant role in determining calcification and dissolution as demonstrated in the "Bio-Chemical Dissolution Processes (BCDP)".

Keywords: Calcium carbonate, Organic matter, Bacterial activity, Bio-Chemical Dissolution Processes (BCDP)

5.1 Introduction

Calcium carbonate dissolution is strongly influenced by the presence and availability of CO_2. Rising of anthropogenic carbon dioxide (CO_2) in atmosphere and ocean acidification could result in increased dissolution of calcium carbonate in marine and coastal environment.

$$CaCO_3 + H_2O + CO_2 \leftrightarrows Ca^{+2} + 2\ HCO_3^-$$

Coral reef is characterized specifically as high productivity and high diversity area, indicating that high variations of pH (8.5 to 7.8) as well as pCO_2 (300 to 800 ppm) during daytime to night time and renowned at risk to ocean acidification. However, there are several questions for ocean acidification. One is to calculate the dissolution of carbonate based on the solubility (Ω) of calcite or aragonite, question is whether dissolution is calculated by only physical-inorganic processes, how is biological processes. Another is to apply idea and/ or results to coral reef ecosystem as well as that of open ocean; because coral reef has so much diversity and large variation of CO_2 concentrations rather than those of open ocean. Therefore, coral reef is not appropriate area to apply straightly idea and/or results of calculation as to ocean acidification. Important question is to determine the dissolution of carbonate based on the solubility (Ω) of calcite or aragonite, because production and dissolution of calcifying organism in coral reef is dominated by biological processes.

The hypothesis is that dissolution is primary determined by biological effect. For examination of this hypothesis, coral rubble is good carbonate materials as interaction between organic matters and inorganic carbonate. Until now many studies on ocean acidification have focused on the physico-chemical aspects. However, biological processes (photosynthesis and respiration) are more important in determining calcification and dissolution.

5.2 Materials and Methods

To understand the carbonate dissolution by changing chemically or biologically; dark and natural illumination experiments were carried out under different level of pCO_2 condition using white coral skeleton, natural rubble and natural rubble with addition of organic matter (glucose and coral mucus) for the simulation as natural reef environment. Partial pressure of carbon dioxide (pCO_2), pH, DO and total alkalinity (A_T) were measured and analyzed carbonate dissolution rates in compare to chemical and biological processes. The details methods and calculation has been described in Chapter 2, 3 & 4.

5.3 Results and Discussion

Rising CO_2 in atmosphere is increasing the levels of pCO_2 in the ocean and increasing rates of carbonate dissolution (Halley et al. 2005) but the magnitude of dissolution rates by chemical interaction is very small (Islam et al. 2012). Natural rubble (NR) is colonized by epilithic and endolithic algal communities and by other heterotrophic organisms as bacteria, foraminifera, nematodes, copepods and other crustaceans. On the other hand white coral skeleton (WCSk) is no attachment of living organism. When photosynthesis is inhibited under dark condition, the respiration of coral rubble associated communities contributed around 80% of carbonate dissolution under elevated pCO_2. Additionally, CO_2 influenced carbonate dissolution in white coral skeleton (WCSk; control), but the magnitude of dissolution is very small which contributed around 20%. This suggests that CO_2 produced by the microbial community respiration intensified dissolution in our experiment. Moulin et al. (1985) reported that the bacterial respiration can induce rapid dissolution of carbonate. In addition of organic matter (glucose and coral mucus); bacterial abundance, respiration rates as well as dissolution rates were further increased. This suggests that availability of organic matter enhanced bacterial growth and with increasing bacterial abundance pCO_2 levels also increased by the respiration and that resulted in dissolution of calcium carbonate (Fig. 5.1).

