# Feeding on pico-and nanoplankton by scleractinian corals from Okinawa

# THESIS

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Shizuoka University

Graduate School of
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Department of Environment and Energy Systems

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沖縄の造礁サンゴによるピコ・ナノプランクトンの捕食

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# General abstract

Coral reefs are complex ecosystems referred as the forest of the sea, maintaining a high biological diversity. They show high gross primary production rate that depends on a rapid nutrient recycling among reef organisms. In the reefs environment successful of corals is due to the efficient nutrients exchange among the coral host, its symbiotic algae, a dinoflagellate algae of the family Symbiodiniaceae, and the environment. The Symbiodiniaceae provide food for the corals by translocating photoassimilates (mainly glucose, glycerol, lipids and amino acids) supporting coral metabolisms and skeleton formation. However, as N is not substantially provided by the Symbiodiniaceae, corals need to feed on plankton to balance their diet by releasing mucus net, composed mainly by dissolved organic carbon (DOC), to trap suspended particles in the seawater. Under recent global warming scenario, the coral-symbiont relationship is strongly affected causing coral bleaching where the corals lose their Symbiodiniaceae (or their pigments) and concomitantly, a decrease in organic matter translocation which results in coral starvation. Therefore, an exogenous food source as plankton may play a crucial role in coral survival under environment stress and bleached conditions. The objective of this study was to understand the feeding strategies of two common scleractinian corals under healthy and bleached conditions. Moreover, the effect of thermal stress was also investigated for the two coral conditions. The study focused on their picoplankton and nanoplankton feeding efficiencies considering that these two plankton size fractions are the most abundant in the reef waters and therefore the most important sources of organic matter for scleractinian corals.

Firstly, I studied the feeding rate of healthy and bleached scleractinian corals, *Montipora digitata* (branching) and *Porites lutea* (massive), which are dominant in

Okinawan reefs and have been subjected to recurring bleaching events. Coral nubbins were incubated for 6 h at normal (27 °C) and stressful (33 °C) seawater temperatures. They were supplied with a natural assemblage of picoplankton and nanoplankton sample near by the corals and concentrated via a tangential flow system. Densities of Bacteria (BA), picocyanobacteria (PCY), picoflagellates (PF), nanoflagellates (NF), and concentration of dissolved organic carbon (DOC) were monitored at the start and end of the incubations. Feeding rates were calculated in terms of cell consumption and converted to carbon units considering the carbon biomass of the different plankton groups. In addition, protein, glucose and glycerol concentrations and Symbiodiniaceae density in coral tissue were monitored. Results of feeding rates of healthy corals showed that *M. digitata* consumed from 87% (27 °C) to 72% (33 °C) more pico-nanoplankton cells and 94% or 70% more organic carbon than *P. lutea*. In terms of plankton preference and carbon incorporation, *M. digitata* consumed mainly NF as major carbon source and *P. lutea* consumed equally on all food items.

I also studied feeding rates of bleached corals and this represents the first study revealing feeding rates of bleached corals under a prolonged heat stress scenario. The combination of bleached condition and high seawater temperature was exercised in incubations to understand bleached coral's responses subjected to a prolonged thermal stress as commonly occurs nowadays in Okinawan reefs. Results showed that bleached and healthy *M. digitata* consumed almost the same amount of carbon, but under thermal stress carbon incorporation widely decreased in bleached corals. Despite the comparatively lower carbon incorporation under thermal stress, *M. digitata* incorporated ~50% more carbon than *P. lutea*. Bleached corals of the two species under thermal stress consumed almost the same amount of cells but *P. lutea* consumed mainly PCY

and PF, which provided relatively less organic matter source but with high C/N ratio. To understand possible changes in feeding strategies, I followed the dissolved organic matter (DOC) fluxes dynamics during incubations. A negative DOC flux in bleached *M. digitata* at 33 °C showed that *M. digitata* needed to uptake DOC from the surrounding seawater to compensate its metabolic coast, therefore decreased plankton capture was due to lack of mucus secretion. Conversely, *P. lutea* continued to release DOC even under the heat stress despite the lower feeding rate. Therefore, I concluded that in *P. lutea* mucus release could serve for another purpose besides plankton trapping. During my incubations, I measured concentrations of glycerol and glucose, calculated their fluxes (in terms of organic carbon) and compare with the incorporation of organic carbon from heterotrophy. Carbon acquisition from heterotrophy respect to dark respiration (consumption of glycerol and/or glucose) varied from 3% to 65% in *M. digitata* and from 7% to 68% in *P. lutea*.

As main conclusion in this research, *M. digitata* was highly dependent on heterotrophy and very efficient at food capture. Nevertheless, its plankton capture performance was substantially diminished by the combination of bleaching and heating. On the other hand, *P. lutea* was comparatively less dependent upon heterotrophy; it utilized organic matter translocated from its endolithic community to produce mucus and was able to maintain certain level of heterotrophy (~7% of dark consumption under thermal stress) with selection of higher C/N ratio food items. Therefore, this coral species is relatively more resistant to bleaching events even at elevated seawater temperatures. this study shows that *P. lutea* is relatively more tolerant to bleaching, having greater chances of survival and recovery. Therefore, this species is more likely to be able to colonize decimated coral reef ecosystems.

# Chapter 1

# General introduction

## 1.1 Coral reef ecosystem

Coral reefs are complex ecosystems offering a variety of microhabitats for the high diversity of organisms that inhabit them. Even coral reefs mostly develop in low nutrient seawaters, they maintain a high gross primary production. (Walker and Wood, 2005). This “coral reef paradox” is mainly the result of a rapid nutrient recycling between the coral host and its symbiotic algae, a dinoflagellate of the family Symbiodiniaceae (LaJeunesse et al., 2018). They are living within bodies of the tiny corals. The Symbiodiniaceae provide food in the form of carbohydrates mainly glucose and glycerol for the coral to support its metabolism including the calcification for skeleton formation (Lesser, 2004). The skeletons of corals display a high variety of geometrical shapes that offers refuges and spaces to support and protect the life of other organisms, therefore reefs are one of the most diverse communities in the word’ sea. Coral reefs represent a significant source of food and livelihood for the people who live along the coastline (around tens of millions of people in over 100 countries worldwide) (van Oppen and Lough, 2009). They support major economic resources for surrounding countries, especially fishing and tourism. Furthermore, they also play a crucial role in stabilizing seashore and moderation of the word climate (Connell, 1978; Lesser, 2004).

## 1.2 Coral physiology

### 1.2.1 Coral structure

Corals are colonial organisms belonging to the phylum *Cnidaria*; the colony is form by the association of many polyps living together (Figure 1). All the polyps are connected by a thin sheet of tissue named coenosarc. The polyps are of cylinder shape displaying a very simple structure forming a sac with two layers, the ectoderm with stinging cells (the nematocysts) and mucus producing cells, and the endoderm that contains the symbiotic Symbiodiniaceae. Corals secrets an exoskeleton by a special tissue layer (the calicoblast) that is located on the base of the polyp. Corals grow by producing new polyps by asexual reproduction and secreting the underneath skeleton resulting in important calcium carbonate structures that protect and shape the reefs. Corals can also sexually reproduce by releasing gametes in the spawning season that may occur once or twice a year during full moon. The coral energy sources are provided mainly by the carbon reach materials that are translocated from their Symbiodiniaceae. Also, most of the corals can capture somehow big plankton using their tentacles that bear the singing cells (nematocysts) surrounding the polyp mouth (Figure 2), however the bacterioplankton seems to be the most important external food source that the corals trap by using mucus nets excreted by specialized cells of the ectoderm (Goldberg, 2018). The polyp size depends on the species (Castro and Huber, 2008). Some coral species live as a single polyp, solitary free-living as the mushroom coral (Family *Fungiidae*). Their polyps can extend up to 30 cm in diameter (Veron, 2000). However, most scleractinian coral species are colonial. Colonial coral polyps are much smaller <3 mm in diameter. Polyp size of some coral species of the family *Poritidae* showed 0.5-

0.7 mm in diameter (Veron, 2000). Coral colonies also vary in size. Some corals form only small colonies. Others may form colonies several meters.

The coral itself is not living alone but it lives in association with a variety or microorganisms that is named “coral holobiont” (Figure 2); (Bourne et al., 2009; Koren and Rosenberg, 2006). Associated microorganisms are virus, bacteria, archaea, fungus, endolithic community, and the unicellular algae Symbiodiniaceae (Sangsawang et al., 2017; Thompson et al., 2015). The Symbiodiniaceae are important microorganism for the coral since they provide up to 90% of the coral nutrition by translocation (Muscatine et al., 1981), (Figure 3). Among associate microorganism of coral, the endolithic community is another very important symbiotic entity in corals. Sangsawang et al., (2017) found that the endolithic are translocated carbon and nitrogen-rich compounds to the coral tissue of *Porites lutea* at both, healthy and bleached conditions (up to 8% of the total fixed carbon by endolithic in healthy corals and 6% in bleached corals). That may support the metabolic coast during bleaching events. In this thesis, I selected two different hermatypic corals: *P. lutea* possess endolithic community, however *M. digitata* does not normally associate with endoliths (Figure 4), and this pattern have important implications for the coral nutrition and survival strategy under stressful conditions.

### 1.2.2 Energy source of coral

Scleractinian corals obtain energy (organic matter) from two main sources: autotrophy and heterotrophy. Autotrophy represents the main source of organic matter (up to 90%) that is fixed by the symbiotic Symbiodiniaceae and translocated to the coral tissues (Grottoli et al., 2006; Muscatine et al., 1981), (Figure 4). Heterotrophy by which corals can obtain organic matter from the outer environment by consuming (feeding on) plankton, bacteriplankton, detritus and taking up dissolved organic matter (Baumann et al., 2014).

The coral host receive organic matter synthetized during photosynthesis by their symbiotic Symbiodiniaceae mainly glycerol, and glucose. Up to 70% of this organic matter is translocated from the Symbiodiniaceae to the host (Yellowlees et al., 2008; Tremblay et al., 2012; Tremblay et al., 2014). This organic matter is used by the coral host to drive physiological processes like respiration and calcification (Muscatine et al., 1981). In return, the Symbiodiniaceae receive a place to live and essential substrates, mainly carbon dioxide ($CO_2$) from coral respiration and ammonium ($NH_4^+$), $NO_3^-$ and $PO_4^{-3}$ from coral waste release (Wooldridge, 2009; Goldberg 2018). Heterotrophic feeding seems to be important to most scleractinian corals, since it provides the corals with nitrogen, phosphorus, and other compounds that cannot be obtained from the translocation of Symbiodiniaceae (Godinot et al., 2011; Houlbrèque and Ferrier-Pagès, 2009; Sebens et al., 1996). The corals must supplement their autotrophic carbon-rich diets with nitrogen- and phosphorus-rich material (Palardy et al., 2006; Titlyanov et al., 2001). Therefore, corals feed on zooplankton using their tentacles to trap and transport the prey to the mouth and stomach (Labarbera, 1984; Palardy et al., 2006) suspended

particulate matter (Anthony and Fabricius, 2000) including bacterioplankton, phytoplankton, and detritus by trapping them using mucus nets (Borell et al., 2008; Ferrier-Pagès et al., 2003; Sorokin, 1973) and dissolved organic matter (Al-Moghrabi et al., 1993; Suzuki and Casareto, 2011). The relative importance of heterotrophy versus autotrophy may vary according to certain conditions. Heterotrophic feeding serves to balance nitrogen and carbon at normal condition, but under environmental stressors as high sea surface temperature, corals bleach. In this process corals lose their Symbiodiniaceae (or their pigments) and, therefore, translocation decreases. Under this condition, heterotrophic feeding may have a more important role in providing corals with external organic matter to balance their metabolic needs (Borell et al., 2008).

## 1.3 Coral feeding strategies

From some time ago several coral species were categorized as carnivores (Ribes et al., 1998; Houlbrèque et al., 2004; Palardy et al., 2005;) since they can trap relatively big zooplankton. Actually, coral polyps expand their tentacles at night to trap their preys when the abundance of zooplankton increases due to the dial vertical migration (Houlbrèque and Ferrier-Pagès, 2009; Lasker, 1979). However, tiny plankton as bacteria, picocyanobacterial, flagellates (pico- and nano- sized) and small diatoms cannot be trapped using tentacles but only by means of mucus nets. This strategy is named suspension feeding. Corals can release a considerable amount of mucus, mainly in the form of DOM, which is then resorbed after being enriched with pico- and nanoplankton (Goldberg, 2018b; Lewis and Price, 1975; Naumann et al., 2009; Ribes et al., 2003), therefore this strategy may play a crucial role in coral feeding, particularly

for coral species with small polyp. At the same time, coral mucus contains nutrient rich compounds as proteins and triglycerides, that bacteria in the surrounding waters can use for their own growth (Ferrier-Pagès et al., 2000; Wild et al., 2004).

## 1.4 Coral food source

Most of the benthic organisms living in the reef lagoons are filter feeders or suspension feeders, therefore plankton and detritus may represent an important food source for the benthic community.

As explained before, corals can feed on zooplankton, phytoplankton and bacterioplankton. Zooplankton in reef lagoons are mainly represented by benthic copepods and amphipods and some other crustaceans, ciliates and larvae of benthic organisms, however those corals with small polyps may not be able to trap big size items. Phytoplankton is rather scarce in the reef lagoons due to low nutrients availability (Sorokin, 1973). Recently the importance of pico- and nanoplankton in reef waters were studied by several researchers confirming that pico and nano sized plankton represents the most important biomass fraction in the lagoon waters (Casareto et al., 2000; Ferrier-Pagès et al., 2000; Lefebvre et al., 2012). More recently studies had shown that corals may take up dissolved organic matter including urea and dissolved free amino acids (DFAA) (Houlbrèque and Ferrier-Pagès, 2009 and references in there).

### 1.4.1 Dissolved organic matter (DOM)

Dissolved organic matter (DOM, <0.2) represents largest stock of organic material on earth. It is used by coral reef creatures. Normally DOM is mostly produced from benthic reef organisms and utilize by bacteria from their growth. (Carlson and Hansell, 2014; Martias et al., 2018). Corals are the major benthic of producer DOM in coral reef (Tanaka et al., 2008). Corals release DOM as a mucus into the seawater which are the major mechanism for trap and ingestion of small food particle like a plankton as pico and nano size (Ferrier-Pagès et al., 2000; Muscatine, 1973). Coral release mucus and re-ingest their exudate by gliding mucus with pico- and nanoplankton associated toward the mouth (Goldberg, 2018) to balance organic matter (carbon and nitrogen). However, mucus release differ depend on coral species or environmental (Goldberg, 2018; Naumann et al., 2010; Tanaka et al., 2009). Mucus production rate of corals were decrease along bleaching events (Glynn et al., 1985). This reflection is likely showing the lack of nutrient content in coral tissue reserves. Yamashiro et al., (2005) found that, lipid composition of coral tissues which are the one composition of coral mucus, were decrease in bleached Okinawa corals, typically the branching corals indicating that. Therefore, when the corals were under stress condition as elevated seawater temperature or bleached, they will produce more mucus to support heterotrophic mode of nutrition, however, in the other case, corals decrease produced mucus and/or uptake more DOM from surrounding seawater than they release to compensated organic carbon and nitrogen in coral tissue. This suggest that dissolved organic carbon and nitrogen may an important source for coral (Suzuki and Casareto, 2011).

### 1.4.2 Living particulate organic matter

The most important living biomass in the reefs waters in the form of particulate organic matter is pico- and nanoplankton (Casareto et al., 2000). In previous years, the study of these tinny organisms was not performed in detail due to the lack of an appropriate methodology, therefore scientists did not consider these planktonic fractions to have important role for the coral food. However, after the development of flow cytometers and advanced fluorescent microscopes, scientist understood that the small plankton (smaller than 3 μm) are dominant in the reef lagoons. Bacteria, picocyanobacteria (mainly of the genus *Synechococcus* and *Prochlorococcus*), and picoeukaryotes are generally in higher abundance in the lagoons than in the open ocean. They can contribute up to 65% or the organic carbon produced by planktonic community in the reefs (Casareto et al., 2006). However, pico-nanoplankton concentration can notably decrease near the reef crest where corals are concentrated, indicating a pattern of plankton depletion by coral feeding (Casareto et al., 2000, 2006).

Following these concepts, I focused my study in the pico- nano sized plankton fraction, taking into account that this fraction should be the most important food source for scleractinian corals.

### 1.4.3 Non – living particulate organic matter (Detrital)

Bacteria, exudation from microbes, protozoans, interstitial invertebrates, microalgae and dead organic matter (detritus) are present in the surface sediments and in suspension in the waters of coral reefs (Houlbrèque and Ferrier-Pagès, 2009). All of these items are potential food sources for corals. Many studies showed that corals are also able to feed on particles trapped in the sediment that can be re-suspended in the water column by convection or tidal currents (Anthony, 1999; Anthony and Fabricius, 2000). *Pocillopora damicornis* and *Acropora millepora* consumed organic matter from the sediment and their consumption rates were in a linear function with sediment load, with an assimilation efficiency of 50-80% (Anthony, 2000). Rosenfeld et al., (1999) found that *Fungia horrida* consumed detritus from sediment during experiments using labeled sediment. *Acropora millepora, Siderastrea radians, Montastrea franksi, Diploria strigose* were also found they were uptake suspended detrital particulate matter (Anthony, 2000; Mills et al., 2004b).

The study of coral feeding on detritus requires field measurements and in situ incubations with a longer time scale, therefore this food source was not considered in the present study.

## 1.5 Pico- and nanoplankton

In coral reef environments, pico-plankton (0.2-2 μm) and nano-plankton (2-20 μm) are the most important components of the particulate of organic matter (Casareto et al., 2000; Charpy and Charpy-Roubaud, 1991). Despite they are of small size compare to other planktonic groups, the have significant implications in the export of organic carbon and nitrogen via consumption by higher trophic level organisms (Ribeiro et al., 2016; Richardson and Jackson, 2007) and export to open ocean via tidal currents. Pico- and nanoplankton includes bacteria, cyanobacteria, flagellates and ciliates (Ferrier-Pagès and Gattuso, 1998; Sorokin, 1991, 1973). Pico- and nanoplankton are the smallest organisms that corals generally consume, as the most abundant particles that are free-living in the water column (Ferrier-Pagès and Gattuso, 1998; Houlbrèque et al., 2004). To build a new tissue, the corals must supplement their phototrophically carbon-rich diets with nitrogen- and phosphorus-rich material (Palardy et al., 2006; Titlyanov et al., 2001). Therefore, corals feed heterotrophically on suspended particulate matter (Anthony and Fabricius, 2000). Few reports were published about coral feeding on pico- and nanoplankton (e.g. Houlbrèque et al., 2004; Picciano and Ferrier-Pagès, 2007; Sorokin, 1973; Tremblay et al., 2011; Wang et al., 2012), however there is no available data on changes of feeding requirements when the corals are subjected to environmental stressor as high seawater temperature, high UV radiation, high nutrient stress, and other derived from climate changes and human impacted reefs.

