博士論文

Factors controlling high productivity of coral reefs, viewed via nitrogen isotope ratios of amino acids in Cnidarians under autotrophic and mixotrophic laboratory breeding

珊瑚礁の高生産性を支えるメカニズム:独立栄養および混合栄養条件で飼育した刺胞動物(サンゴ・イソギンチャク)に含まれるアミノ酸の安定同位体比からの評価

Division of Earth System Science, Graduate School of Environmental Science, Hokkaido University

北海道大学大学院環境科学院地球圏科学専攻

Jincen Li

李謹岑

June 2025

TABLE OF CONTENTS

ABSTRACT

Introduction

Coral reefs are one of the most productive and biologically diverse ecosystems in the modern ocean. Cnidarians, including corals and sea anemones, are the major organisms inhabiting in coral reefs. Theses Cnidarian species host dinoflagellates, known as zooxanthellae, within their polyps, forming the Cnidarian-algal symbiosis. This symbiotic relationship enables Cnidarians to obtain nutrition both autotrophically, through photosynthesis by the zooxanthellae, and heterotrophically, through the capture of external particles or the assimilation of dissolved organic materials. Consequently, Cnidarians exhibit a mixotrophic mode of nutrition, functioning between autotrophy and heterotrophy in marine ecosystems. It is simply thought that this trophic flexibility contributes to the high productivity of coral reefs even in oligotrophic oceans, based on an assumption that symbiotic species benefit energetically from living together. However, little quantitative data is available on the trophic transfer of nutrients and energy within the Cnidarian-algal symbiosis, particularly for (1) the energetic balance between degradation and biomass growth (i.e., a major factor of the efficiency of the efficiency of trophic transfer) of photosynthates from algal symbionts to Cnidarian hosts; (2) the balance between autotrophy and heterotrophy to the nutrient assimilation for Cnidarians (and algae); and (3) the form of the food web pyramid (e.g., biomass size for each of trophic steps) in coral reef ecosystems.

Scientific objectives

We have two scientific objectives in the present study. First, to quantify the energetic balance of photosynthates from algal symbionts to Cnidarian hosts based on the stable nitrogen isotope ratios of amino acids, we used several Cnidarian species including sea anemones, stony corals, zoanthids, and soft corals for the following three studies: (1) determining the trophic position (TP) of Cnidarian holobionts (sum of Cnidarian and symbiotic algae) under an autotrophic condition (i.e., symbiont-derived photosynthate is a solo resource for corals); (2) determining the TP of symbiotic algae (zooxanthellae); and (3) illustrating the effects of symbiosis on the TP for Cnidarians (i.e., consumers) and zooxanthellae (i.e., diets) in holobionts. Second, to evaluate the effect of heterotrophy on the energetic balance to biomass in Cnidarian-algal symbiosis, we used several soft coral species for evaluating their changes in the isotope ratio under a mixotrophic condition (i.e., both symbiont-derived photosynthate and commercial coral foods are resources for corals) for the three following studies: (1) confirming whether or not Cnidarians can feed on zooplankton as foods and illustrating inter- or intra- species diversity in the TP of Cnidarian holobionts; (2) confirming whether or not algae can access such zooplankton-derived protein; (3) determining the relative contribution of heterotrophy to Cnidarian growth under mixotrophic conditions and illustrating whether or not this contribution correlate with amount of foods.

Highly effective energy transfer in the autotroph-heterotroph symbiosis: insights from compound-specific isotope analysis of amino acids (Chapter II)

1. The TP of Cnidarian holobionts (1.5 ± 0.4, mean ± 1σ) is lower than that of general primary consumers (TP = 2.0), such as herbivorous zooplankton, under an autotrophic condition, suggesting that their trophic identity is mixotrophy-like organisms, rather than herbivorous.
2. The zooxanthellae have a TP close to 1.0, indicating that their trophic identity is the primary producer in the present study.
3. The TP of Cnidarian is clearly declined by 0.5 from the TP of 2.0, resulting in that 56% of algal products are transferred into animal hosts (i.e., energetic balance to biomass is 56%). This suggests that Cnidarians employ diverse trophic strategies or represent different evolutionary stages in their utilization of algal-derived products.

Mixotrophy-like characteristics in Cnidarian-algal symbiosis, as a factor in understanding the nutrient flow of coral reef ecosystems (Chapter III)

1. The TP of five Cnidarian species is elevated by an average range from -0.1 to 1.8 under a mixotrophic condition, suggesting that they feed on zooplankton as food resources. The large inter- and intra-diversities in TP observed among Cnidarians indicate significant diversity in their reliance on heterotrophic food.
2. The TP of zooxanthellae remained close to 1.0, suggesting that they do not access the zooplankton-derived protein.

3. The relative contribution of autotrophy and heterotrophy to Cnidarian holobionts is estimated at 65.4% ± 27.2% and 34.6% ± 27.2%, respectively.

CHAPTER I.