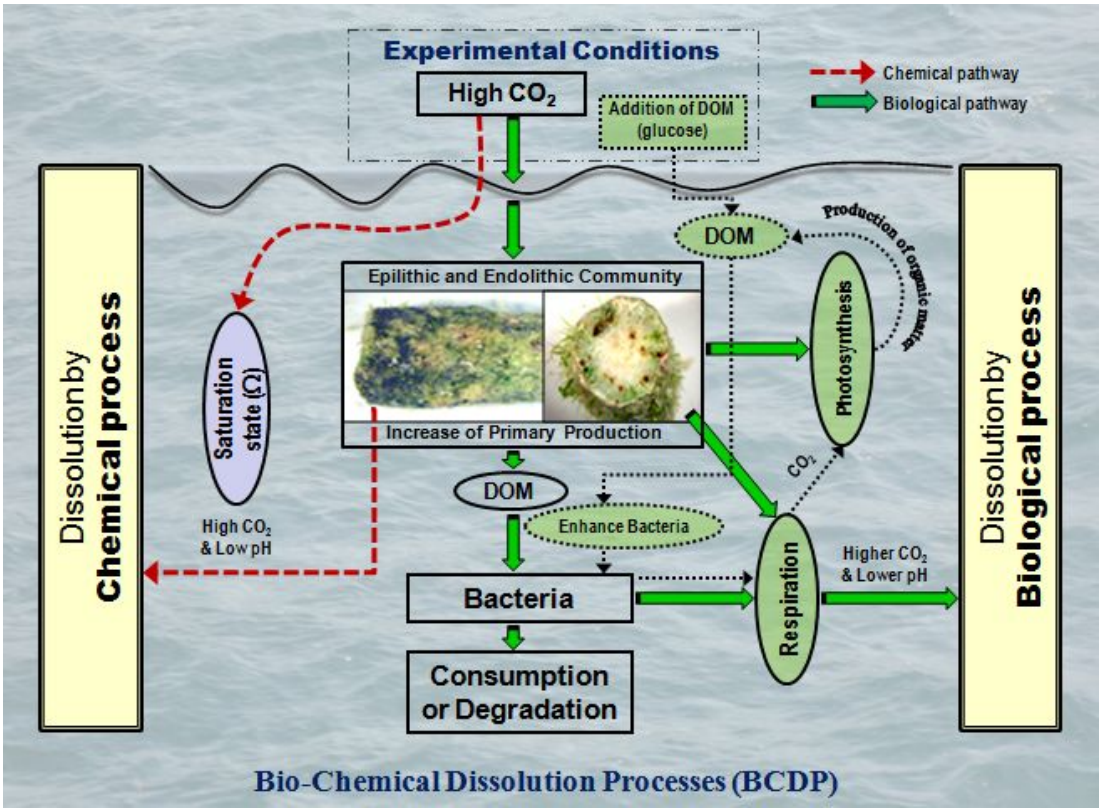

Fig. 5.1 Schematic diagram of "Bio-Chemical Dissolution Processes" (BCDP) showing the relative importance of biological vs. chemical processes to the dissolution of calcium carbonate. Dotted arrow indicates minor contribution for dissolution and solid arrow indicates major contribution for dissolution (Islam et al. 2012)

In case of natural illumination experiment, increased pCO_2 levels may also enhance photosynthesis. However, as the active uptake of carbon dioxide by photosynthesis contribute to the sinks of global atmospheric CO_2. Therefore the dissolution rates were very small at day time (10 times lower than night time) in this experiment. Diurnal variation of carbonate dissolution was also observed by Kinsey (1978), and Barnes and Devereux (1984) in the Great Barrier Reef of Australia. Nevertheless, the active uptake of carbon dioxide by photosynthesis at day time and its active release of carbon dioxide both day and night by the whole community respiration determining calcification and dissolution. These suggest that biological processes (photosynthesis and respiration) has play a crucial role in determining calcification and dissolution.

5.4 Conclusion

In coral reefs ecosystem, calcium carbonate ($CaCO_3$) dissolution is not only governed by physico-chemical processes but also biological processes (photosynthesis and respiration) play a vital role in determining calcification and dissolution as demonstrated in the "Bio-Chemical Dissolution Processes (BCDP)". Moreover, bioavailable organic matter has a potentially important role for calcium carbonate dissolution by enhancing growth of bacteria and their physiological activities.

5.5 References

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Appendices

Appendix Table 1 Measurement of rubble surface area for 24h short incubation [Natural illumination] in 2011