## 1.6 Coral bleaching

Coral bleaching is the most impacting response of scleractinian corals to environmental stresses as elevated seawater temperature and high irradiance. These stresses increased in frequency and widespread on coral reef over the last 20 years (Coles and Brown, 2003; Zhou et al., 2017). During corals bleaching events Symbiodiniaceae in coral tissues was changed morphologically and lost pigmentation (Kuroki and van Woesik, 1999; Suzuki et al., 2015; Yamashiro et al., 2005). Diverse forms of Symbiodiniaceae were observed in tissues of naturally bleached coral during summer (Mise and Hidaka, 2003; Reimer et al., 2007; Suzuki et al., 2015) Thermal stress inflicts damage in chloroplast thylakoid membrane structures by altering their lipid composition (Tchernov et al., 2004) and inducing the production of reactive oxygen species (ROS; Smith et al., 2005), which ultimately destroys chloroplast organization (Salih et al., 1998). Moreover, thermal stress in combination with high light stress may accelerate bleaching. Darkness have also negative effect promoting degradation of the Symbiodiniaceae photosynthetic system (Suwa and Hidaka, 2006).

Under bleaching conditions the photosynthetic activity of Symbiodiniaceae can be greatly reduced (Warner et al., 2002), the energetic and nutritional balance of the symbiotic association may become disrupted, potentially rendering the coral host into a state of starvation, which can lead to changes in biochemical composition and reduced energetic status of the colony (Fitt et al., 1993; Grottoli et al., 2004; Yamashiro et al., 2005). Heterotrophy is a primary source of nitrogen and phosphorus in reef environments (Houlbrèque and Ferrier-Pagès, 2009) and can help corals to overcome starvation under bleaching (Ferrier-Pagès et al., 2010; Grottoli et al., 2006; Tremblay et

al., 2014). The importance of heterotrophy showed be evaluate under those stressful conditions which are now a day highly impacting word coral reef.

## 1.7 Aim and objective

The goal of the present study was to understand the feeding strategies of two important dominant coral species in the Okinawan reef, namely, the branching *Montipora digitata* and the massive *Porites lutea*. It is already known that heterotrophy is of crucial importance for recovery from bleaching event (Grottoli et al., 2006). I thought to study heterotrophy under normal versus stressful condition of high seawater temperature, bleached conditions and the combination of these stresses. Moreover, there are no data on coral heterotrophy for this region. I focused our study on pico-nanoplankton feeding efficiency since these are the main organic matter sources for scleractinian corals. I attempted to answer the following questions:

1. What are the feeding rates, prey preferences, and organic matter assimilation efficiencies of these coral species? I also would like to know if the difference of their polyp size influences feeding efficiency.
2. How much feeding efficiency changes when bleached corals are exposed to extended thermal stress in Okinawan shallows lagoons?
3. In order to understand patterns of dark respiration during our experiment, I measured fluxes of the two main autotrophic products, glycerol and glucose and I compared these values with the amount of organic carbon assimilated by heterotrophy in order to infer how the energy allocation varied under the tested conditions and treatments during our experiments.

## 1.8 Hypothesis

1. *M. digitata* and *P. lutea* would show different heterotrophic requirements and strategies due to the differences in their polyp size and density, including the effects of endolithic communities in *P. lutea* could widely influence the feeding behavior of this coral.
2. The feeding rates of bleached corals under thermal stress conditions will be highly reduced due to the combination of these two conditions.

To test these questions and hypothesis incubation experiments were used.

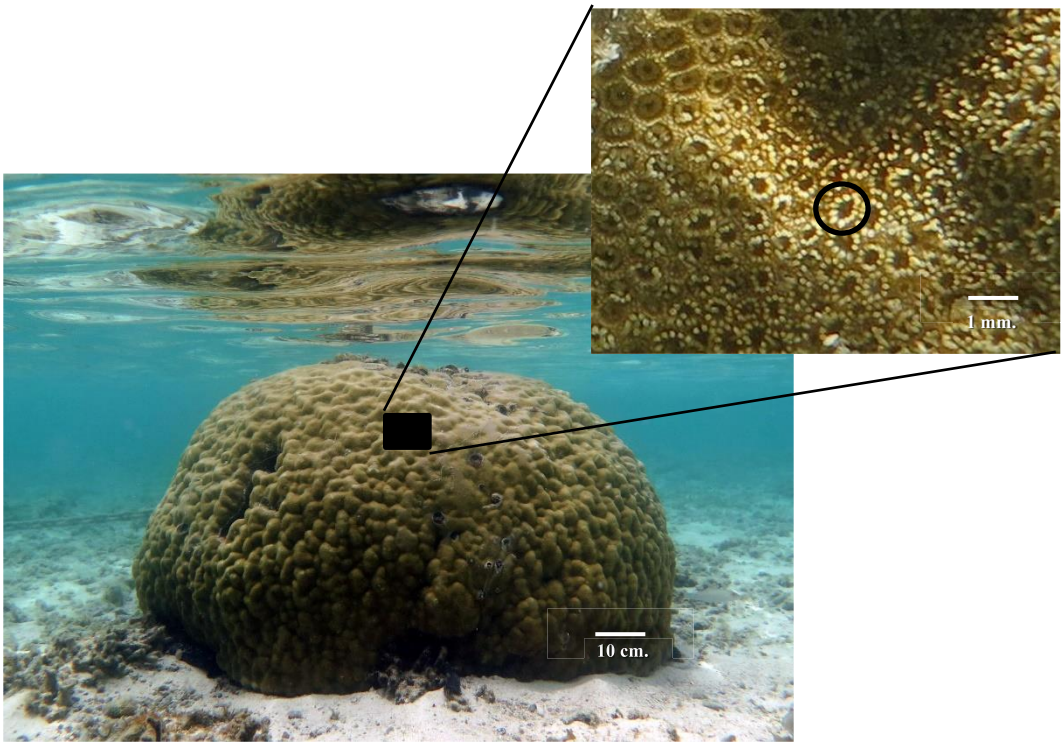



**Figure 1** Characteristic of coral and polyps

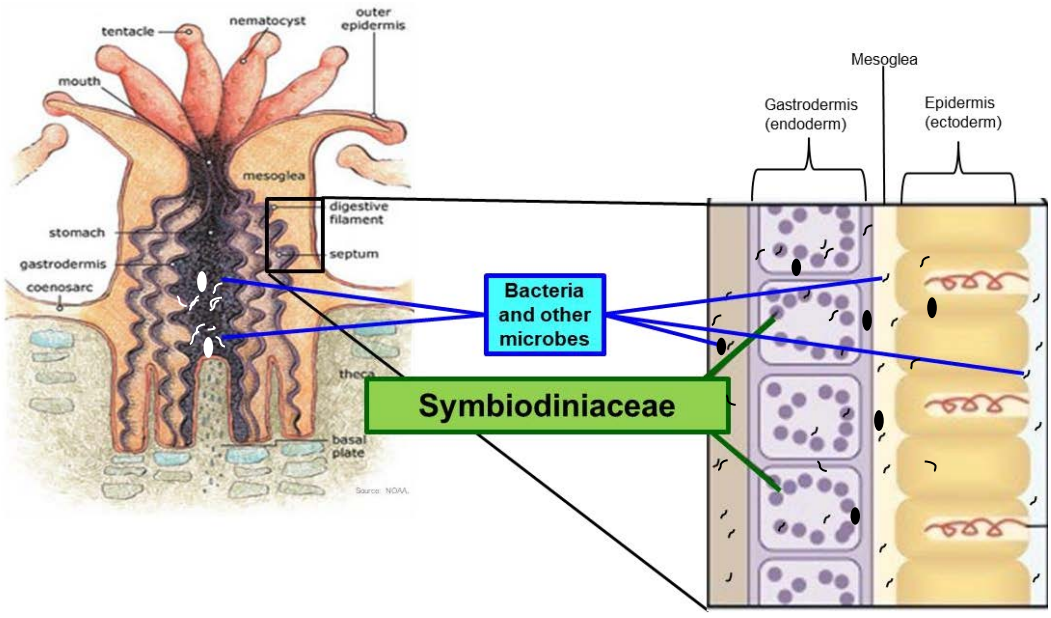



**Figure 2** Characteristic of coral polyp and microorganism communities associated in coral tissue

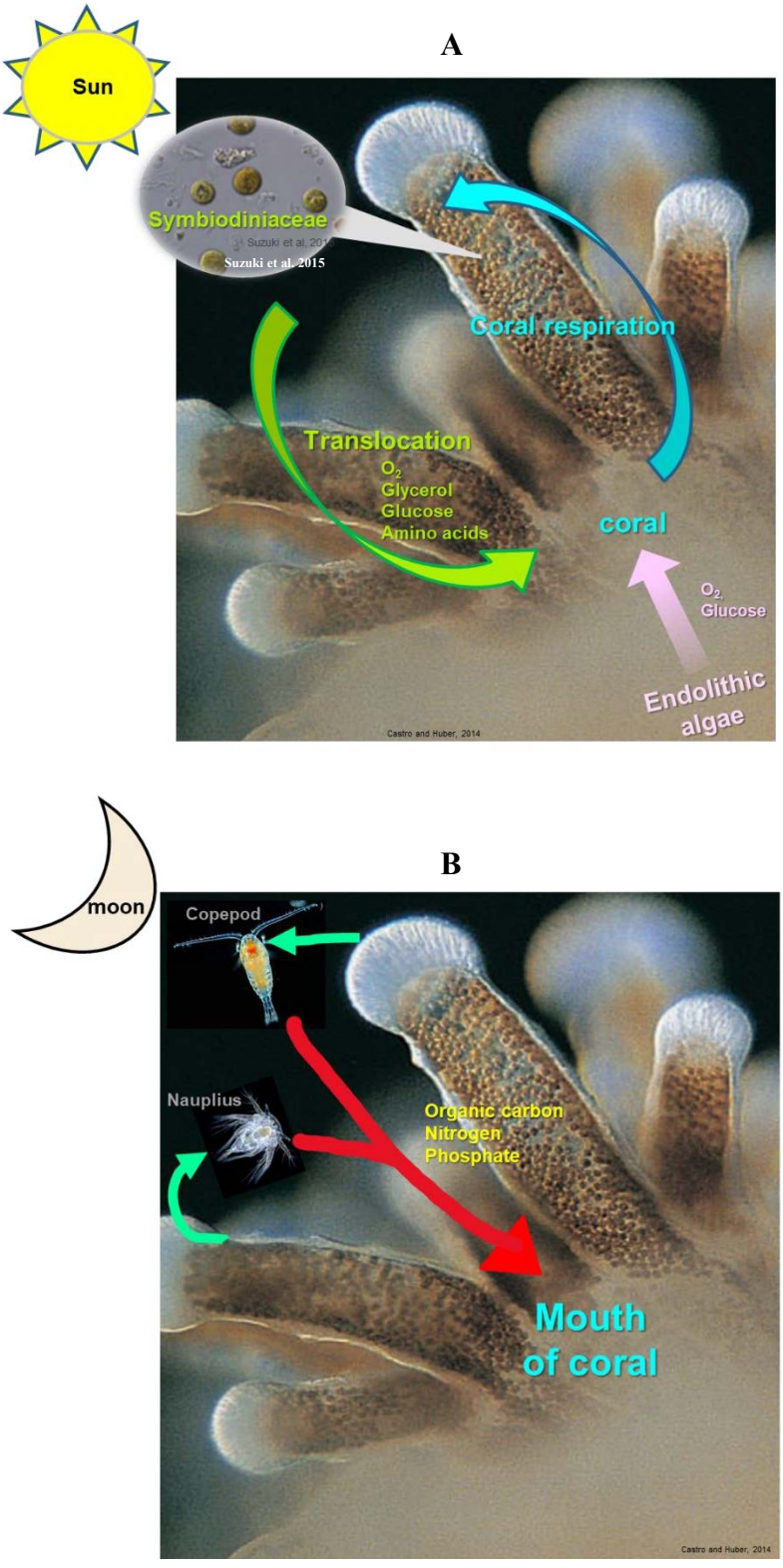



**Figure 3** Coral nutrition of main 2 way (A: autotrophy and B: heterotrophy).


Cross section of a *Porites lutea* colony

Cross section of a *Montipora digitata* colony branch

**Figure 4** Cross section of corals to show the presence or absence of endolithic algae in *Porites lutea* and *Montipora digitata*

# Chapter 2

# Coral feeding on pico- nanoplankton under normal versus thermal stress

## 2.1 Abstract

This study I would like to understand the feeding strategies of healthy and bleached corals under normal and high seawater temperatures. Branching *Montipora digitata* and massive *Porites lutea* were used for these researches. Both coral species are dominant in Okinawan reefs and have been subjected to recurring bleaching events. This study shows for the first-time exercised in our incubations to understand coral responses under combined stresses that commonly occur nowadays in Okinawan reefs. Feeding efficiencies were focused on their picoplankton and nanoplankton feeding efficiencies. These are the most important sources of organic matter for scleractinian corals. Healthy and bleached coral nubbins were incubated for 6 h at normal (27 ºC) and thermal (33 ºC) seawater temperatures. They were supplied with a natural assemblage of picoplankton and nanoplankton concentrated via a tangential flow system. Bacteria (BA), picocyanobacteria (PCY), picoflagellates (PF), nanoflagellates (NF), were monitored at the start and end of the incubations. Results showed that, healthy *M. digitata* at 27 °C and 33 °C more consumed pico-, and nanoplankton cells 87% and 72% and more incorporated organic carbon 94% or 70% than *P. lutea*. In terms of plankton preference and carbon incorporation, *M. digitata* and *P. lutea* consumed mainly NF as major carbon source, which was its major carbon source. In contrast, *P. lutea* consumed only PCY and PF, which provided relatively less carbon. When compared with bleached

corals nubbins, I found that bleached and healthy *M. digitata* consumed almost the same amount of carbon at normal seawater temperature, but under thermal stress carbon incorporation widely decreased in bleached corals. Despite the comparatively lower carbon incorporation under combined stresses, *M. digitata* incorporated ~50% more carbon than *P. lutea*. Under combined stresses, both coral species consumed almost the similar number of pico- and nanoplankton cells but *P. lutea* consumed mainly PCY and PF, which provided relatively less carbon but represents a comparatively high C/N food source. While *M. digitata* consumed mainly BA and NF which are important organic food sauce typically from NF. The study indicated that, the feeding strategies were difference between both coral species to over come the environmental stress.

## 2.2 Introduction

Reef-building corals live in symbiotic relationships with Symbiodiniaceae and other microorganisms. Collectively, these consortia form so-called ‘coral holobionts’ (Bourne et al., 2009; Koren and Rosenberg, 2006; Sangsawang et al., 2017; Thompson et al., 2015). Autotrophy is the main source of organic matter (up to 90%) for Scleractinian corals. It is translocated to the coral host from their Symbiodiniaceae (Baumann et al., 2014; Muscatine et al., 1981) in the form of sugars, glycerol, and amino acids (Tremblay et al., 2014). To build a new tissue, however, the corals must supplement their phototrophically carbon-rich diets with nitrogen- and phosphorus-rich material (Palardy et al., 2006; Titlyanov et al., 2001). Therefore, corals feed heterotrophically on suspended particulate matter (Anthony and Fabricius, 2000) including bacterioplankton, phytoplankton, zooplankton, detritus (Borell et al., 2008; Ferrier-Pagès et al., 2003; Sorokin, 1973). The relative importance of heterotrophy vs. autotrophy may vary according to certain conditions. Baumann et al. (2014) showed that shaded corals obtain >60% of their energy requirements from heterotrophic feeding. For most scleractinian corals, picoplankton and nanoplankton are the most important food sources. Corals trap them by secreting mucus nets, which are then resorbed after being enriched with pico and nanoplankton (Goldberg, 2018). Sorokin (1973) was the first to use labelled bacteria to study coral feeding on bacterioplankton. A study by Houlbrèque et al. (2004) showed that the Symbiodiniaceae coral *Tubastrea aurea* fed on bacteria, picocyanobacteria, picoflagellates, and nanoflagellates. In contrast, the Symbiodiniaceae corals *Galaxea fascicularis* and *Stylophora pistillata* fed mainly on nanoflagellates (80% and 50% of the total ingested carbon and nitrogen, respectively)

whereas bacteria, picocyanobacteria, and picoflagellates contributed only 1-7% of their ingested carbon. Picciano and Ferrier-Pagès (2007) found that the red coral *Corallium rubrum* preyed upon both pico- and nanoplankton, and flagellates constituted their major food source (43-70% of their C and N intake). Wang et al. (2012) showed that the picoplankton capture efficiency of *Stylophora pistillata* was greater than that of *Montipora stellata*. This difference was attributed to the fact that their polyp sizes are dissimilar. The preceding studies provided important information about the organic matter and food, which coral incorporate by heterotrophy when they are not under environmental stress.

At normal environment, Scleractinian corals are almost receive energy photosynthetic products (e.g. sugar and amino acid) from Symbiodiniaceae which are located in their tissue as a main energy source. Recent environmental changes include elevated sea surface temperatures and solar irradiance (Lesser et al., 1990; Lesser and Farrell, 2004) along with diminishing water quality from human impact such as nutrients input (Fabricius, 2005; Møller et al., 2014), contamination from microplastic (Moore, 2008), metal pollution (Prouty et al., 2013). These factors have increased the incidence and severity of coral bleaching worldwide (Coles and Brown, 2003). Bleached corals lose their endosymbiotic Symbiodiniaceae and/or pigments (Baumann et al., 2014; D'Croz et al., 2001; Glynn, 1996; Hoegh-Guldberg, 1999; Suzuki et al., 2015). Therefore, relatively less organic matter is translocated to them. Consequently, their metabolic energy levels decline, their physiology is altered, and their survivorship decreases. Symptoms of coral bleaching include a reduction in calcification (e.g. (Leder et al., 1991; Rodrigues and Grottoli, 2006), increases in the catabolism of reserved materials (Rodrigues and Grottoli, 2007; Schoepf et al., 2015b), and enhanced

heterotrophy (Grottoli et al., 2006). The several studied almost done coral feeding on pico- and nanoplankton in under normal environment (Houlbrèque et al., 2004; Picciano and Ferrier-Pagès, 2007; Sorokin, 1973; Tremblay et al., 2011; Wang et al., 2012). Tremblay et al., (2011) showed that the temperate Mediterranean coral *Cladocora caespitosa* had highly heterogeneous feeding (0.2-200 μm cell diameter) and could survive by heterotrophy alone. However, this is first time for study coral feeding combine stress (bleaching and high seawater temperature) Recurring coral bleaching events were recently observed in the Okinawan coral reefs. They have affected even the most dominant coral species there.

The goal of the present study was to compare feeding rate of healthy and bleached coral between branching corals (*Montipora digitata*) and massive corals (*Porites lutea*) which are dominant coral species in the Okinawan reef. To investigate feeding of healthy and bleached corals under thermal stress. However, there are no data on coral heterotrophy for this region. We focused our study on pico-nanoplankton feeding efficiency since these are the main organic matter sources for scleractinian corals. How does bleaching affect coral feeding rates? How do these two-coral species differ in terms of their feeding strategies? How does thermal stress influence feeding efficiency in bleached coral?