General Introduction

1. Coral reef ecosystems and their benefits to the ocean and humanity

Coral reef ecosystems are among the most productive and biologically diverse ecosystems in the modern ocean, providing essential ecosystem services that support both marine biodiversity and human societies (Groot et al., 2002). These systems serve as critical indicators of ocean health because their high productivity and biodiversity supports diverse trophic interactions among marine organisms and efficient nutrient recycling in food webs. This, in turn, enhances key ecological functions in the ocean, such as nutrient cycling and energy transfer across trophic levels within marine food webs, and provides substantial economic benefits to human beings. For instance, coral reefs support global fisheries by providing a habitat for numerous marine species, such as groupers, lobsters, and snappers, which in turn support the livelihoods of millions of people worldwide (Cinner et al., 2016). Also, coral reefs protect coastal regions by buffering shorelines from wave action and reducing damage from erosion and storms (Ferrario et al., 2014). Besides their ecological importance, coral reefs contribute significantly to the global economy through tourism by attracting millions of visitors to visit tropical coastal regions and generating billions of dollars in revenue (Spalding et al., 2017). However, over the past several decades, increasing anthropogenic pressures, including pollution, overfishing, and global climate change, progressively threaten the long-term stability of coral reefs. These stressors highlight an urgent need for effective conservation and management strategies in coral reefs (Hughes et al., 2017). In this Ph.D. thesis, I mainly focus on the mechanisms by which Cnidarians, the dominant organisms in

coral reef ecosystems, maintain high productivity at the base of coral reef food webs. Understanding these mechanisms is crucial, because high productivity may enhance reef resilience by supporting energy flow, sustaining symbiotic partnerships, and enabling faster recovery of reefs from disturbances.

2. High productivity in coral reefs

Coral reef ecosystems are generally found in shallow waters of the intertropical environments, and such environments are particularly characterized by low levels of nutrients (e.g., nitrogen and phosphorus). Despite growing in these nutrient-poor environments, coral reefs exhibit remarkably high net primary productivity, with estimates ranging from 1800 to 4200 gC/m2/year, in contrast to the open ocean ecosystems, which range from 2 to 400 gC/m2/year (Whittaker and Likens 1975). However, long-term and spatially extensive measurements of reef productivity are relatively scare, with data primarily focused on well-studied Pacific reef flats. Notably, Gladfelter (1985) summarized from approximately 75 studies and several reviews for estimating coral reef productivity with the method by comparing the oxygen concentration differences of water over the reefs. Although these estimates provide useful insights into reef productivity, they do not capture the full geographic distribution of coral reefs globally, with notably fewer studies conducted in the Atlantic and Indian Oceans.

3. Traditional methods for measuring coral reef productivity

Historically, coral reef productivity has been estimated by measuring changes in

oxygen concentrations of reef organisms under light and dark conditions. These measurements are typically conducted either in situ using respirometry chambers placed over reef sections, or in the laboratory using the traditional light and dark bottle method (Kinsey, 1985; Odum & Odum, 1955). Under light conditions, both photosynthesis and respiration occur, whereas under dark conditions, only respiration takes place. Thus, the gross primary productivity (GPP, the rate of total oxygen produced via photosynthesis) and net primary productivity (NPP, calculated as GPP minus the rate of respiration) can be estimated by comparing the oxygen fluxes between the light and dark conditions. These oxygen-based measurements are then converted to carbon fixation rates using established photosynthetic quotients, providing quantitative estimates of reef productivity. Although these methods have provided valuable insights, they also have several limitations. For instance, the data obtained reflects only a snapshot of organism metabolism and fails to capture long-term variations in environments and life cycle dynamics of reef organisms. Furthermore, these methods cannot identify the relative contributions of different primary producers within coral reef communities.

4. 'Coral reef paradox' and potential mechanisms supporting its high productivity

It is known that coral reef ecosystems are often described as paradox: they exhibit a remarkably high primary productivity despite being located in nutrient-poor tropical waters where nitrogen and phosphorus concentrations are generally low. This "coral reef paradox" raises fundamental questions about the mechanisms that allow such

high levels of productivity to be sustained under nutrient-limited conditions (Hatcher, 1990). One of the potential drivers of this paradox may be due to highly effective trophic transfer between many autotroph-heterotroph symbiotic partnerships in coral reefs, particularly those between Cnidarians (e.g., corals and anemones) and their symbiotic algae, zooxanthellae. In this symbiotic partnership, zooxanthellae reside within Cnidarian cells, fixing carbon and translocating a large portion of the photosynthate directly to their Cnidarian hosts (Muscatine and Porter, 1977). In addition to algal-derived photosynthates, Cnidarians can use specialized stinging cells (nematocysts) in their tentacles to capture prey (Houlbrèque & Ferrier-Pagès, 2009). These trophic strategies enable Cnidarian-algal symbiosis to function as mixotrophs, flexibly switching between autotrophic and heterotrophic nutrition to adapt to varying environmental conditions. Given these trophic strategies, we hypothesize that Cnidarian-algal symbiosis may enhance the trophic transfer efficiency from their symbiotic algae to Cnidarian hosts, thereby contributing to the high productivity observed in coral reefs. This direct transfer reflects the tight nutrient cycling between hosts and symbionts, which potentially minimizes energy loss, enhances internal nutrient retention, and ultimately contribute to the high productivity observed in coral reef ecosystems. This hypothesis can be supported by the concept related to the metabolism of organisms. Briefly, in typical oceanic food webs, where no symbiotic relationships exist, the energetic balance of degradation of ammino acids to biomass growth between phytoplankton and zooplankton is approximately 28% (Goto et al., 2018). This is because it is thought that in most grazing processes, around 72% of

diet-derived amino acids are catabolized for life energies, while only 28% contribute to biomass growth of organisms. In contrast, within Cnidarian-algal symbioses, we hypothesize this energetic balance to biomass exceeds 28% due to the direct and efficient transfer of algal-derived photosynthates to Cnidarian hosts. Such enhanced trophic transfer likely results in a greater biomass accumulation in Cnidarians compared to zooplankton in typical oceanic food webs. As a consequence, this process may significantly contribute to the overall high production observed in coral reefs.