24h Short Incubation [Natural illumination]							
		Length	**±SD**	**Diameter**	**±SD**	**Surface area $A = 2\pi r2 + 2\pi rh$**	**±SD**
Ambient		cm		cm		cm^2	
NR_A	1	5.510	0.014	1.000	0.012	18.871	0.044
	2	5.490		1.000		18.809	
	Av	5.500		1.000		18.840	
NR_B	1	5.570	0.057	1.000	0.007	19.060	0.322
	2	5.490		0.990		18.605	
	Av	5.530		0.995		18.832	
NR_C	1	5.590	0.078	0.980	0.021	18.709	0.192
	2	5.480		1.010		18.981	
	Av	5.535		0.995		18.847	
Ambient							
TR_A	1	5.480	0.014	1.010	0.014	18.981	0.244
	2	5.500		0.990		18.636	
	Av	5.490		1.000		18.809	
TR_B	1	5.530	0.035	0.980	0.028	18.525	0.467
	2	5.480		1.020		19.185	
	Av	5.505		1.000		18.856	
TR_C	1	5.550	0.071	0.980	0.028	18.586	0.355
	2	5.450		1.020		19.089	
	Av	5.500		1.000		18.840	
1120 ppm							
NR_A	1	5.510	0.021	1.000	0.007	18.871	0.078
	2	5.540		0.990		18.760	
	Av	5.525		0.995		18.816	
NR_B	1	5.430	0.071	1.030	0.042	19.227	0.641
	2	5.530		0.970		18.320	
	Av	5.480		1.000		18.777	
NR_C	1	5.510	0.021	1.000	0.000	18.871	0.067
	2	5.480		1.000		18.777	
	Av	5.495		1.000		18.824	
1120 ppm							
TR_A	1	5.480	0.035	1.010	0.014	18.981	0.178
	2	5.530		0.990		18.729	
	Av	5.505		1.000		18.856	
TR_B	1	5.490	0.021	0.990	0.014	18.605	0.355
	2	5.520		1.010		19.108	
	Av	5.505		1.000		18.856	
TR_C	1	5.520	0.014	0.990	0.014	18.698	0.245
	2	5.500		1.010		19.044	
	Av	5.510		1.000		18.871	
	Grand Av	**5.507**	**0.025**	**0.999**	**0.012**	**18.835**	**0.175**

Appendix Table 2 Measurement of rubble surface area for 24h Short Incubation [Natural illumination] in 2012

24h Short Incubation [Natural illumination]							
		Length	**±SD**	**Diameter**	**±SD**	**Surface area A=2πr2 + 2πrh**	**±SD**
Ambient		cm		cm		cm2	
NR_A	1	4.100	0.141	1.000	0.012	14.444	0.209
	2	4.300		0.980		14.740	
	Av	4.200		0.990		14.595	
NR_B	1	4.200	0.071	1.000	0.007	14.758	0.104
	2	4.300		0.990		14.906	
	Av	4.250		0.995		14.833	
NR_C	1	4.300	0.141	0.980	0.021	14.740	0.096
	2	4.100		1.010		14.604	
	Av	4.200		0.995		14.676	
Ambient							
NR+G_A	1	4.300	0.071	1.010	0.014	15.239	0.016
	2	4.400		0.990		15.217	
	Av	4.350		1.000		15.229	
NR+G_B	1	4.200	0.071	0.980	0.028	14.432	0.235
	2	4.100		1.020		14.765	
	Av	4.150		1.000		14.601	
NR+G_C	1	4.200	0.071	0.980	0.028	14.432	0.235
	2	4.100		1.020		14.765	
	Av	4.150		1.000		14.601	
Ambient							
NR+M_A	1	4.000	0.141	1.010	0.014	14.287	0.218
	2	4.200		0.990		14.595	
	Av	4.100		1.000		14.444	
NR+M_B	1	4.200	0.071	0.980	0.028	14.432	0.688
	2	4.300		1.020		15.405	
	Av	4.250		1.000		14.915	
NR+M_C	1	4.000	0.071	0.980	0.028	13.817	0.671
	2	4.100		1.020		14.765	
	Av	4.050		1.000		14.287	
1120 ppm							
NR_A	1	4.400	0.071	1.000	0.012	15.386	0.222
	2	4.300		1.000		15.072	
	Av	4.350		1.000		15.229	
NR_B	1	4.300	0.071	1.000	0.007	15.072	0.337
	2	4.200		0.990		14.595	
	Av	4.250		0.995		14.833	
NR_C	1	4.100	0.071	0.980	0.021	14.124	0.564
	2	4.200		1.010		14.921	
	Av	4.150		0.995		14.520	
1120 ppm							
NR+G_A	1	4.300	0.071	1.010	0.014	15.239	0.455
	2	4.200		0.990		14.595	
	Av	4.250		1.000		14.915	
NR+G_B	1	4.400	0.071	0.980	0.028	15.048	0.253
	2	4.300		1.020		15.405	
	Av	4.350		1.000		15.229	
NR+G_C	1	4.100	0.071	0.980	0.028	14.124	0.679
	2	4.200		1.020		15.085	
	Av	4.150		1.000		14.601	
1120 ppm							
NR+M_A	1	4.200	0.071	1.010	0.014	14.921	0.451
	2	4.100		0.990		14.284	
	Av	4.150		1.000		14.601	
NR+M_B	1	4.300	0.141	0.980	0.028	14.740	0.018
	2	4.100		1.020		14.765	
	Av	4.200		1.000		14.758	
NR+M_C	1	4.000	0.141	0.980	0.028	13.817	0.897
	2	4.200		1.020		15.085	
	Av	4.100		1.000		14.444	
	Grand Av	**4.203**	**0.033**	**0.998**	**0.008**	**14.739**	**0.258**