## 2.3 Materials and methods

### 2.3.1 Corals sampling and experimental design

*2.3.1.1 Corals sampling and acclimatization*

The scleractinian corals *Montipora digitata* and *Porites lutea* were sampled at Sesoko Reef, Okinawa, Japan (26°39′ N, 127°51′ E) at low tide (depth 0.5-1 m) during the summer season of 2017. Seawater temperature in the Sesoko reef lagoon varied from 30.1 °C to 32.2 °C (Multiparameter Mini Sonde, OTT, Hydrolab MS5, Ireland) during the sampling period (September 2017 from 12:00 to 16:00), conducted at the low ebb of a spring tide. During September 2017, the degree of heating weeks (DHW) was 13 °C week and the bleaching threshold was 29.5 °C (NOAA Coral Reef Watch Virtual Station in Northern Ryukyu Island, Japan). Coral sampling was conducted under Permit No. 28-75, which was obtained from the Okinawa Prefectural Government. Three mother colonies of healthy *M. digitata* were sampled and divided into three branches each (3 cm long). The same sampling procedure was followed for bleached *M. digitata* from three mother colonies. Healthy fragments of *P. lutea* were obtained from three mother colonies and bleached fragments from other three colonies (Figure 5). Three fragments of 3 $cm^2$ were obtained from each. Some extra fragments from the same colonies were also taken for measurements of biological parameters and symbiont density. Bleached conditions for the two coral species were determined on the bases of Coral Watch Coral Health Chart; http://interpreter.ne.jp/umibe/. An incubation experiment was designed to test feeding rates of these two coral species. The experiment covered three steps as follows: acclimatization after sampling (nine days for fragments exposed to normal temperature and three days for fragments exposed to thermal stress); temperature ramp-up (six days for only fragments exposed to thermal

stress); and a feeding experiment (6 h). The initial time corresponds to the 0 h of the feeding incubation experiment. For acclimatization, coral fragments were attached to a polyethylene net in an aquarium with natural running seawater (flowrate of 10 mL $min^{-1}$), with temperature that fluctuated between 26.8 °C and 27.4 °C, and attenuated natural illumination with maximum 280 µmol $cm^{-2}$ $sec^{-1}$. Coral polyps were active and showed expanded tentacles at night after the 3 days acclimatization period. After this period, some of the healthy and bleached fragments were transferred to other aquaria, where the seawater temperature was gradually increased at a rate of 1 °C per day until it reached 33 °C (temperature ramp-up). Subsequently, the feeding experiment (described in section 2.3.1.2) was performed in dark conditions to enhance heterotrophic feeding by synchronizing the starting time with the dark period during acclimatization.

*2.3.1.2 Feeding experiment design*

After acclimatization and temperature ramp-up period (for those fragments exposed to 33 °C), healthy and bleached coral fragments of the two species were incubated in 800-mL glass bottles with a closed running seawater system flowing through at 6 mL $min^{-1}$. One coral fragment per bottle was set up in the center of the bottle using a polyethylene net. To enhance water movement, stirrers were placed in each bottle. The seawater temperature was set to 27 °C or 33 °C. Both coral species were tested in a 2×2 factorial design (two temperatures (27 °C or 33 °C) and two coral conditions (healthy or bleached) with three replicates per treatment. Controls (incubation bottles with only seawater) were also set up (three replicates per each treatment) to estimate natural planktonic variations (Figure 6 and 7). Thirty incubation bottles (I) were kept in a water bath at normal seawater temperature (27 °C; 15 bottles) and higher seawater

temperature (33 °C; 15 bottles) for 6 h. Temperatures were maintained using thermostats with heaters and coolers set up in the incubation system; monitoring and continuous recording was carried out with in situ sensors (MDS-MkV/T, Alec Electronics, Kobe, Japan).

## 2.3.2 Coral feeding experiment

*2.3.2.1 Preparation of seawater for incubation*

Seawater was sampled from the same sites where the corals were collected before starting the incubation. The seawater was pre-filtered with a 100-µm plankton net to remove microplankton. Pico-nanoplankton were concentrated with a tangential flow filtration system (Vivaflow 50 Crossflow Cassettes, 100K MWCO RC; membrane pore diameter 0.2 µm; Sartorius AG, Göttingen, Germany) to minimize cell damage (Figure 8). Plankton concentration was done to improve efficiency in the measurement of feeding rates in short time. The cell concentration ranges were 5.4 - 10.0 × $10^5$ cells $mL^{-1}$ in the natural seawater and 27.3 - 54.5 × $10^5$ cells $mL^{-1}$ in the concentrated seawater.

*2.3.2.2 Coral feeding rates*

The pico- and nanoplankton including bacteria (BA), picocyanobacteria (PCY), picoflagellates (PF), and nanoflagellates (NF), in the running seawater were measured and recorded before and after the 6 h incubation periods under a fluorescence microscope for each incubation condition (Figure 9). The feeding rates were calculated according to Ribes et al. (1998), Frost (1972) and Houlbrèque et al. (2004). Plankton feeding rates were determined according to plankton growth rates in the control and coral chambers. Briefly, these equations are as follows:

**k** is the growth rate of the pico- and nanoplankton ($h^{-1}$) was calculated as follows:

$$\mathbf{k} = \ln(C_t / C_0)/T_t - T_0 \quad (1)$$

where

$C_0$ is the pico- nanoplankton concentration in the chambers (cells $mL^{-1}$) at the initial time ($T_0$)

$C_t$ is the pico- nanoplankton concentration in the chambers (cells $mL^{-1}$) at the final time ($T_t$)

**g** is the feeding coefficient ($h^{-1}$) was calculated as follows:

$$\mathbf{g} = k_c - k_g \quad (2)$$

where

$k_c$ is the growth rate of the pico- and nanoplankton in the control chamber ($h^{-1}$)

$k_g$ is the growth rate of the pico- and nanoplankton in the coral chamber ($h^{-1}$)

**F** is the feeding rate (cells $h^{-1}$) was calculated as follows:

$$\mathbf{F} = V \times g/N \quad (3)$$

where

V is volume of seawater in the chamber (mL)

g is the feeding coefficient ($h^{-1}$)

N is the final concentration of pico- nanoplankton in the chamber

**C** is the average prey concentration (cells $mL^{-1}$) during the experiment was calculated as follows:

$$\mathbf{C} = C_0[e^{(k-g)(T_t - T_0)} - 1]/(k - g)\,(T_t - T_0) \qquad (4)$$

**I** is the Feeding rate (prey ingested $mg^{-1}$ protein $h^{-1}$) was calculated as follows:

$$\mathbf{I} = FC \qquad (5)$$

The feeding rates were normalized by the branch surface area or by the protein concentration in coral tissues.

*2.3.2.3 Enumeration of the pico- and nanoplankton and calculation of biomass from biovolumes*

To enumerate pico- and nanoplankton, samples were transferred from the incubation chambers into sterile 50-mL tubes, fixed with 25 % w/v glutaraldehyde (1 % final concentration) and kept at 4 °C until analysis. For BA, PCY, PF, and NF, the samples were stained with DAPI (4', 6-diamidino-2-phenylindole) (Porter and Feig, 1980). The 3 mL aliquots of each of these samples were filtered through 0.2-µm black polycarbonate filters to count BA and PCY. Then, 30 mL aliquots were filtered through 0.8-µm black polycarbonate filters to count PF and NF. The filters were mounted onto glass slides and the cells were enumerated under an epifluorescence microscope (Eclipse/E600; Nikon Corp., Tokyo, Japan). Approximately 10–15 fields per sample were counted. The biomasses of the various pico- and nanoplankton groups were calculated from the biovolume to biomass relationships. The average cell dimensions of the picoplankton

and nanoplankton were measured from >30 cells per group. Biovolumes were determined from the average cell dimensions assuming the most similar geometrical shape according to the method of Sun and Liu, (2003). Carbon biomasses were calculated using the biovolume to biomass conversion factors and nitrogen using C/N ratio as BA = 30.2 fg C $cell^{-1}$ and 5.8 fg N $cell^{-1}$ (Fukuda et al., 1998), PCY = 700 fg C $\mu m^{-3}$ and 50 fg N $\mu m^{-3}$ and PF or NF = 220 fg C $\mu m^{-3}$ and 26 fg N $\mu m^{-3}$ (Houlbrèque et al., 2004 and references in there). C/N ratio of each of the plankton groups were calculated according to Fukuda et al., (1998) for bacteria and Houlbrèque et al., (2004) and references in their for other pico- and nanoplankton groups.

## 2.3.3 Coral biological characteristics

*2.3.3.1 Polyp size and density*

Polyps' diameters were measured under a stereomicroscope (C-DSS115, Nikon Corp., Tokyo, Japan). The averages of 30-40 measurements were recorded. Polyp density was determined from polyp counts within an area of 1 $cm^2$.

*2.3.3.2 Symbiodiniaceae density*

Coral tissues were separated from the skeleton using a Waterpik® (Ricoh Elemex Corporation, Aichi, Japan) filled with 3.5% w/v NaCl solution (Johanes and Wiebe, 1970). The extracted tissues were homogenized with a glass homogenizer and centrifuged at 3,000*g* for 15 min. The supernatants were removed, and Symbiodiniaceae pellets were resuspended in the 3.5% w/v NaCl solution. This procedure was repeated three times to remove remaining coral tissue. Symbiodiniaceae were counted with a

Neubauer-line haemocytometer (Erma Inc., Tokyo, Japan) under an ECLIPSE 80i microscope (Nikon Corp., Tokyo, Japan). The data were normalized to the coral surface area ($cm^2$). The surface areas of the coral nubbins were determined by the aluminium foil method (Marsh, 1970).

*2.3.3.3 Protein concentration*

A small portion of coral fragments of about 1 $cm^2$ were grounded in a mortar containing 2 mL of 50 mM Tris-HCl buffer at pH 7.5, then centrifuged at 10,000*g* for 3 min to remove skeleton and insoluble components including broken membranes from Symbiodiniaceae cells. Protein content in the supernatant was determined by the method of Palmer et al. (Palmer et al., 2009); triplicate 25-µL supernatant aliquots were loaded into 96-well microtiter plates and quantified with a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) by measurement absorbance at 562 nm with a multi-detection microplate reader (BioTek Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA). The concentrations were normalized to the surface area of the coral pieces.

## 2.3.4 Statistical analysis

Data were checked for normality using the Kolmogorov-Smirnov test; square root transformation was performed when data were not normally distributed. One-way analysis of variance (ANOVA) was used to test significant differences between coral conditions, two-way ANOVA was used to test significant differences between seawater temperature (normal 27 °C vs. high 33 °C; two levels) and coral conditions (healthy vs.

bleached; two levels), and three-way ANOVA was used to identify differences among coral conditions, temperature treatments, and time (0 h vs. 6 h incubation). Pairwise comparisons with post hoc Tukey's test were used to test level of significant differences ($p < 0.001$, $p < 0.01$, and $p < 0.05$). A $p$ value < 0.05 was considered significant. Minitab v. 14 (Minitab Inc., State College, PA, USA) was used for all statistical analyses.

## 2.4 Results

### 2.4.1 Morphological and biological characteristics of *Montipora digitata* and *Porites lutea* before starting incubation

The initial (before starting the feeding experiment, 0 h) morphological and biological characteristics of the healthy and bleached *M. digitata* and *P. lutea* colonies and their symbiotic Symbiodiniaceae are shown in Table 1. In comparison with *M. digitata,* the polyps of *P. lutea* were twice as large, with concomitantly lower density. The protein concentrations in healthy and bleached of *P. lutea* were 2.24 mg $cm^{-2}$ and 1.55 mg $cm^{-2}$ respectively; these were 5 and 4.4 times higher than in healthy and bleached of *M. digitata*, respectively. Symbiodiniaceae densities in *P. lutea* were also higher densities than in *M. digitata* with 3.5 times in healthy to 3.8 times in bleached.

### 2.4.2 Symbiodiniaceae and protein changes in coral tissue after incubation

Changes in the Symbiodiniaceae densities during incubation are shown in Figure 10. The Symbiodiniaceae densities in the healthy *M. digitata* increased after 6 h incubations at 27 ℃ and 33 ℃; the increase at 33 ℃ was greater than that at 27 ℃, but not in a statistically significant manner. The Symbiodiniaceae densities for healthy *P. lutea* varied among the treatments, but not to a statistically significant level. In bleached nubbins, Symbiodiniaceae cells had similar at the initially and after incubation at both 27 ℃ and 33 ℃. For the bleached *P. lutea* nubbins, the Symbiodiniaceae density substantially but non-significantly decreased at 33 ℃ relative to the initial and 27 ℃ values.

The total protein concentration (mg $cm^{-2}$) in healthy *P. lutea* nubbins was higher than that of healthy *M. digitata* ($p < 0.01$) (Figure 10). In healthy *M. digitata* nubbins, the protein content slightly increased at 27 ℃ and slightly decreased at 33 ℃ relative to the initial value. It did not significantly vary among conditions and treatments. For bleached nubbins, *P. lutea* was higher protein concentration than that of *M. digitata* ($p < 0.01$) (Figure 18). Bleached *M. digitata* were no significant variations in protein concentration among conditions and treatments. For bleached *P. lutea*, the protein concentration was slightly lower in bleached nubbins at 27 ℃ and 27% lower in the bleached nubbins at 33 ℃ than the initial value and were not significant different (Figure 11).

## 2.4.3 Feeding rates

*2.4.3.1 Cell capture and consumption rates*

The feeding rates on different groups of pico-nanoplankton (cells per coral tissue protein per hour) are shown in Figure 12. Healthy *Montipora digitata* in normal seawater temperature (27 °C) preferentially consumed NF, followed by PCY and BA. Bleached fragments showed a greater preference for BA than healthy fragments ($p < 0.01$), followed by NF. In the seawater in which bleached corals were incubated, the PCY and PF concentrations increased (negative values), indicating that the rate of consumption by coral was lower than that of plankton growth (Figure 12A). At 33 °C, the healthy nubbins consumed significantly more BA and NF than the bleached fragments ($p < 0.01$), while the bleached nubbins consumed relatively more PCY than the healthy fragments (Figure 12C). At 27 °C, *Porites lutea* consumed all food sources provided; consumption rates of bleached corals were higher than those of healthy corals (Figure 12B). At 33 °C, both healthy and bleached *P. lutea* consumed food sources in similar quantities, although both of the health conditions preferred PCY (Figure 12D).

Total feeding rates (number of pico-nanoplankton cells) are shown in Figure 13. Higher feeding rates were found in *M. digitata* than in *P. lutea* in all treatments except the bleached fragments at 33 °C, which displayed similar feeding rates. Higher feeding rates were observed at 27 °C in bleached fragments than the healthy ones (42% higher in *M. digitata* and 72% higher in *P. lutea*; $p < 0.01$). In comparison, feeding rates of bleached fragments were lower at 33 °C than those of healthy fragments (79% and 48% for *M. digitata* and *P. lutea*, respectively). Healthy corals showed significantly higher ($p < 0.01$) feeding rates at 33 °C than at 27 °C, while bleached corals showed higher

feeding rates at 27 °C than at 33 °C in both coral species ($p < 0.001$ in *M. digitata*), but this difference was not significant in the case of *P. lutea*.

Table 3 shows the feeding rates of healthy and bleached corals in number of cells consumed per polyp or per $cm^2$ coral surface. At 27 °C, these feeding rates were higher in healthy *M. digitata* than healthy *P. lutea.* However, *M. digitata* incorporated more carbon $cm^{-2}$ than *P. lutea*. At 33 °C, the cell capture rate per healthy nubbin polyp was non-significantly higher in *P. lutea* than it was in *M. digitata.* In terms of cell capture and carbon incorporation per unit surface area, the values for healthy *M. digitata* were substantially higher than those for healthy *P. lutea*. For bleached nubbins, *P. lutea* feeding rates were higher at 27 °C and 33°C than *M. digitata*. Nevertheless, the feeding rates were similar for the bleached nubbins of both species.

*2.4.3.2 Assimilation of organic matter*

Assimilation of organic carbon (ng C $mg^{-1}$ protein $h^{-1}$) are shown in Figure 14. The most important organic carbon source under all conditions for both coral species was NF, with the exception of bleached *P. lutea* at 33 °C, for which PCY was the most important carbon source. In particular, bleached *M. digitata* incorporated significantly ($p < 0.01$) more carbon from NF than their healthy counterparts under a normal temperature; conversely, healthy corals incorporated more carbon from NF than the bleached ones at 33 °C ($p < 0.001$). Bleached *P. lutea* corals incorporated more carbon than the healthy ones from all available food sources at 27 °C. Consumption of BA did not significantly contribute to the incorporation of organic carbon in either coral species.

The total organic carbon and nitrogen assimilated are shown in Figure 15. Carbon assimilated by *M. digitata* was higher than that assimilated by *P. lutea*. At 27 °C, healthy *M. digitata* incorporated 94% more C and N than the healthy *P. lutea,* and the bleached nubbins incorporated 71% and 76% more C and N, respectively, than *P. lutea*. At 33 °C, healthy *M. digitata* incorporated more organic C and N (~70% and 76%, respectively) than healthy *P. lutea*. Despite the comparatively lower C and N incorporation by the bleached nubbins, *M. digitata* incorporated ~50% and ~63% more C and N, respectively, than *P. lutea* under thermal stress. Under thermal stress (33 °C), healthy *M. digitata* assimilated three times more C ($p < 0.001$) and N ($p < 0.01$) than bleached. At normal temperature (27 °C), C and N assimilation were higher in the bleached nubbins compared with the healthy ones, but not statistically different. For *P. lutea*, the patterns were similar, except the differences between treatments and conditions were not statistically significant. The C/N ratios reflected the coral feeding preferences under each condition: under thermal stress, healthy *M. digitata* only consumed NF, and thereby represents a comparatively low C/N ratio; however, bleached nubbins under thermal stress consumed low amounts of food but also incorporated PCY, which represents a comparatively high C/N food source (Table 2). Under thermal stress, bleached *P. lutea* consumed mainly PCY, which are characterized by a relatively high C/N ratio. Therefore, both corals under bleached conditions and thermal stress preferred higher C/N-ratio foods to overcome the decrease in carbon-rich resources from translocation.

Table 3 shows the feeding rates of healthy and bleached corals in term of carbon assimilation per polyp or per $cm^2$ coral surface. Healthy *P. lutea* incorporated more cells than *M. digitata.* Whileas that, bleached *M. digitata* incorporated more carbon $cm^{-2}$ than

bleached *P. lutea*. Even if *P. lutea* incorporated more cells than *M. digitata,* but the latter incorporated more carbon than the former when normalized carbon per cm per h. These results reflect the shift in food preferences from nanoplankton to picoplankton for *P. lutea.* Picoplankton did not constitute important carbon sources (Supplementary Tables 4 and 5).

## 2.5 Discussion

Healthy corals living in well-illuminated waters obtain most of their metabolic requirements from autotrophy, but heterotrophy plays a crucial role in supplementing the carbon-rich autotrophic diet with nitrogen and phosphorus (Goldberg, 2018). The survival and recovery of bleached corals may be totally or partially contingent on heterotrophy to compensate for the lack of organic matter resulting from the loss of Symbiodiniaceae (Hughes and Grottoli, 2013; Schoepf et al., 2015b). Heterotrophy may also change according to environmental conditions and coral health. Our experimental configuration enabled us to measure feeding rates in both healthy and bleached coral conditions and under normal (27 °C) and stressful (33 °C) seawater temperatures. Pico- and nanoplankton also represent the main carbon and nitrogen sources there. They play crucial roles in the organic carbon and nitrogen flow through the food web. They also link the planktonic and benthic environments (Casareto et al., 2006, 2000; Ferrier-Pagès and Gattuso, 1998; Ribes et al., 2003). Recently, it was shown that corals can feed on pico-nanoplankton (Houlbrèque et al., 2004; Picciano and Ferrier-Pagès, 2007; Tremblay et al., 2011). The use of concentrated plankton enabled the efficient measurement of feeding rates within a short time frame (6 h). In this way, changes in

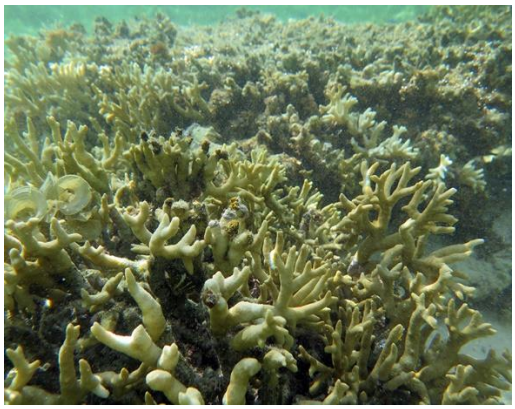
coral condition due to chamber enclosing effects were avoided. Despite the seawater concentration, the plankton densities in our experimental chambers still corresponded with those normally observed in Okinawan reef waters (Casareto et al., 2006). Therefore, our feeding rate estimates were comparable to those found under natural conditions.