5. Potential of trophic transfer efficiency (E_{troph}) in assessing primary production of endosymbiotic algae in coral reefs

Over the past few decades, there are some significant improvements in mentioned measurement techniques. For instance, in situ chamber techniques have been refined to improve spatial resolution, while developments in autonomous sensor deployments now allow for continuous oxygen and pH monitoring over diel and seasonal cycles (e.g., Falter et al., 2012; Long et al., 2013). More recently, satellite remote sensing and autonomous in situ sensors have expanded our understanding to evaluate primary productivity across broader spatial and temporal scales (Barnes et al., 2015; Vahtmäe et al., 2022). These techniques are particularly effective for measuring the productivity of free-living primary producers, such as phytoplankton, turf algae, and macroalgae. However, they are not capable of detecting the photosynthetic activity of endosymbiotic algae (e.g., zooxanthellae), which reside within Cnidarian hosts. In general, trophic transfer efficiency (E_{troph}) is one of the crucial factors in estimating

primary production because the production at a given trophic level can be inferred if the production at the preceding trophic level and the transfer efficiency between them are known. However, at this moment, we cannot estimate primary production in coral reefs accurately because we have no idea about the E_{troph} from primary producers to primary consumers. Currently, directly quantifying E_{troph} in coral reef ecosystems is challenging due to the complexity of food web structures and intricate metabolic processes within organisms. However, as one of the major factors of trophic transfer efficiency, the energetic balance between metabolic degradation and biomass production within Cnidarian-algal symbiosis can be evaluated by comparing of stable nitrogen isotope ratios ($^{15}N/^{14}N$) of amino acids (AAs) with compound-specific analysis of amino acids (CISA-AA) between species involved in trophic interactions (Goto et al., 2018), or, is, more simply, the illustration of the trophic position (TP, which represents the position of organisms in food webs, TP = 1 for primary producers, TP = 2 for primary consumers, and TP = 3 for secondary consumers in the traditional knowledge) estimated with the isotope ratios between two types of AAs (trophic and source) within a single consumer species in food webs (e.g., Chikaraishi et al. 2009).

6. Objectives

This Ph.D. thesis is composed of two studies: 1) quantifying the energetic balance to biomass (i.e., a major factor of E_{troph}) of photosynthates from algal symbionts to Cnidarians within symbiosis, and 2) quantifying the balance between autotrophy and heterotrophy to the nutrient assimilation for Cnidarians (and algae), illustrating the

form of the food web pyramid (e.g., biomass size for each of trophic steps) in coral reef ecosystems. For the first study, we evaluate stable nitrogen isotope ratios of amino acids for several Cnidarian species that bred under an autotrophic condition in a laboratory aquarium to quantify the energetic balance to biomass for the utilization of algal products in Cnidarian hosts. This study will contribute to a better understanding of the ecological and evolutional effects of symbiosis on Earth (e.g., the evolution of photoautotrophs from the view of productivity). For the second study, we quantify the contribution of heterotrophy-derived nutrition to several Cnidarian species that bred under a mixotrophic condition. This study will contribute to a better understanding of the nutritional flexibility on Cnidarians in wild environments (e.g., trophic strategy and bleaching resistance in coral reefs).

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CHAPTER II.

Highly effective energy transfer in the autotroph-heterotroph symbiosis: insights from compound-specific isotope analysis of amino acids

2-1 Quantifying the energetic balance between amino acid degradation and biomass growth in Cnidarian-algal symbiosis: a major factor of trophic transfer efficiency

Abstract

Many species establish symbiotic partnerships with different organisms to benefit energetically from living together to fit into environments, and such symbiosis is frequently found in the modern ocean as well as throughout Earth's history. For example, coral reef ecosystems represent one of the most productive and biologically diverse ecosystems in the modern ocean, which is simply explained by an assumption that many autotroph-heterotroph symbiotic species found in there increase the efficiency of trophic transfer from primary producers (i.e., algal symbionts) to primary consumers (i.e., animal hosts), and that such high efficiency may be propagated to entire food webs. However, this high efficiency has not been proved and illustrated with quantitative data in the trophic transfer of photosynthates from algal symbionts to animal hosts. In the present study, we measured the energetic balance between degradation and biomass growth, which is a major factor of the trophic transfer efficiency (E_{troph}), based on the analysis of stable nitrogen isotope ratios of amino acids for several species including sea anemones, stony corals, zoanthids, and soft corals, that were bred under an autotrophic condition in a laboratory aquarium. The results confirm that the symbiosis indeed provides highly effective energy transfer of algal photosynthates to animal hosts, and allow us to quantify that the energy transfer in the symbiosis increases by approximately twice of

that when non-symbiotic herbivorous species feed on primary producers. These results induce a new perspective that the effective energy transfer driven by symbiosis is a critical factor controlling the development of highly productive and biologically diverse ecosystems in the modern ocean as well as throughout Earth's history.