Appendix Table 3 Measurement of rubble surface area for 4 day's long incubation in 2011

4 day's long Incubation [Dark]							
		Length	±SD	Diameter	±SD	Surface area $A=2\pi r2 + 2\pi rh$	±SD
Ambient		cm		cm		cm^2	
NR_A	1	5.490	0.014	1.000	0.000	18.809	0.044
	2	5.510		1.000		18.871	
	Av	5.500		1.000		18.840	
NR_B	1	5.500	0.007	1.000	0.007	18.840	0.166
	2	5.490		0.990		18.605	
	Av	5.495		0.995		18.722	
NR_C	1	5.480	0.078	0.980	0.021	18.371	0.678
	2	5.590		1.010		19.330	
	Av	5.535		0.995		18.847	
Ambient							
NR+G_A	1	5.480	0.014	1.010	0.014	18.981	0.244
	2	5.500		0.990		18.636	
	Av	5.490		1.000		18.809	
NR+G_B	1	5.530	0.035	0.980	0.028	18.525	0.467
	2	5.480		1.020		19.185	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.550	0.071	0.980	0.028	18.586	0.355
	2	5.450		1.020		19.089	
	Av	5.500		1.000		18.840	
520 ppm							
NR_A	1	5.520	0.007	1.000	0.007	18.903	0.123
	2	5.530		0.990		18.729	
	Av	5.525		0.995		18.816	
NR_B	1	5.440	0.078	1.030	0.042	19.260	0.621
	2	5.550		0.970		18.381	
	Av	5.495		1.000		18.824	
NR_C	1	5.520	0.021	1.000	0.000	18.903	0.067
	2	5.490		1.000		18.809	
	Av	5.505		1.000		18.856	
520 ppm							
NR+G_A	1	5.500	0.021	1.010	0.014	19.044	0.223
	2	5.530		0.990		18.729	
	Av	5.515		1.000		18.887	
NR+G_B	1	5.490	0.021	0.990	0.014	18.605	0.355
	2	5.520		1.010		19.108	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.500	0.021	0.990	0.014	18.636	0.356
	2	5.530		1.010		19.139	
	Av	5.515		1.000		18.887	
720 ppm							
NR_A	1	5.520	0.028	0.990	0.014	18.698	0.200
	2	5.480		1.010		18.981	
	Av	5.500		1.000		18.840	
NR_B	1	5.490	0.057	1.000	0.007	18.809	0.032
	2	5.570		0.990		18.854	
	Av	5.530		0.995		18.832	
NR_C	1	5.600	0.092	0.980	0.021	18.740	0.148
	2	5.470		1.010		18.949	
	Av	5.535		0.995		18.847	
720 ppm							
NR+G_A	1	5.490	0.014	1.010	0.014	19.013	0.244
	2	5.510		0.990		18.667	
	Av	5.500		1.000		18.840	
NR+G_B	1	5.540	0.049	0.980	0.028	18.556	0.422
	2	5.470		1.020		19.153	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.560	0.085	0.980	0.028	18.617	0.311
	2	5.440		1.020		19.057	
	Av	5.500		1.000		18.840	
1120 ppm							
NR_A	1	5.500	0.035	1.000	0.007	18.840	0.034
	2	5.550		0.990		18.791	
	Av	5.525		0.995		18.816	
NR_B	1	5.450	0.042	1.030	0.042	19.292	0.730
	2	5.510		0.970		18.260	
	Av	5.480		1.000		18.777	
NR_C	1	5.500	0.007	1.000	0.000	18.840	0.022
	2	5.490		1.000		18.809	
	Av	5.495		1.000		18.824	
1120 ppm							
NR+G_A	1	5.490	0.021	1.010	0.014	19.013	0.222
	2	5.520		0.990		18.698	
	Av	5.505		1.000		18.856	
NR+G_B	1	5.500	0.007	0.990	0.014	18.636	0.311
	2	5.510		1.010		19.076	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.530	0.014	0.990	0.014	18.729	0.245
	2	5.510		1.010		19.076	
	Av	5.520		1.000		18.903	
	Grand Av	**5.508**	**0.027**	**0.999**	**0.012**	**18.839**	**0.199**

Appendix Table 4 Measurement of rubble surface area for 4 day's long Incubation in 2012