Two species of scleractinian corals that are very common in Okinawan waters (Hongo and Yamano, 2013) were evaluated in the present study: the branching *Montipora digitata* and the massive *Porites lutea*. They both have rather small polyps. However, the polyps of *P. lutea* are twice the size of those of *M. digitata* whereas the protein content in *P. lutea* is ~5× greater than that in *M. digitata*. These morphophysiological differences may reflect the variations in food requirement, the particle size preference, and/or feeding efficiency between these two species. Heterotrophy may also change according to environmental conditions and coral health.

Based on the number of cells consumed or organic carbon incorporated per coral protein unit, *M. digitata* consumed more pico-nanoplankton cells (72-87 % in healthy or 29-74 % in bleached) and more carbon (70-94 % in healthy or 50-71 % in bleached) than healthy *P. lutea.* However, *M. digitata* consumed mainly NF, which is an important carbon source, whereas *P. lutea* consumed only PCY and PF, both of which are comparatively poorer carbon sources. These results answer our question (1) related to feeding rates in terms of prey selection and organic carbon assimilation. All treatment combinations of *P. lutea* incorporated more cells per polyp than the healthy *M. digitata* nubbins at normal temperature possibly because this species possesses relatively large polyps. Nevertheless, *M. digitata* consumed mainly NF, which is an important carbon

source. In contrast, *P. lutea* consumed equal amounts of all plankton across the size spectrum. These results answer second part of our question (1) related to feeding implications due to differences in polyp size and density. Under the combination of bleaching and thermal stress, *M. digitata* feeding dramatically decreased but its food preference shifted towards NF. At normal temperature, bleached corals consumed more cells and incorporated more carbon than did healthy corals. *M. digitata* consumed mainly NF, which is an important carbon source. In contrast, *P. lutea* consumed equal amounts of all plankton across the size spectrum but under combined bleaching and thermal stress, they consumed only pico plankton, which are not important carbon sources. These results answer second part and third part of our question. The relatively high feeding rates of *M. digitata*, particularly with NF as the main food source, indicate that this species depends upon heterotrophy to overcome bleaching.

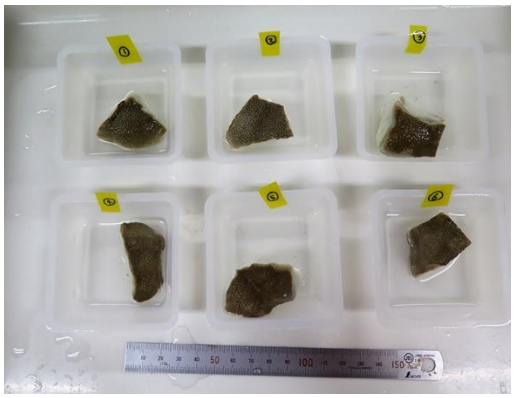
An important aspect of *P. lutea* coral is the possession of endolithic community. (Sangsawang et al., 2017) reported the translocation of organic carbon and nitrogen from the endolithic community to the tissues of both healthy *P. lutea.* This supply of organic matter from the endolithic community of *P. lutea* explains its low feeding rates compared to those for *M. digitata.*



branching *Montipora digitata*

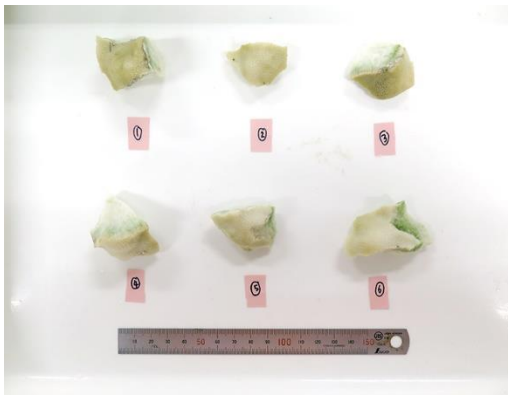
massive *Porites lutea*

healthy *M. digitata*



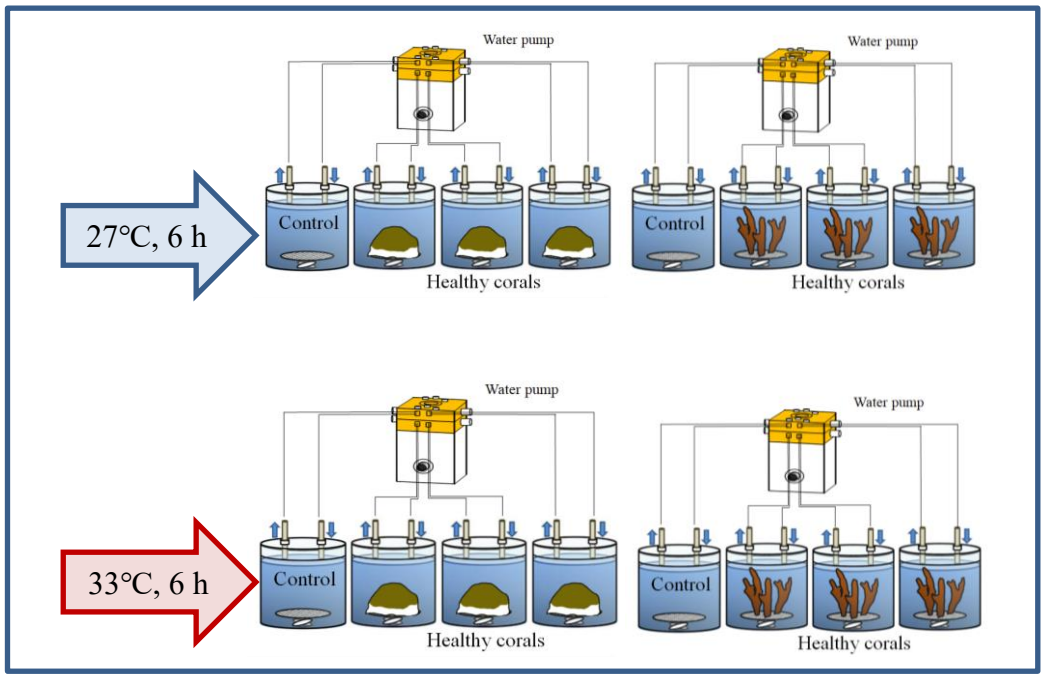
healthy *P. lutea*

bleached *M. digitata*



bleached *P. lutea*

**Figure 5** Healthy and bleached coral nubbins used during incubation experiment



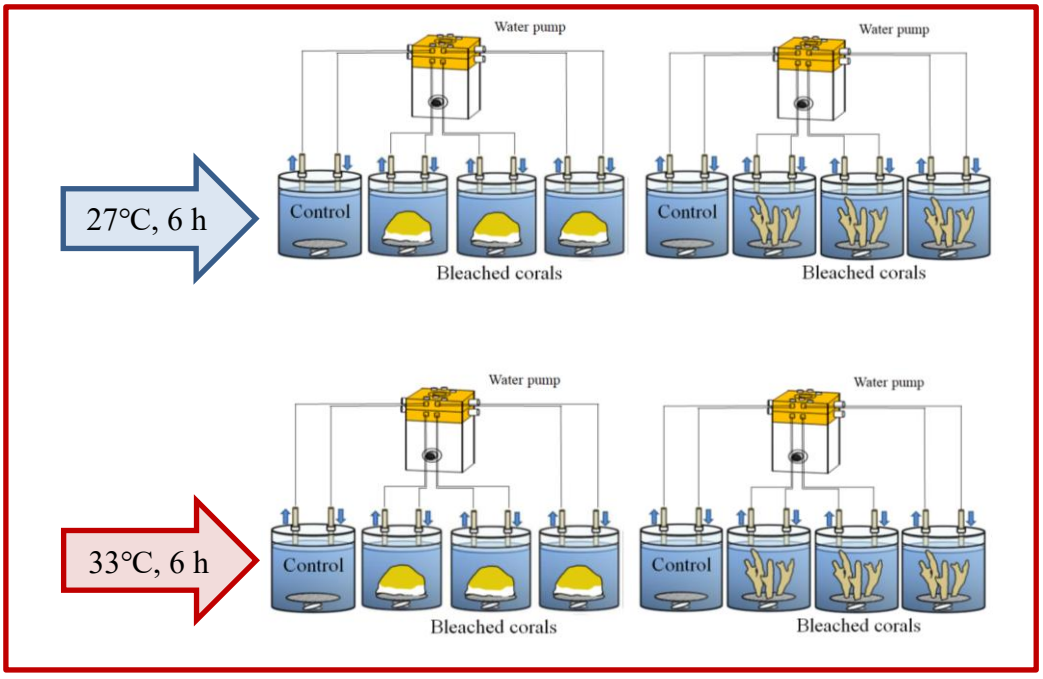




**Figure 6** Incubation experiment design

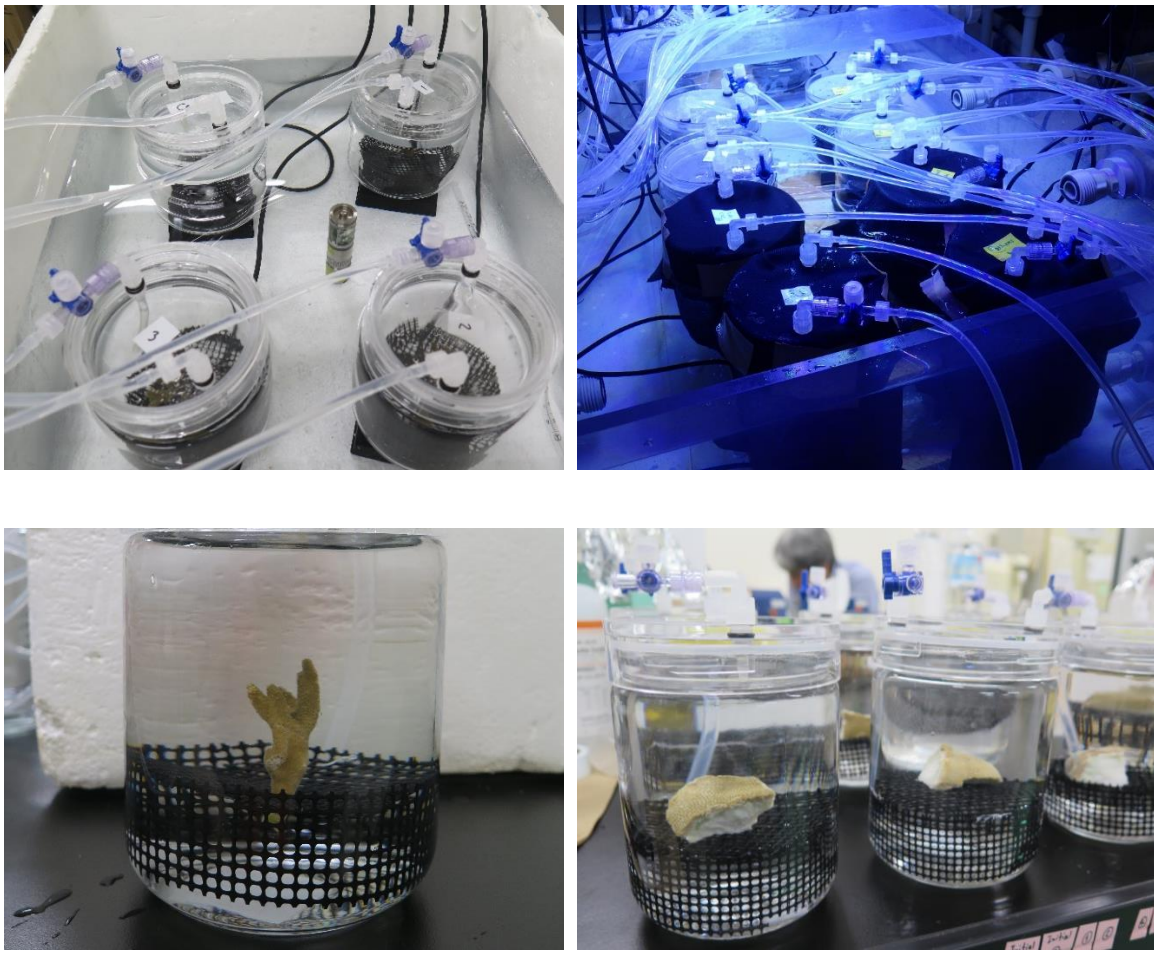


**Figure 7** Incubation system for coral feeding experiment

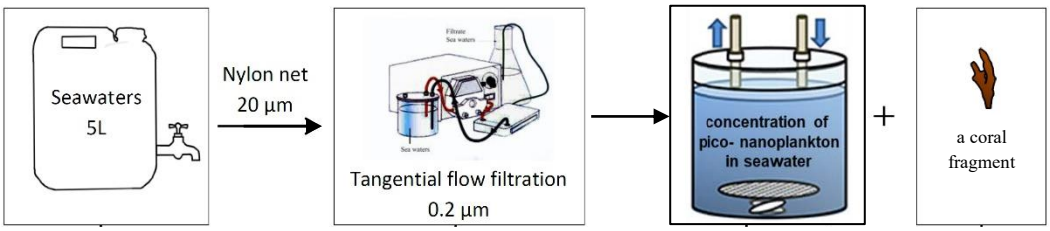




**Figure 8** Tangential flow filtrated system and preparation of seawater for incubation process.

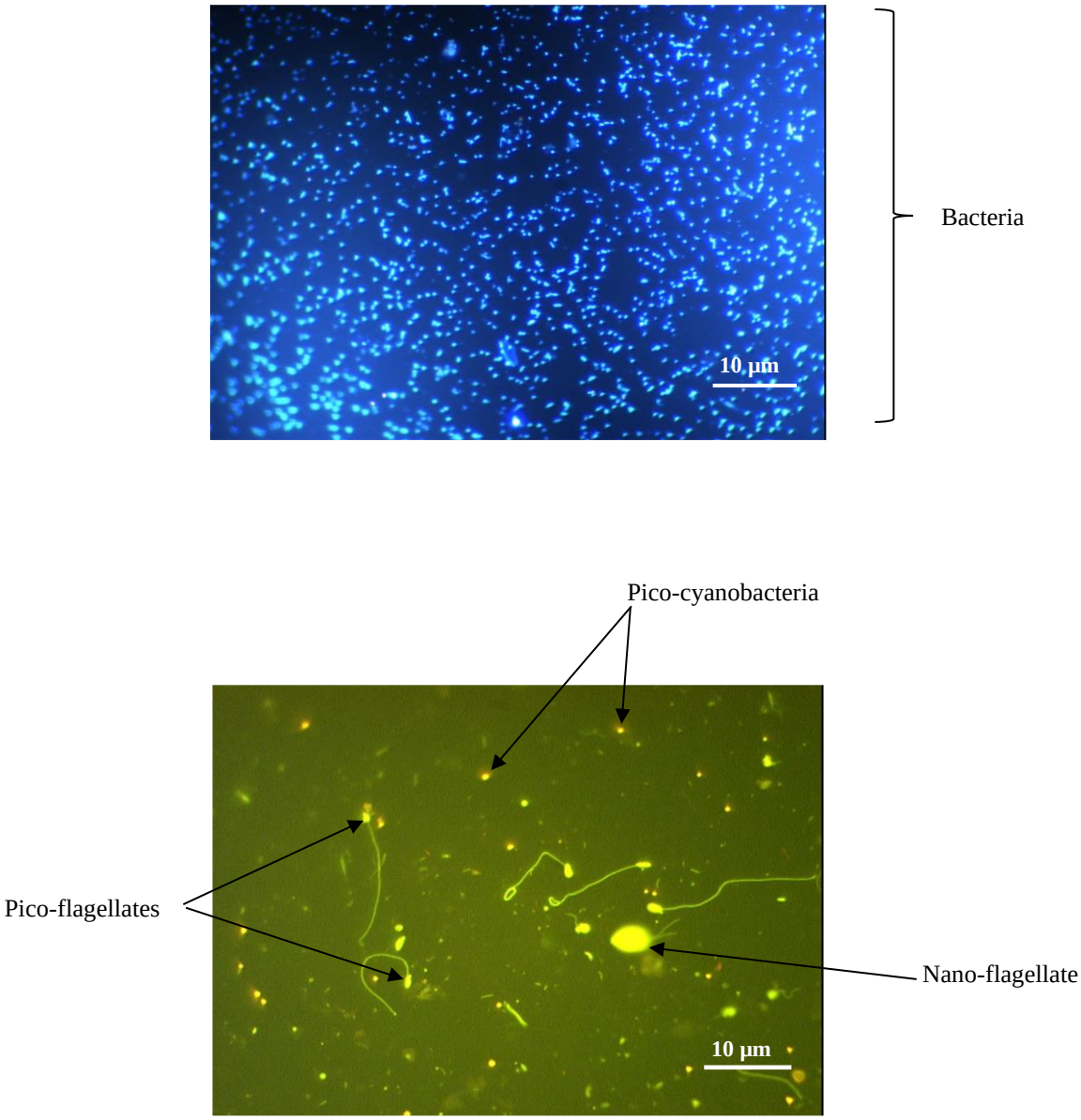



**Figure 9** Character of pico- and nanoplankton groups under fluorescence microscope

**Table 1** Morphological and biological characteristics of healthy and bleached corals before incubation (means ± SD).

| Corals condition | Polyp size (mm) | Polyp density ($cm^{-2}$) | Protein (mg $cm^{-2}$) | Symbiodiniaceae (cells $cm^{-2}$) |
|---|---|---|---|---|
| Healthy corals | | | | |
| *M. digitata* | 0.51 ± 0.08 | 63.64 ± 4.70 | 0.43 ± 0.1 | 0.69 ± 0.1 $\times 10^6$ |
| *P. lutea* | 1.17 ± 0.22 | 49.09 ± 3.75 | 0.35 ± 0.0 | 0.37 ± 0.1 $\times 10^6$ |
| Bleached corals | | | | |
| *M. digitata* | 0.51 ± 0.08 | 63.64 ± 4.70 | 2.24 ± 0.1 | 2.41 ± 0.0 $\times 10^6$ |
| *P. lutea* | 1.17 ± 0.22 | 49.09 ± 3.75 | 1.55 ± 0.4 | 1.40 ± 0.7 $\times 10^6$ |
| Replicates (n) | 30-40 | 10-11 | 9 | 6 |