Keywords

Nitrogen isotopic composition; mixotrophy; coral; anemone; zooxanthellae; productivity; trophic position; endosymbiosis; trophic transfer; food web

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This section was originally published as; Li et al. (2025) Highly effective energy transfer in the autotroph-heterotroph symbiosis: insights from compound-specific isotope analysis of amino acids.

Prog Earth Planet Sci, 12 (1), 13

1. Introduction

Symbiosis is an intimate and long-term association between different species in which they live together to benefit energetically from each other to fit into environments (e.g., Paracer and Ahmadjian 2000). They involve several ecologically diverse types such as mutualism, commensalism, and parasitism, in the interaction between symbionts and hosts (e.g., Dales 1957; Addicott and Freedman 1984). For instance, the symbiotic relationship between cyanobacteria (the earliest organisms to release oxygen by photosynthesis) and eukaryotes gave rise to the evolution of oxygenic photosynthetic eukaryotes (i.e., Glaucophyta, Rhodophyta, and Chlorophyta) (e.g., Archibald 2015). This photosynthetic ability of eukaryotes is also later transferred to other lineages of heterotrophic eukaryotes through eukaryote-eukaryote symbiosis (i.e., evolving to Haptophyta, Heterokontophyta, Dinoflagellata, etc.) that has contributed as photoautotrophs to the overwhelming majority of primary production in Earth's history (e.g., Sánchez-Baracaldo et al. 2021). Even in the modern ocean, symbiotic partnerships between autotrophs and heterotrophs are frequently found in highly productive ecosystems. We therefore have a common interest in how the symbiosis and its energy efficiency from symbiont to host species are linked to the ecological and evolutionary development of productive ecosystems in Earth's history.

Coral reef ecosystems are one of such highly productive ecosystems, which are composed of many autotroph-heterotroph symbiotic species (e.g., sea anemones, stony corals, zoanthids, soft corals, jellyfish, clams, and foraminiferans): algal

symbionts may provide photosynthates to eukaryotic hosts, while the eukaryotic hosts may support the algal symbionts with habitats and nutrients (e.g., Tremblay et al. 2012; Tremblay et al. 2016; Yellowlees et al. 2008). Indeed, coral reef ecosystems show a high net primary productivity (1800 – 4200 gC/m^2/year) compared to the open ocean ecosystems (2 – 400 gC/m^2/year) (e.g.,Whittaker and Likens 1975). Such 'high productivity' may be attributable to the close symbiotic partnerships between algal symbionts (commonly referred to as zooxanthellae) and Cnidarian species (e.g., sea anemones and corals). As the general system in the autotroph-heterotroph symbiosis, Cnidarian-algal symbiosis may promote the high efficiency of trophic transfer of photosynthates (including energy fixed) from algal symbionts to Cnidarians, and Cnidarians may supply the amount of energy to high trophic level organisms in food webs (e.g., De Goeij et al. 2013). However, such high efficiency and its diversity in the trophic transfer have not been proven and quantified.

Based on the trophodynamic concept (which illustrates the cycle of energy and nutrients in the food web pyramid) proposed by Lindeman's pioneering works, it has been believed that the efficiency of trophic transfer from one trophic level to the next is approximately 10% (e.g., Lindeman 1942; Pauly and Christensen 1995). The size of the efficiency may be highly diverse between biomes (e.g., tropical and temperate) and even within a single biome, related to many factors, including ecological properties such as biomass size and species diversity, anthropogenic activities such as fishing and climate change, and natural environmental dynamics at multiple levels from daily to annual changes (e.g., Eddy et al. 2021; Ishikawa, 2018). One of the

potential tools to evaluate the energetic balance to biomass (i.e., a major factor of efficiency of trophic transfer) is the comparison of stable isotope ratios of nitrogen ($^{15}N/^{14}N$) of amino acids (AAs) between two species in a trophic interaction of food webs (e.g., Goto et al. 2018) or, is, more simply, the illustration of the trophic position (TP, which is sometimes called the energetic position of organisms in food webs, TP = 1 for primary producer, TP = 2 for primary consumer, and TP = 3 for secondary consumer in the traditional knowledge) estimated with the isotope ratios between two types of AAs (trophic and source) within a single consumer species in food webs (e.g., Chikaraishi et al. 2009). Indeed, the trophic interaction results in a variable enrichment in ^{15}N for AAs of organisms in food webs (e.g., Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007), such that glutamic acid (a trophic amino acid) has a large enrichment in ^{15}N whereas phenylalanine (a source amino acid) has a little enrichment in ^{15}N from diet to consumer species (e.g., the enrichment is approximately +8.0‰ and +0.4‰ as the $\delta^{15}N$ values, respectively) (e.g., Chikaraishi et al. 2009).