4 day's long Incubation [Natural Illumination]							
		Length	±SD	Diameter	±SD	Surface area $A=2\pi r2 + 2\pi rh$	±SD
Ambient		cm		cm		cm^2	
NR_A	1	5.520	0.028	0.990	0.014	18.698	0.200
	2	5.480		1.010		18.981	
	Av	5.500		1.000		18.840	
NR_B	1	5.490	0.057	1.000	0.007	18.809	0.032
	2	5.570		0.990		18.854	
	Av	5.530		0.995		18.832	
NR_C	1	5.600	0.092	0.980	0.021	18.740	0.148
	2	5.470		1.010		18.949	
	Av	5.535		0.995		18.847	
Ambient							
NR+G_A	1	5.490	0.014	1.010	0.014	19.013	0.244
	2	5.510		0.990		18.667	
	Av	5.500		1.000		18.840	
NR+G_B	1	5.540	0.049	0.980	0.028	18.556	0.422
	2	5.470		1.020		19.153	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.560	0.085	0.980	0.028	18.617	0.311
	2	5.440		1.020		19.057	
	Av	5.500		1.000		18.840	
520 ppm							
NR_A	1	5.500	0.035	1.000	0.007	18.840	0.034
	2	5.550		0.990		18.791	
	Av	5.525		0.995		18.816	
NR_B	1	5.450	0.042	1.030	0.042	19.292	0.730
	2	5.510		0.970		18.260	
	Av	5.480		1.000		18.777	
NR_C	1	5.500	0.007	1.000	0.000	18.840	0.022
	2	5.490		1.000		18.809	
	Av	5.495		1.000		18.824	
520 ppm							
NR+G_A	1	5.490	0.021	1.010	0.014	19.013	0.222
	2	5.520		0.990		18.698	
	Av	5.505		1.000		18.856	
NR+G_B	1	5.500	0.007	0.990	0.014	18.636	0.311
	2	5.510		1.010		19.076	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.530	0.014	0.990	0.014	18.729	0.245
	2	5.510		1.010		19.076	
	Av	5.520		1.000		18.903	
720 ppm							
NR_A	1	5.530	0.021	1.000	0.000	18.934	0.067
	2	5.500		1.000		18.840	
	Av	5.515		1.000		18.887	
NR_B	1	5.500	0.007	1.000	0.007	18.840	0.166
	2	5.490		0.990		18.605	
	Av	5.495		0.995		18.722	
NR_C	1	5.480	0.078	0.980	0.021	18.371	0.678
	2	5.590		1.010		19.330	
	Av	5.535		0.995		18.847	
720 ppm							
NR+G_A	1	5.480	0.014	1.010	0.014	18.981	0.244
	2	5.500		0.990		18.636	
	Av	5.490		1.000		18.809	
NR+G_B	1	5.530	0.035	0.980	0.028	18.525	0.467
	2	5.480		1.020		19.185	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.550	0.071	0.980	0.028	18.586	0.355
	2	5.450		1.020		19.089	
	Av	5.500		1.000		18.840	
1120 ppm							
NR_A	1	5.520	0.007	1.000	0.007	18.903	0.123
	2	5.530		0.990		18.729	
	Av	5.525		0.995		18.816	
NR_B	1	5.440	0.078	1.030	0.042	19.260	0.621
	2	5.550		0.970		18.381	
	Av	5.495		1.000		18.824	
NR_C	1	5.520	0.021	1.000	0.000	18.903	0.067
	2	5.490		1.000		18.809	
	Av	5.505		1.000		18.856	
1120 ppm							
NR+G_A	1	5.500	0.021	1.010	0.014	19.044	0.223
	2	5.530		0.990		18.729	
	Av	5.515		1.000		18.887	
NR+G_B	1	5.490	0.021	0.990	0.014	18.605	0.355
	2	5.520		1.010		19.108	
	Av	5.505		1.000		18.856	
NR+G_C	1	5.500	0.021	0.990	0.014	18.636	0.356
	2	5.530		1.010		19.139	
	Av	5.515		1.000		18.887	
	Grand Av	**5.508**	**0.027**	**0.999**	**0.012**	**18.841**	**0.198**

Chapter 1

General Introduction

- Background of Research
- Ocean acidification
- Biological impacts
- Importance of coral rubble
- Objectives of research
- Research hypothesis
- References

Chapter 2

Methodology and Measurements

- Study area and collection of samples
- Experimental design
- Preparation of incubation experiments
- Laboratory measurements
- Calculation and analysis
- References

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Chapter 3

Contribution of coral rubble associated microbial community to the dissolution of calcium carbonate under high pCO_2

Chapter 4

The availability of organic matter accelerates carbonate dissolution by influencing bacterial activity under elevated pCO_2

Chapter 5

Bio-Chemical Dissolution Processes (BCDP):
A novel view of carbonate dissolution in coral reef ecosystems

Appendice