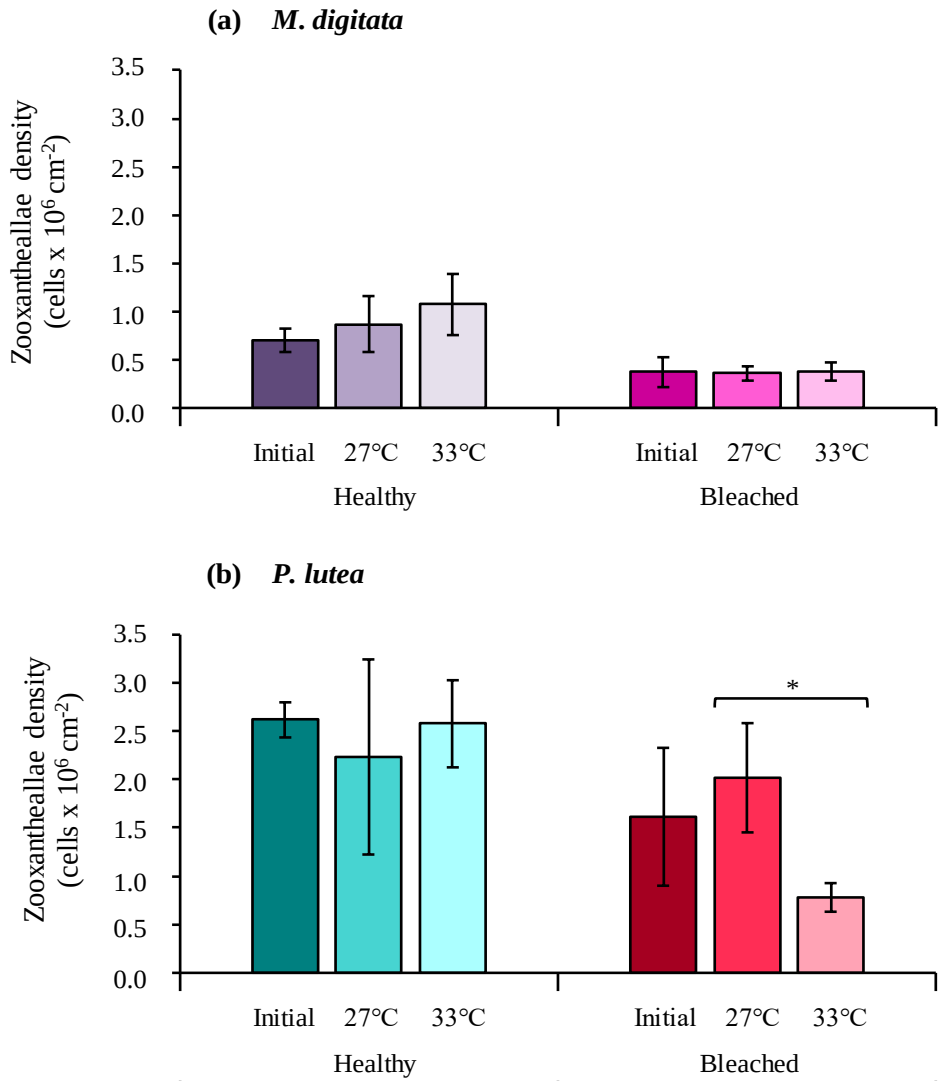



**Figure 10** Symbiodiniaceae densities of (a) *M. digitata* and (b) *P. lutea* before (initial) and after 6 h incubation at normal (27 ℃) and high (33 ℃) seawater temperatures. Data are means ± SD, n = 6 for each treatment. One-way ANOVA and post hoc Tukey's test were applied to identify the differences among temperature treatments, * = $p < 0.05$

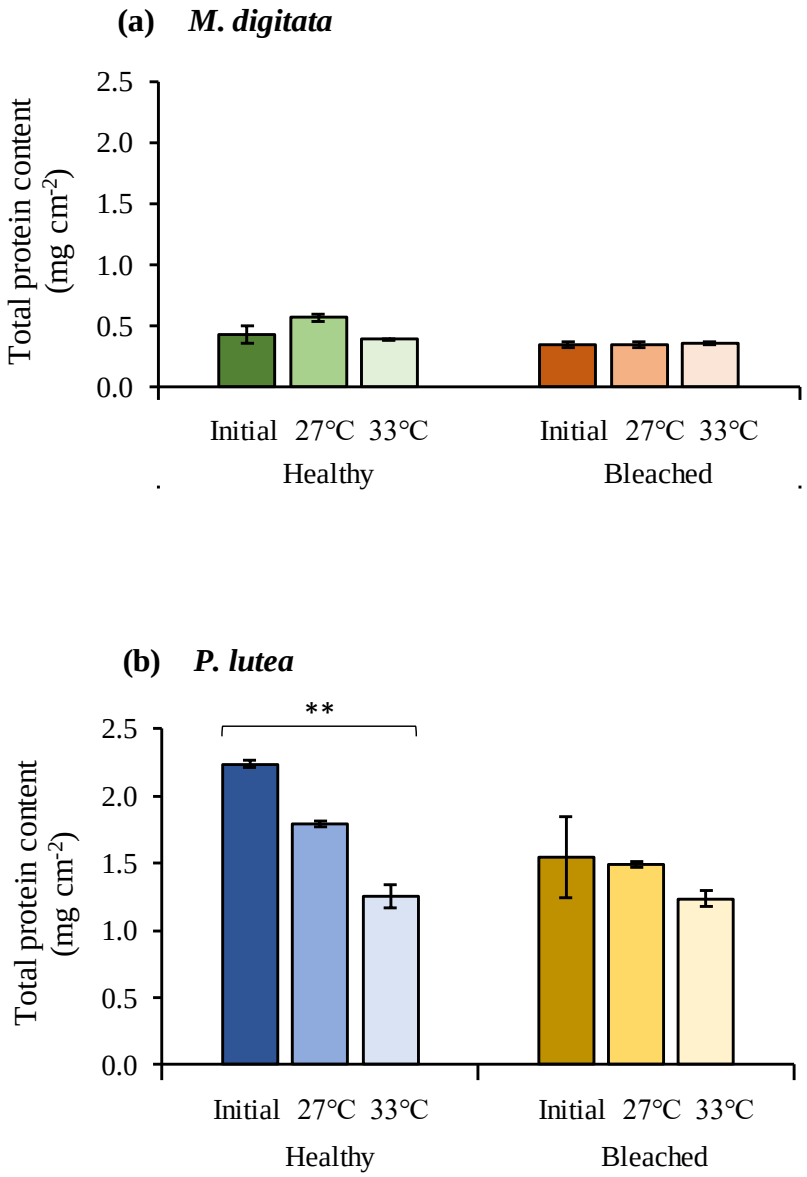



**Figure 11** Total protein concentration of corals tissue: (a) M. digitata and (b) P. lutea before (initial) and after 6 h incubation at normal (27 °C) and high (33 °C) seawater temperatures. Data are means ± SD, n = 9 for each treatment. One-way ANOVA and post hoc Tukey's test were applied to identify the differences among temperature treatments, ** = $p < 0.01$

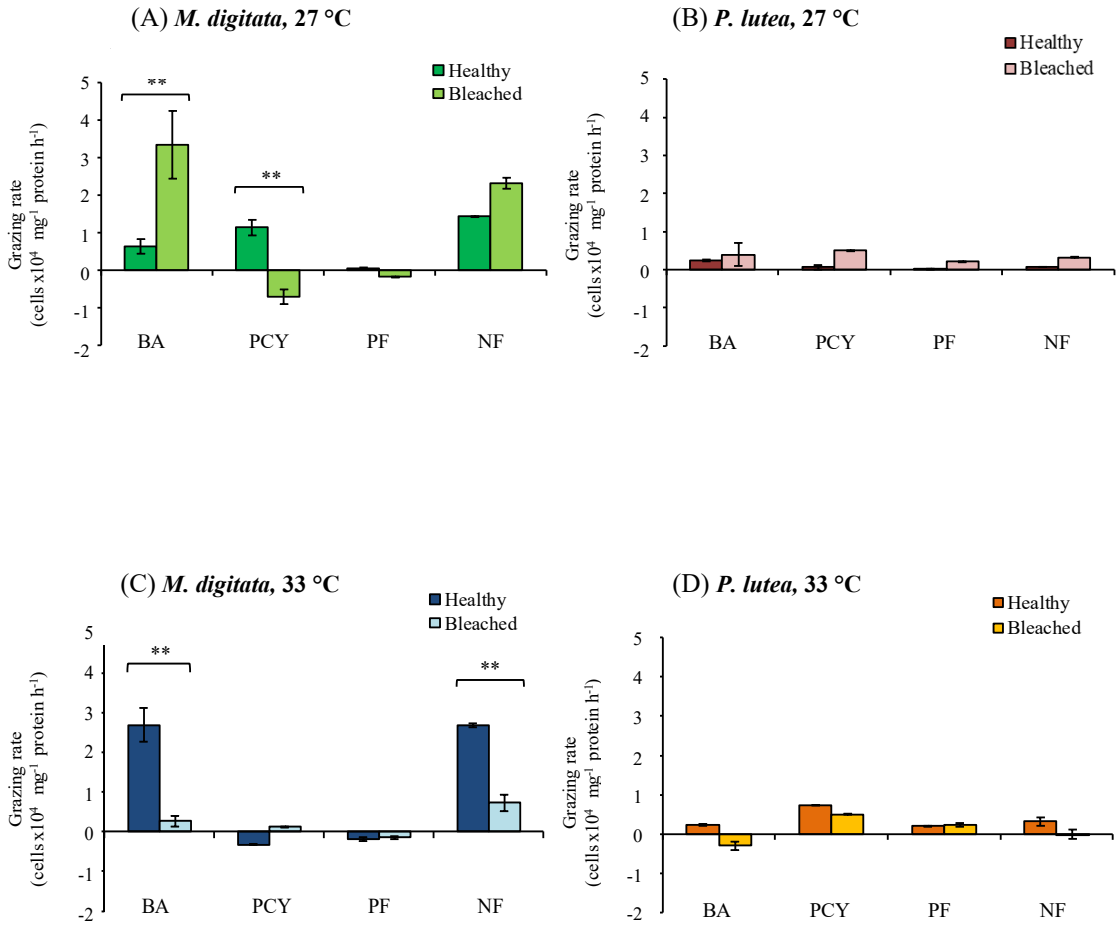



**Figure 12** Feeding rates of corals nubbins in number each group of cells (pico-nanoplankton) consumed per mg protein (in coral tissue) per hour at 27 °C or 33 °C. Data are means ± SD, n = 3 for each treatment. One-way ANOVA and post hoc Tukey's test were applied to identify the differences among temperature treatments, * $p < 0.05$. BA = bacteria, PCY = picocyanobacteria, PF = picoflagellates, and NF = nanoflagellates.

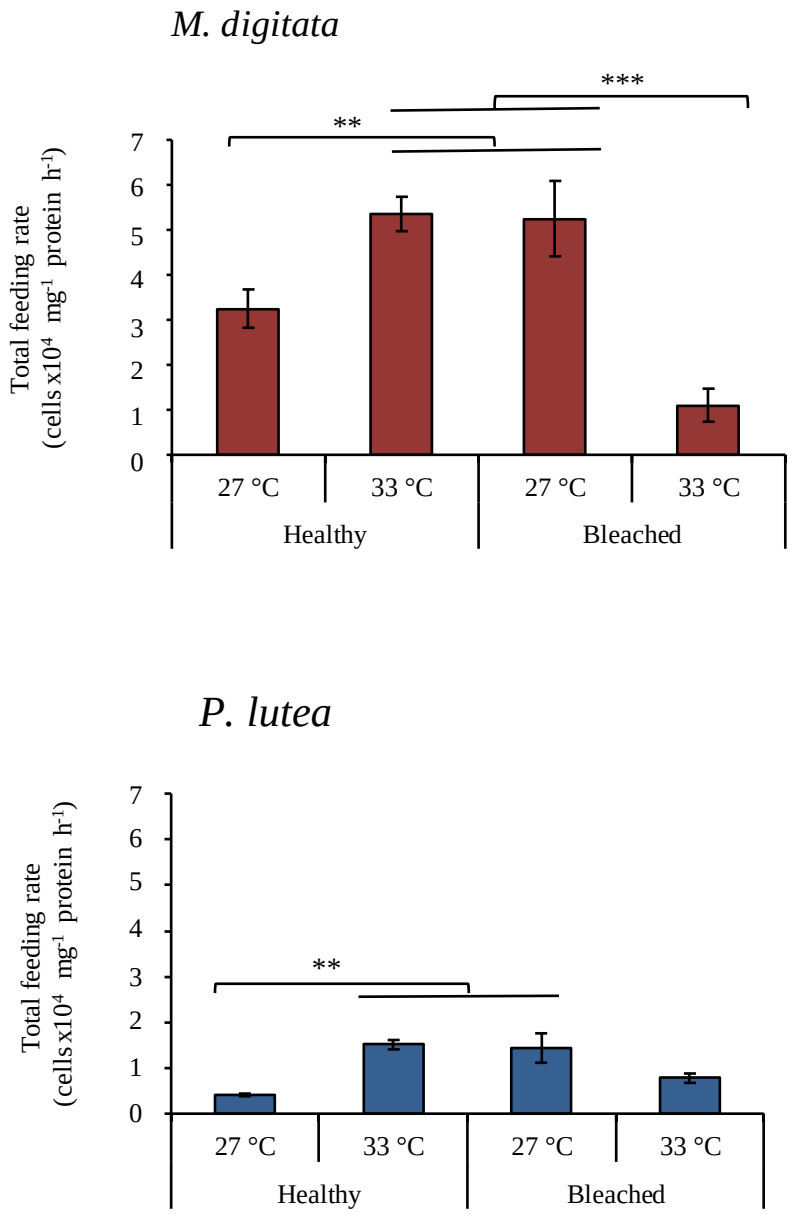



**Figure 13** Feeding rates of coral nubbins in total number of cells (pico- nanoplankton) consumed per mg protein (in coral tissue) per hour in at 27 °C and 33 °C**.** Data are means ± SD, n = 3 for each treatment. Two-way ANOVA and post hoc Tukey's test were applied to identify the differences among coral species and temperature treatments, ** $p < 0.01$, *** $p < 0.001$.

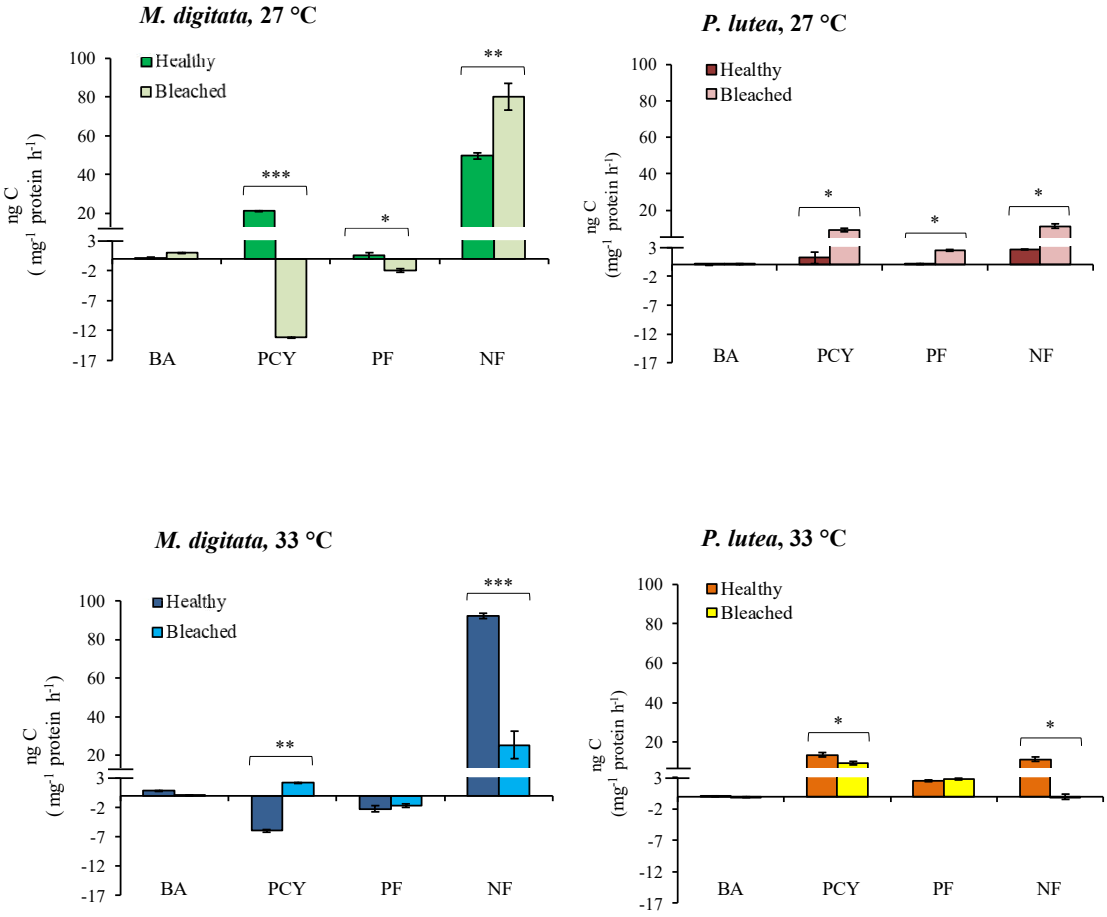



**Figure 14** Carbon assimilated of coral feeding by pico- and nanoplankton groups in ng C per protein (in coral tissue) per hour of corals at 27 ºC and 33 °C. Data are means ± SD, n = 3 for each treatment. One-way ANOVA and post hoc Tukey's test were applied to identify the differences among temperature treatments, * $p < 0.05$, **. $p < 0.01$, ***. $p < 0.001$.

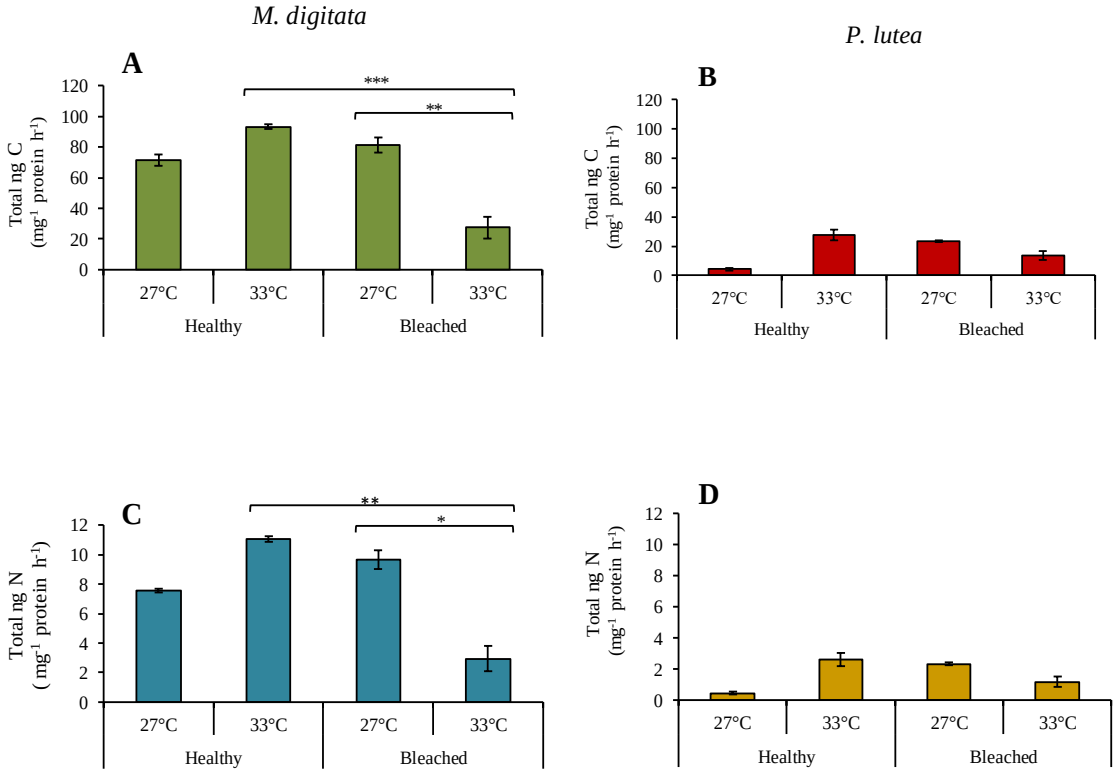



**Figure 15** Total ng carbon (A, B) and nitrogen (C, D) assimilated of corals feeding per protein (in coral tissue) per hour (picoplankton + nanoplankton) at 27 °C and at 33 °C. Data are means ± SD, n = 3 for each treatment. Two-way ANOVA and post hoc Tukey's test were applied to identify the differences among coral species and temperature treatments, * $p < 0.05$, **. $p < 0.01$, ***. $p < 0.001$.