The size of enrichment in ^{15}N is primarily controlled with (1) the types of AAs (e.g., Chikaraishi et al. 2009) and (2) the flux of deamination (e.g., Takizawa et al. 2020) and its isotopic fractionation factor (α) specific for individual AAs (e.g., α = 0.9938 for glutamic acid) (e.g., Goto et al. 2018). Based on this knowledge, the isotope ratios of AAs allow us to evaluate the energetic balance to biomass between organisms in food webs (e.g., Goto et al. 2018; Takizawa et al. 2020). For instance, in the former way (i.e., the comparison between two species), the enrichment in ^{15}N of

glutamic acid between two species in a trophic interaction can be explained by an energetic balance such that 72% of diet-derived glutamic acid is degraded to produce life energy while the other 28% is used for biomass growth (i.e., the efficiency of energy transfer is 28%) if the enrichment is +8.0‰ as the $\delta^{15}N$ values (e.g., Goto et al. 2018). Moreover, in the latter way (i.e., the illustration of TP), a difference in the isotope ratios between trophic (e.g., glutamic acid) and source (e.g., phenylalanine) AAs within a single consumer species estimates the TP of the species, which can account for energetic balance that 72% of diet-derived glutamic acid is degraded (i.e., the efficiency of energy transfer is also 28%) if the TP estimated is 2.0 for herbivores species.

In the present study, we determined the TP based on the isotope ratios ($\delta^{15}N$, ‰ relative to AIR) of glutamic acid and phenylalanine for six Cnidarian species including four Hexacorallia (3 sea anemone, 3 stony coral, 5 zoanthid, and 4 discosoma specimens) and two Octocorallia (11 soft coral specimens) classes that were bred under an autotrophic condition in a laboratory aquarium. Based on the TP, we calculated the energetic balance to biomass from algal symbionts to Cnidaria and discussed the ecological and evolutionary aspects of the symbiosis in Earth's history. The TP was estimated with the following equations (1) and (2) (e.g., Chikaraishi et al. 2009) :

$$TP = [(\Delta\delta^{15}N - 3.4) / 7.6] + 1 \quad (1)$$

$$\Delta\delta^{15}N = \delta^{15}N_{Glu} - \delta^{15}N_{Phe} \quad (2)$$

In these equations, −3.4‰ and 7.6‰ representing the β value and trophic discrimination factor (TDF) respectively, which are major factors determining TP. The β value represents the isotopic difference ($\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$) between glutamic acid and Phe value in primary producers, reflecting the baseline isotopic signature at the lowest trophic level in food webs. Here, the β value used as −3.4‰ is specifically to aquatic primary producers, such as cyanobacteria and algae (Chikaraishi et al., 2010), which is distinct from significantly higher values that reported for terrestrial C_3 plants (+8.4‰). The TDF of 7.6‰ represents the average increase in the isotopic difference ($\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$) per trophic level, primarily caused by isotopic fractionation during amino acid deamination, where ^{14}N is preferentially lost during catabolism and ^{15}N is retained in newly synthesized biomass. This TDF has been empirically validated across diverse aquatic food webs (Chikaraishi et al., 2009). Thus, these parameters (β = −3.4‰, TDF = 7.6‰) were specifically chosen for accurately estimating the trophic positions of marine organisms and their symbiotic algae within coral reef ecosystems.

The energetic balance to biomass was estimated with the following equations (3) and (4) (e.g., Goto et al. 2018):

$$\Delta\delta^{15}N = 1000 \times [\, F^{(0.9938-1)} - 1] \quad (3)$$

$$\text{Efficiency of trophic transfer} = 100 \times F \quad (4)$$

These equations are derived from the Rayleigh isotopic fractionation model, a widely used approach to quantify energetic balance between amino acid degradation (catabolism) and biomass synthesis (anabolism). In equation (3), the left side ($\Delta\delta^{15}N$)

represents the isotopic enrichment (in units of per mil, ‰) of $\delta^{15}N$ observed in amino acids during the transfer from a food source to a consumer organism. This enrichment arises due to isotopic fractionation occurring during amino acid metabolism, specifically when amino acids are deaminated (removal of amino groups) to generate metabolic energy or to be assimilated into biomass. In the right side of equation (3) the exponent (0.9938−1) represents the isotopic fractionation factor ($\alpha = 0.9938$), an empirically determined parameter representing the isotopic discrimination during the transamination (specifically, deamination) of amino acids. For instance, glutamic acid exhibits an α value of 0.9938 under constant enzymatic activity in vitro (Goto et al., 2018). Using this isotopic fractionation factor, it can be calculated that achieving an 8.0‰ increase in $\delta^{15}N$ of glutamic acid corresponds to approximately 28% of glutamic acid being converted into aspartic acid and incorporated into biomass, while the remaining 72% is catabolized into α-ketoglutaric acid to generate metabolic energy. Thus, this estimation can be explained by the observed isotopic enrichment ($\Delta\delta^{15}N$) directly reflects the ratio between amino acids used for biomass growth (F) and those catabolized for energy ($1-F$).

2. Materials and methods

2.1 Aquarium for breeding Cnidarian species

An aquarium used in the present study consists of a “breeding tank” (54 L, 600 mm in length, 300 mm in height, and 300 mm in depth) and a “filtrating tank” (35 L, 520 mm in length, 260 mm in height, and 260 mm in depth) (Fig 2-1-1). Seawater is

circulated in these tanks: wastewater is overflowed and is fallen into the filtrating tank, while filtrated water is pumped up to the breeding tank. In the filtrating tank, waste (NH_3) in the water is oxidized to nitrate (NO_3^-) with ammonia-oxidizing bacteria and nitrate bacteria, and the nitrate produced and ammonia remaining are absorbed with chemical absorbents. In the breeding tank, the nitrate remained is denitrified to dinitrogen gas (N_2) at the anoxic layer of sands. The seawater is partly replaced with new artificial seawater by 5 L/month. The seawater is thus kept clean (no detection of ammonia and nitrate) for the experiment term. The temperature of the seawater is set at 26ºC (±0.2ºC). The light was provided with two UV-LED lamps (Lightening Master 24 DC Sapphire blue, 20 W, B.R.S-Best Reef Systems) at 130 mm upper from water surface in the breeding tank. The lighting condition is set as an 8 h light: 16 h dark cycle in a day.

2.2 Cnidarian species

Four Hexacorallia including the sea anemones *Radianthus crispus* (*n* = 1, number of individuals), *Entacmaea quadricolor* (*n* = 2), the discosoma *Discosoma* sp. (*n* = 4), the stony coral *Duncanopsammia axifuga* sp. (*n* = 3), and the zoanthid *Zoanthus* sp. (*n* = 5), and two Octocorallia including the star polyp *Pachyclavularia violacea* sp. (*n* = 8) and the leather corals *Sarcophyton* sp. (*n* = 3) were used in the present study (Fig 2-1-2, Table 2-1-1), here, *n* represents the number of individual specimens subjected to isotope analysis. These Cnidaria were bred for at least two months in the aquarium (breeding tank) before the isotope analysis. For five Cnidaria specimens (Red and green of *Zoanthus* sp., red of *Discosoma* sp., Centre light of *Pachyclavularia violacea*

sp., and yellow of *Sarcophyton* sp., Supplementary Table 1), polyps were put into a petri dish that was filled with artificial seawater, and cut into several pieces to release the symbiotic algae. The symbiotic algae (approximately 10 μm diameter) were purified by filtration with 25 μm (Polypropylene Net Filters, Merck Millipore Ltd) and 12 μm (Nuclepore^{TM} Track-etch Membrane, Whatman^{TM}) pore-sized filters, and the symbiotic algae filtered were concentrated with a manual centrifuge at 1200 rpm for 10 s under great care against breaking the cell of algae, which are confirmed with a microscope. Notably, we did not include technical or biological replication for each Cnidarian specimen, this is primarily because theses Cnidarians in the aquarium originated from asexually propagated colonies, meaning they are genetically identical and we thus expected they show minimal individual-level variation in measurement. Also, the stable nitrogen isotope analysis of amino acids used in this study provides high analytical precision, with an established estimation error of ± 0.1–0.2 in TP estimates (Chikaraishi et al., 2009; Nielsen et al., 2015).

2.3 Isotope analysis

The Cnidaria collected and symbiotic algae purified were prepared for the analysis of stable isotope ratios ($\delta^{15}N$, ‰ relative to AIR) of amino acids, following the procedure described in the previous study. In brief, the samples were hydrolyzed with 12 N HCl at 110°C overnight (> 12 h). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. Derivatizations were then performed sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4). The $\delta^{15}N$ values of the *N*-

pivaloyl/isopropyl (Pv/OiPr) esters of amino acids were determined with a gas chromatograph - isotope ratio mass spectrometer (GC-IRMS) using a 7890B GC (Agilent Technologies) coupled to a DeltaV IRMS through combustion (950°C) and reduction (550°C) furnaces via a GC-C/TC III interface (Thermo Fisher Scientific) (e.g., Takizawa and Chikaraishi 2021). The derivatives of amino acids were separated on an Agilent Ultra-2 capillary column (50 m length, 320 μm i.d., 0.5 μm film thickness). The carrier gas (He) flow rate was controlled using a constant flow mode at 1.4 mL/min. The $\delta^{15}N$ values were expressed relative to the isotope ratio of AIR, on scales normalized to known $\delta^{15}N$ values of the reference amino acids (Indiana University; Shoko Science Co.). The 1σ standard deviation for the reference amino acid analysis was 0.4 – 0.7‰.