**Table 2** C:N ratio of incorporated organic matter by *Montipora digitata* and *Porites lutea* (mean ± SD, n = 3).

| Coral species | Healthy | | Bleached | |
|---|---|---|---|---|
| | 27 °C | 33 °C | 27 °C | 33 °C |
| *M. digitata* | 9.6 ± 0.2 | 8.4 ± 0.01 | 8.4 ± 0.02 | 9.4 ± 0.4 |
| *P. lutea* | 9.3 ± 0.8 | 10.6 ± 0.4 | 10.0 ± 0.1 | 11.8 ± 0.7 |

**Table 3** Total feeding of pico- nanoplankton cells consumed, or organic carbon incorporated of pico- nanoplankton by *M. digitata* and *P. lutea* represent per coral polyp or per $cm^2$ of coral tissue. Values indicate mean ± SD, n = 3

| | ***M. digitata*** | ***P. lutea*** |
|---|---|---|
| **Cells polyp$^{-1}$ h$^{-1}$** | | |
| Healthy - 27 ℃ | 287.7 ± 46.4 | 148.7 ± 36.9 |
| Healthy - 33 ℃ | 326.4 ± 38.7 | 386.3 ± 43.2 |
| Bleached - 27 ℃ | 299.8 ± 25.6 | 317.3 ± 44.8 |
| Bleached - 33 ℃ | 63.2 ± 26.6 | 186.3 ± 126.7 |
| **Cells cm$^{-2}$ h$^{-1}$** | | |
| Healthy - 27 ℃ | 1.8 ± 0.3 x$10^4$ | 0.7 ± 0.1 x$10^4$ |
| Healthy - 33 ℃ | 2.0 ± 0.2 x$10^4$ | 0.8 ± 0.2 x$10^4$ |
| Bleached - 27 ℃ | 1.9 ± 0.2 ×$10^4$ | 2.1 ± 0.6 ×$10^4$ |
| Bleached - 33 ℃ | 0.4 ± 0.2 ×$10^4$ | 0.9 ± 0.4 ×$10^4$ |
| **ng C polyp$^{-1}$ h$^{-1}$** | | |
| Healthy - 27 ℃ | 0.22 ± 0.2 | 0.11 ± 0.02 |
| Healthy - 33 ℃ | 0.56 ± 0.02 | 0.39 ± 0.1 |
| Bleached - 27 ℃ | 0.36 ± 0.01 | 0.45 ± 0.03 |
| Bleached - 33 ℃ | 0.18 ± 0.1 | 0.09 ± 0.02 |
| **ng C cm$^{-2}$ h$^{-1}$** | | |
| Healthy - 27 ℃ | 14.18 ± 15.6 | 5.68 ± 0.2 |
| Healthy - 33 ℃ | 36.19 ± 1.0 | 22.26 ± 1.2 |
| Bleached - 27 ℃ | 27.46 ± 2.10 | 19.10 ± 5.7 |
| Bleached - 33 ℃ | 15.00 ± 3.0 | 4.62 ± 6.7 |

# Chapter 3

# Dissolved organic carbon (DOC) flux from *M. digitata* and *P. lutea*

## 3.1 Abstract

Scleractinian coral secrete mucus (mainly dissolve organic carbon, DOC) to trap plankton especially pico- and nano- size from the seawater. Dissolved organic carbon (DOC) were monitored at the initial and final incubation experiment of healthy and bleached coral at normal (27 ºC) and stressful (33 ºC) seawater temperatures about 6 h and supplied by natural assemblages of pico-nanoplankton concentrated via tangential flow system. This researched would like to understand coral efficiency and capture pico- and nanoplankton from dissolved organic matter (DOC) fluxes dynamics during incubations. The result shown a negative DOC flux in bleached *M. digitata* at 33 °C showed that decreased plankton capture was due to reduced mucus secretion. Conversely, *P. lutea* continued to release DOC even under the combined stress. Therefore, I concluded that mucus release in *P. lutea* could serve for another purpose besides plankton trapping. Moreover, protein catabolism was observed in *P. lutea* under the combined stresses. *M. digitata* was highly dependent on heterotrophy and very efficient at food capture. Nevertheless, its plankton capture performance was substantially diminished by the combination of bleaching and heating. On the other hand, *P. lutea* was comparatively less dependent on heterotrophy and was able to catabolize stored materials (proteins).

## 3.2 Introduction

In coral reef environments, pico-plankton (0.2-2 μm) and nano-plankton (2-20 μm) are the most important components of the particulate of organic matter (Casareto et al., 2006; Charpy, 2005). Many hard and soft corals release mucus to trap small size of particulate matter as pico- and nanoplankton from surrounding sea water for feeding, which are a source of reef (Naumann et al., 2009). When corals produce mucus, almost dissolves in the sea water immediately about 56% to 80% of coral mucus production. At the same time, bactreoplankton can utilize mucus release from coral release as a food source (Ducklow and Mitchell, 1979; Wild et al., 2004). This is important for converting dissolve nutrient into particulate biomass  Therefore, coral mucus can act as an energy-rich (DOC/DON) for the microbial loop in food web to higher consumer levels (Bythell and Wild, 2011), such as, sponge (Rix et al., 2016), cnidarians (Coffroth, 1990; Naumann et al., 2009). Coral mucus can be degraded in the water column (Moriarty et al, 1985). Scleractinian coral also produce mucus under environment stress to protect them from coral disease, dehydration, UV radiation, pollutants, oil spill, sedimentation, salinity or temperature changes (Bythell and Wild, 2011). Moreover, corals also may absorb dissolve organic matter (DOM) such as dissolved free amino acids (DFAA) under certain conditions, especially starvation. In this study, I wished to understand a role of dissolved organic carbon flux in feeding efficiency of pico-, nanoplankton by both healthy and bleached corals.

## 3.3 Methods

The dissolved organic carbon (DOC) concentrations in seawater were collect before and after 6h incubation experiments. Thirty milliliters seawater from each incubation chamber was collected using a glass syringe directly connected to a 25-mm diameter glass fiber filter set (Whatman GF/F; Toyo Roshi Kaisha Ltd., Bunkyo-ku, Tokyo, Japan). The seawater samples were filtered and directly dispensed into brown glass vials (Shinomura et al., 2005). They were preserved at -20 °C until analysis (Figure 16). The glass fiber filters were pre-combusted at 500 °C for 4 h. The DOC concentrations were measured with a TOC-LCPH auto analyzer (Shimadzu Corp., Kyoto, Japan) by the high-temperature combustion catalytic oxidation method (Suzuki et al., 1992). The analytical precision of the DOC measurement was within 0.95%. The intercept and slope of the calibration curve were determined for the total blank. An international certified reference material (deep Sargasso Sea water; Hansell Lab, University of Miami, Coral Gables, FL, USA) was used as a reference material for the DOC measurements. DOC concentrations were calculated by subtracting the value of the intercept and dividing the difference by the slope of the calibration curve (Shinomura et al., 2005).

## 3.4 Results

The dissolved organic carbon (DOC) concentrations at the initial time (raw seawater), after 6 h incubation (final) in the control chambers (only seawater), or in the coral chambers and DOC fluxes (ΔDOC or **δ**DOC) are shown in Table 4, Figure 17. DOC fluxes in the control chambers represent the contributions of the planktonic community to the incubated seawater. DOC fluxes in the coral incubations represent the contributions of both, the corals and the planktonic community, to the incubated seawater. Finally, **δ**DOC represents the contributions of only the corals to the incubated seawater. DOC contributions of healthy *M. digitata* were similar at both, 27 °C and 33 °C (9.2 ± 0.8 µmol $L^{-1}$ and 10.0 ± 1.2 µmol $L^{-1}$, respectively). The highest DOC contribution (17.1 ± 1.0 µmol $L^{-1}$) was observed for bleached nubbins at 27 °C. Nevertheless, a negative flux (-18.9 ± 2.0 µmol $L^{-1}$) was observed for the bleached nubbins at 33 °C; therefore, these corals were taking up DOC from the surrounding seawater. For *P. lutea*, higher DOC contributions were observed in the bleached coral compared with the healthy coral, at both 27 °C and 33 °C. The healthy nubbins at 33 °C contributed with less DOC (5.4 ± 4.9 µmol $L^{-1}$) than those at 27 °C (12.3 ± 7.4 µmol $L^{-1}$).

To test for a possible association between DOC release (as mucus) and pico-nanoplankton trapping efficiency, the correlations between feeding rates (cells × $10^5$ $mg^{-1}$ protein) and the DOC flux (µmol $mg^{-1}$ protein) are presented in Figure 18. A positive significant correlation was observed for *M. digitata* ($r = 0.887$, $n = 12$, $p < 0.05$). However, no correlation was found for *P. lutea* ($r = 0.100$, $n = 12$). These results

suggest that feeding efficiencies depend on the amount of DOC release for *M. digitata* but not for *P. lutea.*

## 3.5 Discussion

Corals produce mucus nets to trap pico and nanosized particles (Muscatine, 1973). Corals release substantial quantities of mucus into seawater. Fifty to eighty percent of it dissolves and serves as food for bacteria, thereby enhancing their growth and that of the pico- and nanoflagellates, which graze upon them (Wild et al., 2004). Mucus enriched with pico- and nanoplankton in the form of dissolved organic matter is re-incorporated by coral polyps. Therefore, I monitored changes in DOC production and fluxes during the incubations to determine the role of DOC (as the main component of coral mucus) in coral food capture.

The DOC fluxes for *M. digitata* agreed well with the cell capture ability of this coral. The bleached nubbins incubated at normal temperature had the highest positive DOC fluxes (net release) followed by the healthy nubbins incubated at 33 °C. This is in agreement with Tremblay et al., (2012) in which *Stylophora pistillata* under heat stress showed positive POC fluxes against net uptake in control corals. However, in our incubations, a negative DOC flux (net uptake) was only observed in bleached *M. digitata* incubated at 33 °C. This shows that these fragments required more energy source than those obtained solely from the autotrophy plus plankton feeding. This phenomenon accounts for the substantial decline in its feeding rate from 93.1 ng C $mg^{-1}$ protein $h^{-1}$ in healthy corals at 33 °C to 27.5 ng C $mg^{-1}$ protein $h^{-1}$ in bleached corals, with thermal stress. These data suggest that *M. digitata* strongly depends upon mucus

production for heterotrophy as shown in Figure 18, which exemplified the positive correlation between prey capture and DOC fluxes. On the contrary, bleached *P. lutea* released higher amounts of DOC, compared with the healthy samples, at both temperatures. However, feeding efficiency (both cell capture and carbon incorporation) in bleached *P. lutea* under high seawater temperature was very low. Moreover, there was no correlation between the feeding rates and DOC fluxes under these conditions. *P. lutea* may not depend upon DOC release for effective prey capture, and it may release mucus under stress for reasons other than predation. For example, Brown and Bythell reported that coral mucus quenches harmful reactive oxygen species and Wooldridge suggested that POC release could be a strategy of the host to avoid photo-inhibition of its symbionts (Brown and Bythell, 2005; Wooldridge, 2009b). These stimuli might also have induced DOC release from bleached *P. lutea,* under heat stress conditions. Other authors also found substantial differences in DOC fluxes among different coral species. Fitt et al., (2009) found a drastic mucus reduction in tissues of *S. pistillata* subjected to heat stress and further death during recovery in contrast with high mucus content in tissues of *Porites cylindrica* subjected to heat stress. This shows that fluxes of DOC in corals under different stressful conditions may vary and different coral species may show different responses, therefore DOC fluxes may reflect a species-specific physiological response.

**Figure 16** DOC concentration of seawater were collected before and after 6h incubation experiment

**Table 4** Concentrations of dissolved organic carbon (DOC; µmol $L^{-1}$) for the initial seawater samples and those incubated (control or coral incubation), for healthy and bleached corals at normal (27 °C) and high (33 °C) seawater temperatures (means ± SD, n = 3). Differences between coral conditions and temperatures were tested by two-way ANOVA and post hoc Tukey's test. **a** indicates statistical difference with **b** at $p < 0.01$

| Corals and condition | initial DOC (seawater) | final DOC (seawater) control | final DOC (coral) | ΔDOC seawater (control-initial) | ΔDOC coral (coral-initial) | δDOC (ΔDOC seawater-ΔDOC coral) |
|---|---|---|---|---|---|---|
| *M. digitata* | | | | | | |
| 27 °C Healthy | 90.2 ± 3.9 | 135.6 ± 1.9 | 144.8 ± 2.2 | 45.3 ± 2.1 | 54.6 ± 1.9 | 9.2 ± 0.8 |
| 27 °C Bleached | 99.8 ± 2.0 | 127.5 ± 5.0 | 144.7 ± 5.7 | 27.8 ± 3.0 | 44.9 ± 3.8 | 17.1 ± 1.0[a] |
| 33 °C Healthy | 106.7 ± 2.1 | 134.8 ± 3.1 | 144.8 ± 2.1 | 28.1 ± 3.2 | 38.1 ± 2.9 | 10.0 ± 1.2[a] |
| 33 °C Bleached | 99.8 ± 2.0 | 140.2 ± 1.2 | 121.2 ± 1.0 | 40.4 ± 2.0 | 21.4 ± 3.0 | -18.9 ± 2.0[b] |
| *P. lutea* | | | | | | |
| 27 °C Healthy | 76.0 ± 2.3 | 93.5 ± 0.7 | 105.9 ± 7.9 | 17.6 ± 2.5 | 29.9 ± 9.7 | 12.3 ± 7.4 |
| 27 °C Bleached | 78.3 ± 2.1 | 141.1 ± 1.9 | 163.4 ± 13.4 | 62.8 ± 3.1 | 85.1 ± 14.4 | 22.2 ± 15.0 |
| 33 °C Healthy | 76.0 ± 2.3 | 103.3 ± 4.3 | 108.8 ± 8.5 | 27.4 ± 4.5 | 32.8 ± 9.3 | 5.4 ± 4.9 |
| 33 °C Bleached | 78.3 ± 2.1 | 114.4 ± 4.1 | 136.9 ± 3.4 | 36.1 ± 3.2 | 58.6 ± 2.5 | 22.5 ± 5.6 |

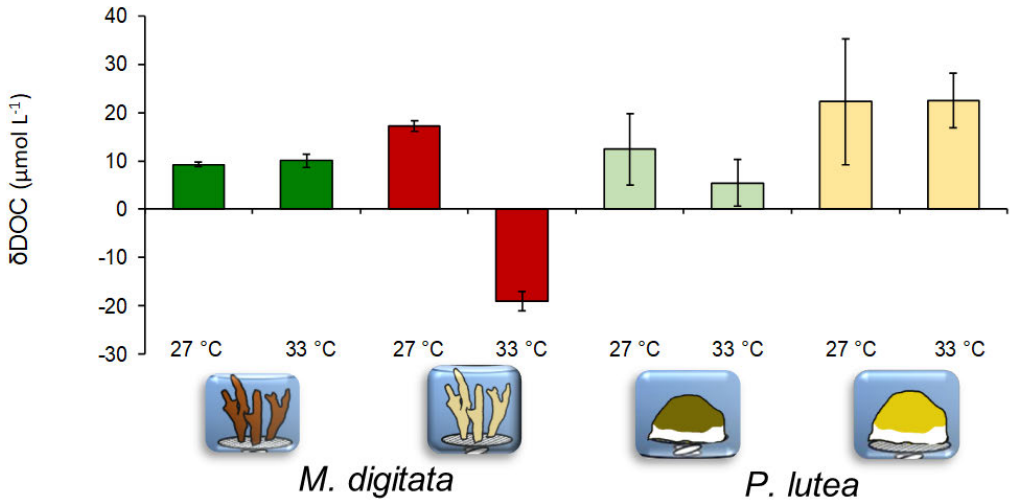



**Figure 17** Flux of dissolved organic carbon (DOC; µmol $L^{-1}$) as the difference between before and after incubation for healthy and bleached *M. digitata* and *P. lutea* nubbins at normal (27 °C) and high (33 °C) seawater temperatures. Incubation time was 6 h.

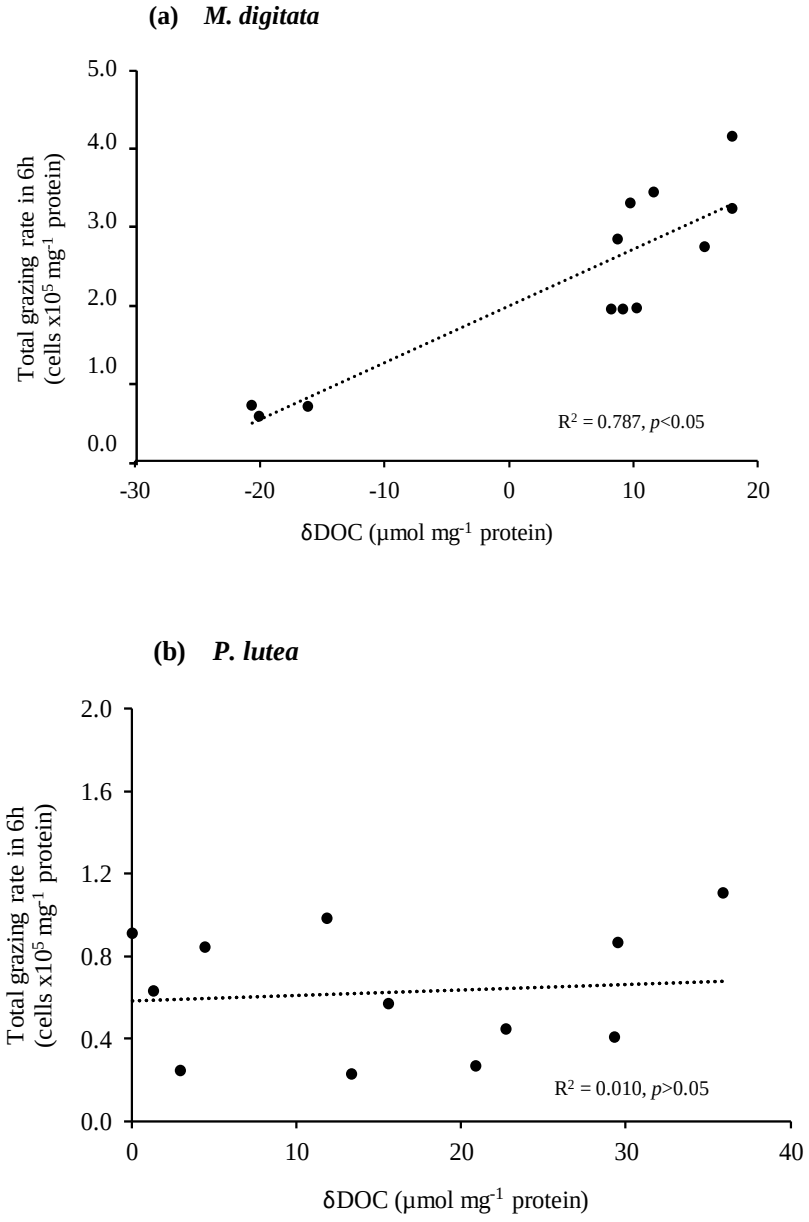



**Figure 18** Correlations between total feeding rate and δDOC for (a) *M. digitata* and (b) *P. lutea* (n = 12 for each species).