3. Results

Differences in the $\delta^{15}N$ value between glutamic acid and phenylalanine ($\Delta\delta^{15}N$) correlate with the TP of organisms if we did not care about the efficiency of trophic transfer, such that 3.4‰, 11.0‰, and 18.6‰ for the $\Delta\delta^{15}N$ values generally represent primary producers (TP = 1, photoautotrophs), primary consumers (TP = 2, herbivores), and secondary consumers (TP = 3), respectively, with a 7.6‰ elevation from diet to consumer species (e.g., Chikaraishi et al. 2009). For instance, the giant clam *Tridacna crocea* hosts zooxanthellae (symbiotic algae) within their mantle tissues, in which the $\Delta\delta^{15}N$ values elevated from algae (2.9‰) to clam (10.6‰), illustrating an autotroph-heterotroph relationship in this symbiosis (e.g., Maeda et al. 2012). On the other hand, the sea slug *Plakobranchus ocellatus* has kleptoplasts (i.e.,

retains intact chloroplasts derived from diet algae), and their 5.6‰ for the $\Delta\delta^{15}N$ values indicate mixotrophy in their trophic identity (e.g., Maeda et al. 2012). In the present study, we found a significant variation in the $\Delta\delta^{15}N$ value ranging from 2.8‰ to 12.5‰, which corresponds to the TP ranging from 0.9 to 2.2 for these Cnidarian species that grew autotrophically (i.e., symbiont-derived photosynthate is a solo resource) in the laboratory aquarium (Fig. 2-1-3). In Hexacorallia, the sea anemones *Radianthus* and *Entacmaea* sp., the stony corals *Duncanopsammia* sp., the zoanthid (order *Zoantharia*), and the discosoma *Discosoma* sp. have the TP of 1.2 ± 0.1, 1.6 ± 0.1, 1.9 ± 0.1, and 1.5 ± 0.1, respectively, with little variation within a species. In contrast, in Octocorallia, the star polyp *Pachyclavularia* sp. and the leather coral *Sarcophyton* sp. have the TP of 1.3 ± 0.5 and 1.7 ± 0.2, and thus show a larger variation in the TP within a species than Hexacorallia. No substantial difference in the TP is found between Hexacorallia (TP = 1.6 ± 0.3, n = 15) and Octocorallia (1.4 ± 0.4, n = 11) examined (t-test: t = 1.15, df = 17, p > 0.1). Thus, the TP of Cnidarian species (1.5, as the median value for all specimens examined) is frequently lower than that of general herbivores (i.e., TP = 2) (one sample t-test: t = −6.66, df = 25, p < 0.0001) and exhibits a significant inter- and intra-diversity within the species. On the other hand, we cannot find such a considerable variation in the $\Delta\delta^{15}N$ value for symbiotic algae purified from three Hexacorallia and two Octocorallia specimens. Their $\Delta\delta^{15}N$ values ranging from 3.0‰ to 4.2‰, which corresponds to the TP ranging from 1.0 to 1.1, indicate that their trophic identity is surly autotrophy, as substantially the same values for the reference green and red algae (4.1‰ and 3.6‰, respectively, for the $\Delta\delta^{15}N$

values) (t-test: $t = -0.48$, $df = 2$, $p > 0.1$).

4. Discussion

4.1 Trophic transfer from algal symbionts to animal hosts

In the species in the autotroph-heterotroph symbiosis, the equations (1) – (4) illustrate the correlation between the following three factors: energetic balance to biomass from animal symbionts to animal hosts; the $\Delta\delta^{15}N$ values of host species; and the TP of host species. For example, the efficiency is 100%, 28%, and 9% (i.e., F = 1.00, 0.28, and 0.09) if the $\Delta\delta^{15}N$ value is 3.4‰, 11.0‰, and 18.4‰ (i.e., TP = 1, 2, and 3) for the host species, respectively. The energetic balance to biomass has thus a negative correlation with the $\Delta\delta^{15}N$ values and TP of host species. Notably, the TP estimated for the host species can differ dramatically from the traditional trophic position of herbivorous species in food webs. In the present study, the TP of Cnidarian species was estimated as 1.5 (as the median value), ranging from 0.9 (*Pachyclavularia violacea* (Centre light)) to 2.2 (*Pachyclavularia violacea* (Fluorescent green)). This variation observed in TP among individuals reflects intrinsic species- or morph-specific differences in algal symbionts (Muscatine and Porter, 1977; Baker, 2003). For example, even within the same species of *Pachyclavularia violacea*, the difference between Centre light and Fluorescent green morphs may be attributed to variations in the abundance or activity of algal symbionts, or differences in nitrogen recycling efficiency. The median value (1.5) was used for TP instead of the mean value to avoid skewness caused by extreme values.

These results reveal that the TP of Cnidarian species examined (i.e., TP = 1.5) is lower than that of general primary consumers (TP = 2.0) such as herbivorous zooplankton. The equation (3) gives us that the energetic balance to biomass of the Cnidarian species examined is approximately 56% (which corresponds to the median $\Delta\delta^{15}N$ values, mean: 58%, 1σ deviation: 25%, Fig. 2-1-4) for using algal products in the symbiosis, which is increased by approximately twice compared to when herbivores feed on phytoplankton in the general grazing process of food webs. Notably, the median $\Delta\delta^{15}N$ value was used for the calculation of energetic balance to biomass to minimize the influence of extreme values caused by inherent species-specific variations in $\delta^{15}N$, thus providing a more representative estimate of Cnidarians. Nevertheless, the mean-based energetic balance to biomass was calculated as 58% (± 25%, 1σ deviation), indicating only a minor difference between mean and median estimates.