# Chapter 4

# Organic carbon acquisition from heterotrophy vs. respiration of autotrophyc products (glucose and glycerol)

## 4.1 Abstract

Autotrophy is the main nutrition source for Scleractinian corals: they can receive up to 95% of their nutrition via translocation for their Symbiodiniaceae in the form of sugars, glycerol, and amino acids (Tremblay et al., 2014). However, recent environmental changes including elevated sea-surface water temperatures and strong solar irradiance along with diminishing water quality had resulted in an increase of the incidence and severity of coral bleaching worldwide (Coles and Brown, 2003). Bleached corals lose their endosymbiotic Symbiodiniaceae and/or pigments (Baumann et al., 2014; D'Croz et al., 2001; Glynn, 1996; Hoegh-Guldberg, 1999; Suzuki et al., 2015). Therefore, relatively less organic matter is translocated to them. Consequently, their metabolic energy levels decline, their physiology is altered, and their survivorship decreases. Symptoms of coral bleaching include a reduction in calcification (Leder et al., 1991; Rodrigues and Grottoli, 2006), increases in the catabolism of reserved materials (Rodrigues and Grottoli, 2007; Schoepf et al., 2015a), and enhanced heterotrophy (Grottoli et al., 2006) The aim of this chapter was to evaluate how much heterotrophy represents when compare with the consumption (respiration) of autotrophyc products during the dark respiration period. In order to evaluate dark respiration glycerol and glucose concentrations were measured in coral tissues over the 6 h of our feeding experiment under dark conditions. The fluxes of photosynthatic products was compared

with the carbon incorporation via heterotrophic feeding. The result show that, glycerol concentration in both healthy and bleached corals at 33 °C decreased than those at the initial and at 27°C. Glucose concentrations in *M. digitata* showed similar pattern with glycerol. Conversely in *P. lutea* showed glycerol increased at all conditions, particularly under thermal stress. Net carbon flux by heterotrophy (%), with respect to dark respiration of carbon from autotrophic products was varied from 3% to 65% in *M. digitata* and from 7% to 68% in *P. lutea*. Due to both of coral species differences of shape and differences of coral tissue in thickness, I suggest the hypotheses that thick coral tissue as *P. lutea* have energy reserved from photosynthates higher than those thin coral tissue as *M. digitata.* Moreover, *P. lutea* was obtain the other energy from endolithic algae translocation which are located inside skeletons but did not found in skeletons of *M. digitata.* All symbiont composition of *P. lutea* indicated that massive and encrusting coral are survive environmental stress events better than branching corals.

## 4.2 Introduction

Symbiodiniaceae synthesized organic carbon that is translocated to coral tissue for methabolism and growth (Muscatine et al., 1981; Tremblay et al., 2012; Yellowlees et al., 2008). Glycerol and glucose are the two main carbon sources from photosynthsis (Tremblay et al., 2012; Yamashiro et al., 2005). Historically, glycerol was consider to be the main component to in translocated from photosynthetically fix carbon by Symbiodiniaceae to their scleractinian corals (Battey and Patton, 1987; Muscatine and Cernichiari, 1969). However, recent studies show that glucose is the most important organic carbon translocatied to the host in normal condition (Burriesci et al., 2012; Molina et al., 2017). However, glycerol translocation increases when the corals are subjected to stressful conditions as strong irradiance and/or high seawater temperature. Duc Nguyen et al., (2018) found that glycerol concentration increased when *M. digitata* was exposed to light and high temperature seawater enhancing the growth and antimicrobial properties of certain Vibrio bacteria. According to Burriesci et al. (2012) glucose early production in host tissue but not glycerol after exposure to light and $^{13}$C-bicarbonate suggesting that glucose is the major translocated metabolite in dinoflagellate–cnidarian symbiosis and that the release of glycerol from isolated algae may be part of a stress response. Molina et al., (2017) found that glucose significantly decrease in bleached sea anemone *Exaiptasia pallida* together with a significant increase in glycerol, suggesting that bleached sea anemones degrade lipids to compensate for the loss of symbionts. The above studies were done in experiments under light to measure photosynthesis products. However, a few research was done to study the respiration of photoassimilates, particularly during the dark period

(Wooldridge, 2013).. Inthis chapter glycerol and glucose were measure in order to understand organic matter respiration (consumption) and compare with gain of organic matter from heterotrophy. The main objective was to evaluate how much heterotorphy represents when compare with respiration during dark hours.

## 4.3 Materials and methods

### 4.3.1 Glycerol and glucose concentration from coral tissue

Glycerol and glucose concentrations were measured at the beginning and at the end of the incubation experiments (corals nubbins from chapter 2). A small part of coral nubbins was ground in a mortar containing 2 mL of 50 mM Tris-HCl buffer at pH 7.5 and centrifuged at 10,000 × *g* for 3 min. After that Triplicate 25-µL supernatant aliquots were loaded into 96-well microtiter plates and quantified with a Pierce$^{TM}$ BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Glycerol and glucose were measured with Glycerol Colorimetric Assay Kit (Cayman Chemical, MI, USA) and Glucose Colorimetric Assay Kit (Cayman Chemical) by measuring absorbances at 540 and 510 nm following manufacturer's protocol (glycerol and glucose respectively). The concentrations were normalized to the surface area of the coral pieces.

### 4.3.2 Estimation of organic carbon fluxes

In order to estimate consumption of energy reserves during the 6 h incubations, I measured concentrations of the two most important products from Symbiodiniaceae, glycerol and glucose (Battey and Patton, 1987, 1984; Burriesci et al., 2012; Molina et al., 2017; Suescún-Bolívar et al., 2016) that are translocated to the coral tissues (Figure 4). On the basis of the molecular weights and the atomic carbon weights of glycerol and glucose, we calculated the organic carbon fluxes of these two products and further compared with the carbon incorporation via heterotrophic feeding. Fluxes were calculated as the difference between final and initial concentrations (positive values for increased and negative values for decreased concentrations). Carbon calculations were done using glycerol ($C_3H_5(OH)_3$ : 36/92) and glucose ($C_6H_{12}O_6$ : 72/180) as reference.

### 4.3.3 Statistical analysis

Data were checked for normality using the Kolmogorov-Smirnov test; square root transformation was performed when data were not normally distributed. ANOVA was performed to identify any significant differences among experimental conditions. Post hoc Tukey's test was used to compare differences when ANOVA showed statistically significant effects. Minitab v. 14 (Minitab Inc., State College, PA, USA) was used for all statistical analyses.

## 4.4 Results

### 4.4.1 Glycerol concentrations

Variations of the glycerol concentrations during the incubations (µg $cm^{-2}$) are shown in Figure 19. The initial concentrations of glycerol in the healthy *M. digitata* were 2.0 times (16.3 ± 5.1 µg $cm^{-2}$, $p < 0.05$) higher than those in the bleached nubbins. Under thermal stress (33 °C) the glycerol concentrations significantly decreased (7.7 ± 2.4 µg $cm^{-2}$, $p < 0.05$), with respect to the initial and final concentrations under normal (27 °C) temperature conditions. Bleached nubbins showed fluctuations during the incubations, but no statistically significant changes were observed. In *P. lutea,* the initial concentration of glycerol was 3 times higher than in the bleached nubbins; in healthy nubbins the concentrations significantly decreased (18.7±3.2 µg $cm^{-2}$, $p < 0.001$) under thermal stress, whereas bleached nubbins showed fluctuations but not significant changes.

### 4.4.2 Glucose concentrations

At the initial stage of the experiment, the glucose concentrations of healthy *M. digitata* almost double those of the bleached. During the incubations, glucose concentrations decreased, but no significant differences were noted among the conditions ($p > 0.05$). Conversely, glucose in *P. lutea* increased during the incubations, particularly those under thermal stress incubations with both, healthy, and bleached conditions (Figure 20).

### 4.4.3 Net organic carbon flux from autotrophy and carbon acquisition from heterotrophy

In healthy *Montipora digitata*, fluxes of organic carbon from glycerol were negative (consumption) and more pronounced (1.4 times higher) for corals under thermal stress. However, in bleached nubbins, positive fluxes of carbon from glycerol were observed at 27 °C (Figure 21A). Carbon from glucose was consumed under both temperature treatments and for both coral conditions. In *Porites lutea*, the pattern was quite different, with positive fluxes of carbon from glucose in all treatments, and consumption of glycerol for both coral conditions under thermal stress (Figure 21B).

In *M. digitata* the carbon acquisition by heterotrophy (%) with respect to consumption of carbon from autotrophic products (Table 5) was high (65%) for bleached fragments at 27 °C but very low (3%) under thermal stress. The carbon contribution from heterotrophy was similar (6%) in healthy corals at both seawater temperatures. In thermally stressed *P. lutea*, carbon acquisition from heterotrophy was 68% in healthy corals, but only 7% in bleached corals. However, under normal temperature, fluxes of autotrophic products were not detected in healthy and bleached *P. lutea* within our incubation time.

## 4.5 Discussion

During incubations, glycerol and glucose were measured, calculated their fluxes (in terms of organic carbon) and compare with the incorporation of organic carbon from heterotrophy. Glucose is the main autotrophic product of Symbiodiniaceae (Burriesci et al., 2012) and is easily consumed by the host; meanwhile, glycerol is produced by Symbiodiniaceae when they are subjected to osmotic stress (Mayfield and Gates, 2007; Seibt and Schlichter, 2001). The results showed that glycerol was metabolized during incubations of both corals in the healthy conditions and when they were subjected to thermal stress; however, glycerol increased in bleached corals under normal temperature conditions. Molina et al. (2017) also observed similar patterns, and they suggested that corals may degrade lipids to glycerol to compensate for the loss of Symbiodiniaceae. Conversely, glucose was mainly consumed during incubations of *M. digitata* under heat stress. By the contrary, glucose was incorporated along incubations under any of the conditions in *P. lutea*. As the incubations were performed under dark conditions, the incorporation of glucose by *P. lutea* was not due to translocation from Symbiodiniaceae, but most probably from translocation from their endolithic community. Sangsawang et al. (2017) demonstrated that the endolithic community of *P. lutea* collected in the same reef, translocated their photo assimilates to the coral tissue under both, healthy and bleached conditions, during extended periods of time covering also dark hours. The huge increase of glucose in *P. lutea* tissues during incubations was attributed to endolith translocations.

In terms of net carbon fluxes, substantial consumption of photoassimilates during the dark period was found in healthy *M. digitata* at any temperature and bleached *M.*

*digitata* under thermal stress; however, carbon incorporation by heterotrophy for both corals condition at 33 °C did not represent more than 3% to 6% of this consumption. Conversely, bleached corals at normal temperatures consumed lower levels of photoassimilates; therefore, carbon incorporation by heterotrophy appeared to be higher, accounting for 65% of the total. Photoassimilates of *P. lutea* were consumed only under thermal stress conditions; this consumption was higher in bleached corals. Carbon incorporation by heterotrophy was high in healthy corals (68%); however bleached corals under thermal stress incorporated 7% with respect to glycerol consumption. Respiration of photoassimilates was very high in *M. digitata*; however, the metabolic cost in *P. lutea* was very low owing to glucose incorporation. Other studies reported comparable values of contribution of heterotrophy (in % of respiration) as 28.7 % and 15.9 %, for *Oculina patagonica* and *Turbinaria reniformis*, respectively(Tremblay et al., 2011), and from 10 to 30 % for *Styllophora pistillata* (Tremblay et al., 2014). These data suggest that bleached *P. lutea* under thermal stress may face its metabolic coasts in a more successful manner. Taken together, these results answer the second question (2) raised in the introduction about how bleached corals under thermal stress may modify their feeding strategy.

The relatively high feeding rates of *M. digitata*, indicate that this species depends upon heterotrophy to overcome bleaching. However, when bleaching was combined with high seawater temperature, *M. digitata* could not produce enough mucus for particle trapping; therefore, its capture ability was dramatically reduced. Moreover, bleaching with high temperature forced *M. digitata* to uptake DOC from the surrounding waters to compensate for the lack of energy resources. *P. lutea* was relatively less dependent on heterotrophy, if considering the important difference in

particle capture and organic matter incorporation when compared to *M. digitata*. However, *P. lutea* incorporated glucose most probably via translocation form endoliths that help the coral to be relatively independent from heterotrophy. Bleached *P. lutea* continued to secrete mucus, but most probably for purposes other than particle capture. These results answer questions (2) and (3) set out in the introduction, related to the responses of feeding behaviors when the corals are bleached and subjected to thermal stress and how the energy allocation vary under these conditions.

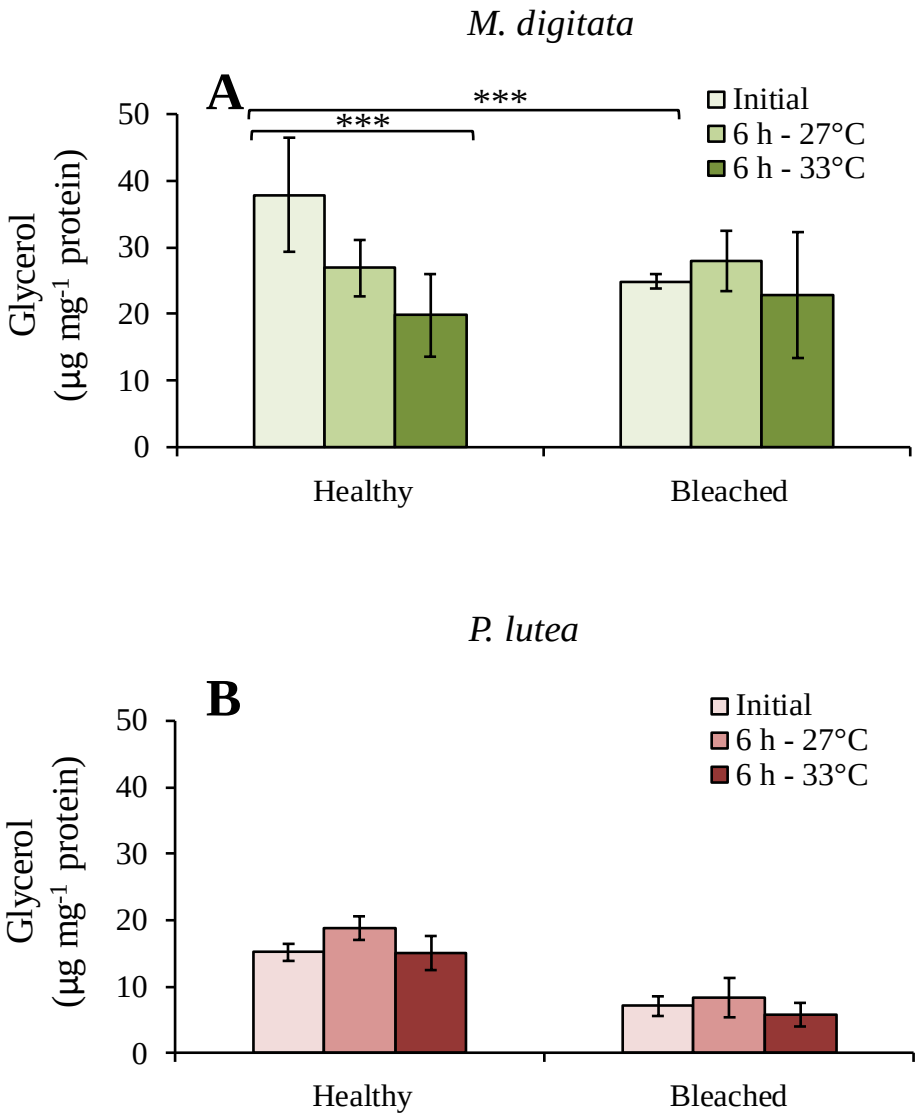



**Figure 19** Glycerol concentrations (µg $mg^{-1}$ protein) in the healthy and bleached *M. digitata* and *P. lutea* at initial (0 h) and after 6 h incubations at 27 ℃ and 33 ℃ of seawater temperature. Data are the means ± SD, n = 6 for each treatment. Three-way ANOVA and post hoc Tukey's test were applied to identify the differences among coral conditions, the temperature treatments, and incubation time, *** $p < 0.001$.

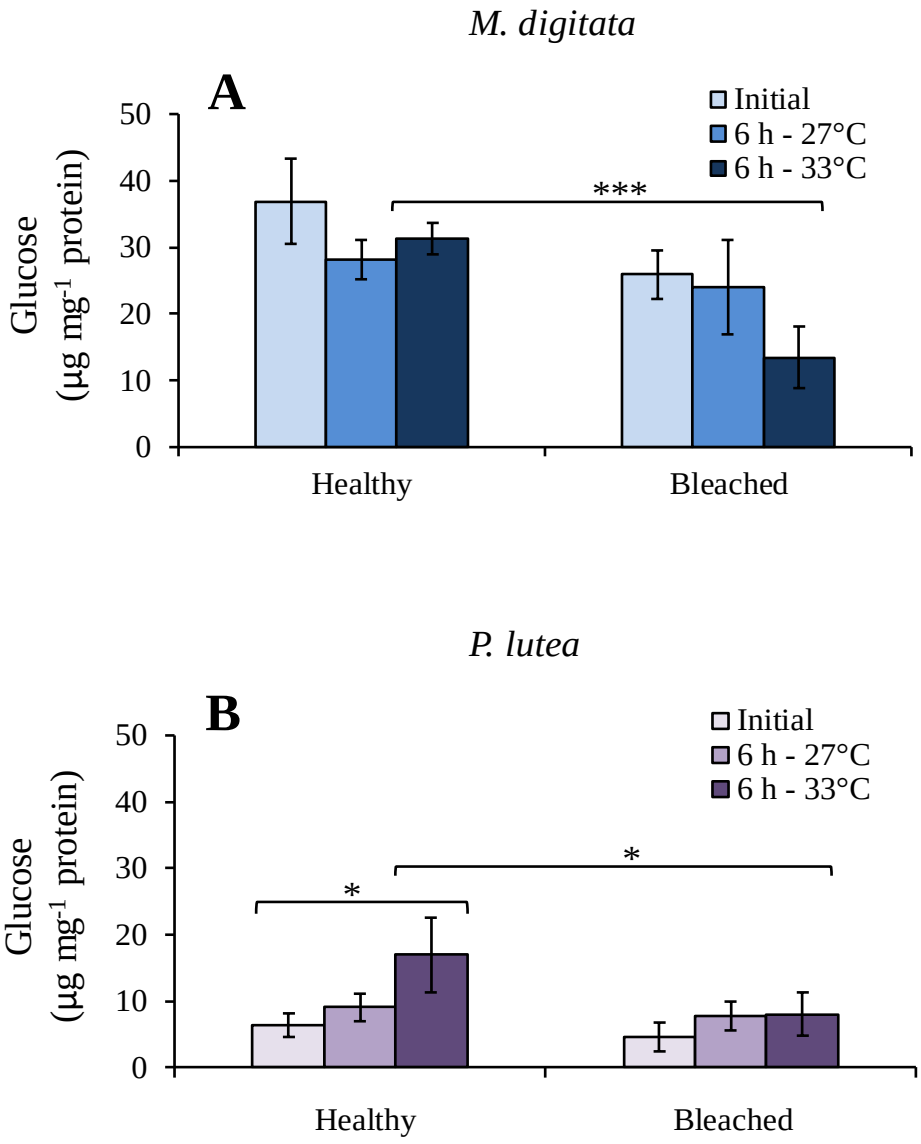



**Figure 20** Glucose concentrations (µg $mg^{-1}$ protein) in the healthy and bleached *M. digitata* and *P. lutea* at initial (0 h) and after 6 h incubations at 27 °C and 33 °C of seawater temperature. Data are the means ± SD, n = 6 for each treatment. Three-way ANOVA and post hoc Tukey's test were applied to identify the differences among coral conditions, the temperature treatments, and incubation time * $p < 0.05$; *** $p < 0.001$.

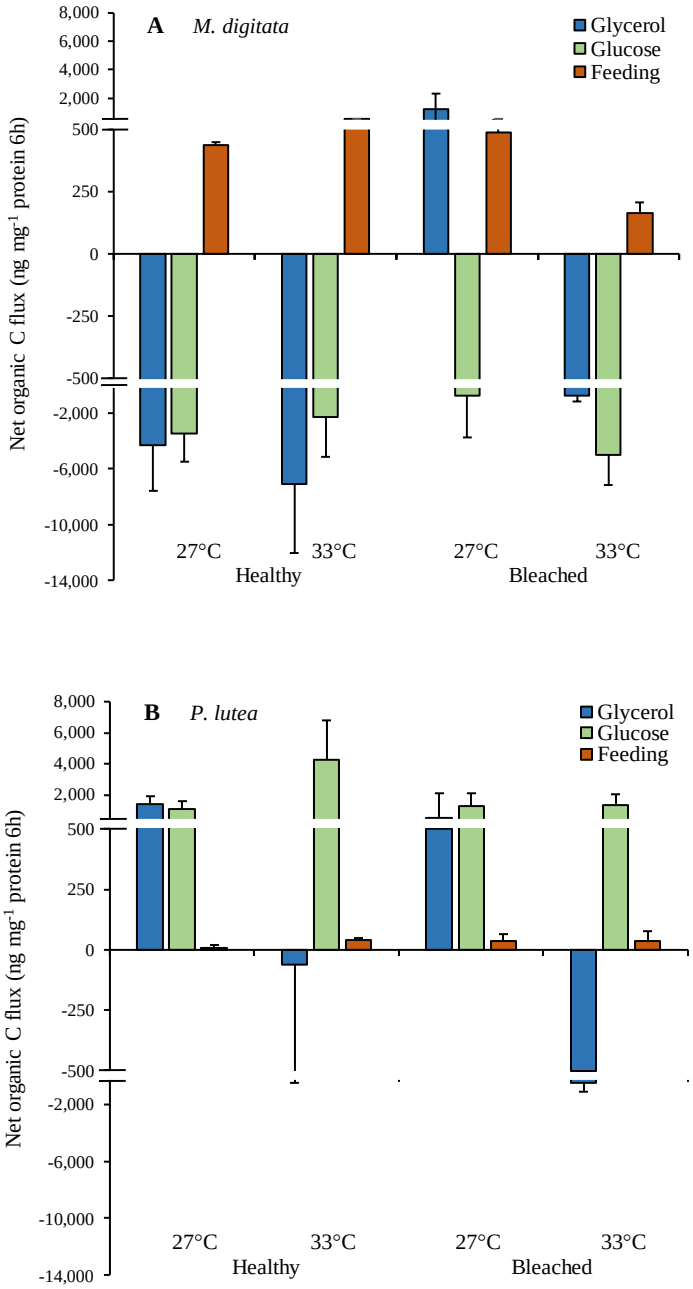



**Figure 21** Net fluxes of organic carbon from glycerol and glucose and organic carbon acquisition from heterotrophy during 6 h incubations of (A) *M. digitata* and (B) *P. lutea*. Data are mean ± SD, n = 6 for each condition.

**Table 5** Net fluxes of organic carbon from glycerol and glucose and organic carbon acquisition from heterotrophy (% of glycerol and/or glucose consumption) during 6 h incubations of *M. digitata* and *P. lutea* at normal (27 °C) and high (33 °C) seawater temperatures. Data are mean ± SD for each condition, n = 6 for glucose and glycerol; n = 3 for heterotrophy. Negative values indicate consumption.

| Coral species | ng $mg^{-1}$ protein 6 h | Healthy 27°C | Healthy 33°C | Bleached 27°C | Bleached 33°C |
|---|---|---|---|---|---|
| *M. digitata* | C of glycerol | -4,308 ± 3,251 | -7,100 ± 4,938 | 1,209 ± 1,279 | -799 ± 417 |
| | C of glucose | -3,493 ± 1,976 | -2,267 ± 2,883 | -752 ± 3,023 | -5,009 ± 2,138 |
| | C of feeding | 439 ± 11 | 558 ± 8 | 486 ± 65 | 165 ± 42 |
| | % * | 6 | 6 | 65 | 3 |
| *P. lutea* | C of glycerol | 1,420 ± 464 | -60 ± 53 | 500 ± 1,576 | -504 ± 642 |
| | C of glucose | 1,057 ± 553 | 4,233 ± 2,572 | 1,266 ± 822 | 1,357 ± 686 |
| | C of feeding | 6 ± 1 | 41 ± 5 | 34 ± 0.7 | 35 ± 0.3 |
| | % * | | 68 | | 7 |

*% acquisition with respect to respiration of autotrophic products (glycerol and/or glucose)

# Chapter 5

# General conclusions

## 5.1 Heterotrophic feeding of coral

The present study revealed different strategies and dependences of the two selected corals species on heterotrophy. *M. digitata* appeared highly dependent upon heterotrophy. It was highly efficient at food capturing, however under thermal stresses, bleached coral's capture efficiency decreased and needed to uptake DOC from the surrounding environment to compensate its daily metabolic requirements. *P. lutea* was comparatively less dependent upon heterotrophy, it may utilize the organic matter translocated from the endolithic community to produce mucus and was able to maintain certain level of heterotrophy (~11% of dark consumption) with selecting higher C/N ratio food items. Therefore, this coral species is relatively more resistant to bleaching events even at elevated seawater temperatures.

In Okinawan waters the corals *M. digitata* and *P. lutea* represent important components of the reefs; therefore, their survivorship may have implications for the coral reef resilience in this area. While *M. digitata* appears to be more dependent on heterotrophy, *P. lutea* may thrive under these adverse conditions due to its strategy to face its metabolic coasts thanks to a potential exogenous supply or organic matter from their endolithic communities. In this way, *P. lutea* is relatively more tolerant to bleaching, having greater chances of survival and recovery. Therefore, this species is more likely to be able to colonize decimated coral reef ecosystems.

## 5.2 New findings (research)

Trophic ecology of corals is an important field that is becoming more frequently studied and that has tremendous implications in the current climate change scenario. However, new finding on this study is feeding efficiency of corals in relation with bleaching tolerance. Pico- and nanoplankton feeding was the main point to investigate between the corals *Montipora digitata* and *Porites lutea*. My results showed complete differences for heterotrophic feeding behavior between these coral species, because dependences of the two selected corals species on heterotrophy, or quantitative relationship between DOC and heterotrophic feeding showed differences. *M. digitata* appeared highly dependent upon heterotrophy. It was highly efficient at food capturing, however under the combined stress treatments, the coral's capture efficiency decreased, and it needed to uptake DOC from the surrounding environment to compensate its daily metabolic requirements. While as that *P. lutea* was comparatively less dependent upon heterotrophy, it utilized the organic matter translocated from the endolithic community to produce mucus and was able to maintain certain level of heterotrophy by selecting higher C/N ratio food items. Overall, *P. lutea* is relatively more resistant to bleaching events even at elevated seawater temperatures, therefore this coral may have greater chances of survival and recovery.

## 5.3 Future research

Findings in this greatly contribute to the understanding of coral feeding and survival strategies under the actual scenario of high seawater temperature and extended bleaching periods. Particularly in Okinawa reefs, these two-coral species represent important components of the reefs, therefore their survivorship may have tremendous implication for the coral reef resilience in this area.

Our incubation was set-up under dark condition considering that heterotrophy is enhanced during the night; experiments during the day (light period) need to be performed to compare feeding rates under these two conditions. Moreover, Studies on coral feeding in different habitats such as shaded area and deep corals (mesophotic areas) with lower illumination. More understand on the mechanism of bigger plankton trapping using tentacles: especially what coral condition induce this feeding strategy. Further studies should be done to compare feeding rates and uptake of organic matter from heterotrophy vs autotrophy of corals growing in well illuminated areas vs. those corals adapted to shaded areas.

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# Appendix I

**Supplementary Table 1.** sea surface temperature at Sesoko Island during corals sampling for feeding experiments on 21 September 2017.

| Time | °C | time | °C |
|---|---|---|---|
| 0:00 | 28.417 | 12:00* | 30.016 |
| 0:30 | 28.407 | 12:30* | 30.407 |
| 1:00 | 28.329 | 13:00* | 31.078 |
| 1:30 | 28.247 | 13:30* | 31.757 |
| 2:00 | 28.186 | 14:00* | 32.328 |
| 2:30 | 28.112 | 14:30* | 32.691 |
| 3:00 | 27.903 | 15:00* | 33.043 |
| 3:30 | 27.905 | 15:30* | 33.331 |
| 4:00 | 27.862 | 16:00* | 32.659 |
| 4:30 | 27.693 | 16:30 | 32.892 |
| 5:00 | 28.037 | 17:00 | 32.799 |
| 5:30 | 28.144 | 17:30 | 32.418 |
| 6:00 | 28.308 | 18:00 | 30.181 |
| 6:30 | 28.283 | 18:30 | 29.869 |
| 7:00 | 28.271 | 19:00 | 29.4 |
| 7:30 | 28.155 | 19:30 | 28.904 |
| 8:00 | 28.166 | 20:00 | 28.75 |
| 8:30 | 28.371 | 20:30 | 28.782 |
| 9:00 | 28.455 | 21:00 | 28.797 |
| 9:30 | 28.593 | 21:30 | 28.633 |
| 10:00 | 28.719 | 22:00 | 28.692 |
| 10:30 | 28.836 | 22:30 | 28.715 |
| 11:00 | 29.037 | 23:00 | 28.589 |
| 11:30 | 29.456 | 23:30 | 28.544 |

# Appendix II

**Supplementary Table 2.** Growth rate per hour of pico and nanoplankton after 6h incubation. (mean±SE, n=3 for each incubation conditions) BA=bacteria, PCY=pico-cyanobacteria, PF=picoflagellates, NF= nanoflagellates

| **Growth rate** | **27°C** | | | | **33°C** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Healthy** | | **Bleached** | | **Healthy** | | **Bleached** | |
| | **Control** | **Coral** | **Control** | **Coral** | **Control** | **Coral** | **Control** | **Coral** |
| ***M. digitata*** | | | | | | | | |
| BA | -0.06±0.03 | -0.09±0.01 | -0.06±0.02 | 0.07±0.00 | 0.06±0.01 | -0.01±0.001 | -0.07±0.01 | -0.07±0.002 |
| PCY | -0.04±0.02 | -0.09±0.01 | 0.19±0.01 | 0.32±0.01 | 0.17±0.02 | 0.21±0.02 | 0.38±0.01 | 0.22±0.02 |
| PF | 0.04±0.02 | 0.04±0.001 | 0.16±0.02 | 0.12±0.002 | 0.03±0.01 | 0.08±0.003 | 0.06±0.02 | 0.10±0.002 |
| NF | 0.05±0.01 | 0.01±0.02 | -0.03±0.01 | -0.08±0.00 | -0.05±0.03 | -0.07±0.06 | -0.11±0.01 | -0.05±0.01 |
| ***P. lutea*** | | | | | | | | |
| BA | -0.06±0.02 | -0.10±0.01 | -0.12±0.04 | -0.11±0.01 | -0.05±0.01 | -0.09±0.02 | -0.12±0.06 | -0.10±0.01 |
| PCY | -0.18±0.09 | -0.21±0.03 | 0.05±0.03 | -0.06±0.05 | 0.08±0.01 | -0.05±0.001 | -0.21±0.06 | -0.22±0.05 |
| PF | 0.04±0.03 | 0.06±0.01 | -0.13±0.02 | -0.16±0.001 | -0.03±0.001 | -0.05±0.001 | -0.16±0.07 | -0.04±0.001 |
| NF | -0.03±0.001 | -0.04±0.01 | -0.15±0.06 | -0.15±0.06 | -0.19±0.02 | -0.22±0.01 | -0.15±0.06 | -0.19±0.04 |

# Appendix III

**Supplementary Table 3.** Feeding rate expressed in number of cells per polyp per hour after 6h incubation of *M. digitata* and *P. lutea* at normal (27℃), high (33℃) seawater temperature on healthy or bleached according to groups of pico– and nanoplankton. (mean±SE, n=3) BA=bacteria, PCY=pico-cyanobacteria, PF=picoflagellates, NF= nanoflagellates.

| Feeding rate | 27°C | | 33°C | |
|---|---|---|---|---|
| | Healthy | Bleached | Healthy | Bleached |
| **Cells polyp$^{-1}$ h$^{-1}$** | | | | |
| *M. digitata* | | | | |
| BA | 56.23±20.89 | 175.24±0.06 | 161.88±31.18 | 14.92±10.56 |
| PCY | 99.95±22.99 | -62.08±11.35 | -19.91±1.11 | 6.87±0.001 |
| PF | 4.38±2.51 | -9.06±0.61 | -11.80±3.22 | -8.61±1.78 |
| NF | 127.22±0.02 | 124.56±13.63 | 164.59±3.23 | 41.48±14.30 |
| *P. lutea* | | | | |
| BA | 87.58±12.03 | -24.10±32.90 | 61.38±6.77 | -71.83±32.41 |
| PCY | 24.21±24.21 | 151.95±2.70 | 187.37±0.00 | 127.33±3.80 |
| PF | 8.14±0.66 | 65.64±4.40 | 53.87±4.24 | 58.99±13.35 |
| NF | 28.84±0.001 | 99.78±4.86 | 83.73±32.19 | -0.65±77.24 |

# Appendix IV

**Supplementary Table 4.** Feeding rate expressed in number of **cells per cm$^2$ per hour** after 6h incubation of *M. digitata* and *P. lutea* at normal (27°C), high (33°C) seawater temperature on healthy or bleached according to groups of pico– and nanoplankton. (mean±SE, n=3) BA=bacteria, PCY=pico-cyanobacteria, PF=picoflagellates, NF= nanoflagellates.

| Feeding rate | 27°C | | 33°C | |
|---|---|---|---|---|
| | Healthy | Bleached | Healthy | Bleached |
| *M. digitata* | | | | |
| BA | 3.56±1.32x10$^3$ | 11.09±0.001 x10$^3$ | 10.25±1.97 x10$^3$ | 0.94±0.66 x10$^3$ |
| PCY | 6.33±1.45 x10$^3$ | -3.93±0.71 x10$^3$ | -1.26±0.07 x10$^3$ | 0.43±0.002 x10$^3$ |
| PF | 0.27±0.15 x10$^3$ | -0.57±0.03 x10$^3$ | -0.74±0.20 x10$^3$ | -0.54±0.11 x10$^3$ |
| NF | 8.05±0.00 x10$^3$ | 7.88±0.86 x10$^3$ | 10.42±0.20 x10$^3$ | 2.62±0.90 x10$^3$ |
| *P. lutea* | | | | |
| BA | 4.29±0.001 x10$^3$ | 5.97±5.54 x10$^3$ | 3.01±0.33 x10$^3$ | -3.52±1.59 x10$^3$ |
| PCY | 1.18±1.18 x10$^3$ | 7.45±0.13 x10$^3$ | 9.19±0.002 x10$^3$ | 6.25±0.18 x10$^3$ |
| PF | 0.39±0.03 x10$^3$ | 3.22±0.21 x10$^3$ | 2.64±0.20 x10$^3$ | 2.89±0.65 x10$^3$ |
| NF | 1.41±0.00 x10$^3$ | 4.89±0.23 x10$^3$ | 4.11±1.58 x10$^3$ | -0.03±1.69 x10$^3$ |

# Appendix V

**Supplementary Table 5.** Feeding rate expressed **ng C per polyp per hour** after 6h incubation of *M. digitata* and *P. lutea* at normal (27℃), high (33℃) seawater temperature on healthy or bleached according to groups of pico– and nanoplankton. (mean±SE, n=3) BA=bacteria, PCY=pico-cyanobacteria, PF=picoflagellates, NF= nanoflagellates.

| Feeding rate | 27°C | | 33°C | |
|---|---|---|---|---|
| | Healthy | Bleached | Healthy | Bleached |
| *M. digitata* | | | | |
| BA | 0.001±0.0001 | 0.004±0.001 | 0.002±0.0001 | 0.000±0.00002 |
| PCY | 0.018±0.0001 | -0.011±0.00001 | -0.004±0.0003 | 0.001±0.0001 |
| PF | 0.005±0.0002 | -0.011±0.00002 | -0.014±0.00004 | -0.010±0.00002 |
| NF | 0.196±0.24 | 0.361±0.03 | 0.560±0.01 | 0.183±0.09 |
| *P. lutea* | | | | |
| BA | 0.002±0.00002 | 0.003±0.002 | 0.002±0.001 | -0.002±0.001 |
| PCY | 0.004±0.004 | 0.028±0.0002 | 0.034±0.00001 | 0.023±0.001 |
| PF | 0.009±0.002 | 0.079±0.005 | 0.065±0.005 | 0.071±0.016 |
| NF | 0.098±0.00001 | 0.339±0.017 | 0.285±0.010 | -0.002±0.117 |

# Appendix VI

**Supplementary Table 6.** Feeding rate expressed **ng C per cm² per hour** after 6h incubation of *M. digitata* and *P. lutea* at normal (27℃), high (33℃) seawater temperature on healthy or bleached according to groups of pico– and nanoplankton. (mean±SE, n=3) BA=bacteria, PCY=pico-cyanobacteria, PF=picoflagellates, NF= nanoflagellates.

| Feeding rate | 27°C | | 33°C | |
|---|---|---|---|---|
| | Healthy | Bleached | Healthy | Bleached |
| *M. digitata* | | | | |
| BA | 0.08±0.03 | 0.24±0.00001 | 0.23±0.04 | 0.02±0.01 |
| PCY | 1.16±0.27 | -0.44±0.15 | -0.23±0.01 | 0.08±0.0002 |
| PF | 0.03±0.19 | -0.69±0.04 | -0.90±0.24 | -0.65±0.14 |
| NF | 12.61±15.18 | 27.22±1.93 | 35.97±0.71 | 14.90±2.71 |
| *P. lutea* | | | | |
| BA | 0.09±0.01 | 0.07±0.01 | 0.13±0.12 | -0.08±0.03 |
| PCY | 0.22±0.22 | 1.68±0.0001 | 1.37±0.02 | 1.14±0.03 |
| PF | 0.48±0.04 | 3.17±0.25 | 3.87±0.26 | 3.47±0.79 |
| NF | 4.88±0.0002 | 14.18±5.45 | 16.90±0.82 | -0.11±5.85 |