It is known that the energetic balance to biomass from one trophic level to the next is potentially highly variable and related to many factors, including anthropogenic activities and natural environmental dynamics (e.g., Eddy et al. 2021). Our results reveal that the symbiosis is another critical factor for considerably enhancing the efficiency of trophic transfer, in which energy is directly transferred from algal symbionts to host species with less losses compared to typical phytoplankton-zooplankton combinations at the base levels of food webs, which will affect all other higher hierarchies in the entire food web of ecosystems. For example, if 28% (± 3%, 1σ deviation) for the energetic balance to biomass is simply acceptable to many types

of ecosystems, biomass size of primary consumers is systematically reduced to 28% of that of the primary producers. However, the increase of biomass at the second level of food webs by twice from 28% of that of the primary producers for normal (i.e., many types of ecosystems) to 56% of that of the algal symbionts for host species can be propagated to all other higher levels in the entire food web. It is known that coral reef ecosystems have been considered hotspots of biodiversity and productivity (e.g., Whittaker and Likens 1975). Based on our results, we predict that the high energetic balance to biomass derived from autotroph-heterotroph symbiosis is a critical factor that provides, at least partly, such high production in the coral reef ecosystems.

4.2 Possible factors on the trophic transfer

The size of the efficiency of trophic transfer in food webs may be highly variable depending on multiple ecological factors, in which the mixotrophy and biomass-diversity of food webs are highly contributable (e.g., Ishikawa, 2018; Ward and Follows 2016). It is known that mixotrophy is defined as the combined use of photosynthetic and heterotrophic nutrients within a single organism, thereby suggesting that such flexibility in the nutrient has a positive effect on enhancing the trophic transfer of biomass to organisms at high trophic levels in food webs (Ward and Follows 2016). In contrast, it is also thought that high biomass diversity in food webs increases the degree of edibility and omnivory of organisms at high trophic levels in the food webs, thereby suggesting that such biomass diversity apparently has a negative effect on enhancing trophic transfer in food webs (Ishikawa, 2018). In the present study, we reveal that the autotroph-heterotroph symbiosis increases the

energetic balance to biomass at the bases of food webs. Thus, our results propose that, not only mixotrophy (i.e., positive effect) and biomass-diversity (i.e., negative effect) suggested in the previous studies, but also symbiosis has a positive effect to enhance the energy transfer from one trophic level to the next in food webs, particularly for explaining high productivity in coral reef ecosystems. It is noted that we should carefully discuss the other factors, calcification and heterotrophy of Cnidarian species, particularly for corals, to illustrate the energy transfer in the symbiosis. Several species of the Cnidaria form a large amount of external calcium carbonate skeletons. It may be expected that the growth rate of Cnidarian species is highly dependent on the calcification because large energy may be required for producing calcium carbonate skeletons. Moreover, heterotrophy is frequently found in many Cnidarian species (i.e., zooxanthellae is not the solo nutrient source), similar to mixotrophic organisms. This allows us to think that the production rate of calcium carbonate and the heterotrophy may have negative and positive effects, respectively, to enhance the energy transfer to organisms at high trophic levels in the ecosystems. In addition, all Cnidarian species examined in the present study are collected from an aquarium breeding under autotrophic conditions. Thus, in further studies, evaluating these factors (i.e., heterotrophy and calcification) in the symbiosis will contribute to reducing errors in quantifying the energetic balance to biomass of autotroph-heterotroph symbiosis in wild ecosystems.

4.3 Ecological and evolutionary aspects

The energetic balance to biomass in the Cnidarian-algal symbiosis (revealed in the

present study) can be implied for considering the coevolution between photoautotrophs and biomass size in Earth's history. It is agreed that the symbiosis of eukaryotes with photoautotrophs is a major driver of the evolution of oxygenic photosynthetic eukaryotes (e.g., Delwiche 1999; Gould 2012) and may cause a significant increase in the size of biomass at the base of food webs from past to modern ocean (e.g., McCarthy et al. 2013). Photosynthetic eukaryotes have evolved via the endosymbiotic events, which are caused by three major stages (Fig. 2-1-5) including initial stage: heterotrophic eukaryotes feed on (i.e., engulfed) and digest photosynthetic species, middle stage: these two species form symbiotic partnership, and final stage: such symbiotic partnership transforms to endosymbiosis that photosynthetic species are enslaved toward chloroplasts in the eukaryotic cells (e.g., Delwiche 1999; Gould 2012). These endosymbiotic events resulted in a large diversity of photosynthetic eukaryotes (e.g., Rhodophyta and Chlorophyta via primary, Cryptophyta and Chlorarachniophyta via secondary, and Dinoflagellata via tertiary endosymbiosis) (e.g., Delwiche 1999). Because the TP of a giant clam and symbiotic algae is 2.0 and 1.0, respectively (e.g., Maeda et al. 2012), the TP of heterotrophic eukaryotes is considered to be 2.0 (i.e., 28 % for the efficiency of trophic transfer) at the initial stage of endosymbiosis. In contrast, based on the evidence that many studies (e.g., Chikaraishi et al. 2009; McCarthy et al. 2007) reported 1.0 for the TP of photosynthetic eukaryotes, the TP of symbiotic eukaryotes should be declined from 2.0 to 1.0 (i.e., from 28% to 100% for the efficiency of trophic transfer) from the middle to the final stages of endosymbiosis. Indeed, our

results illustrated that the TP of Cnidarian species is 1.5 as the median value, which is clearly declined by 0.5 from the TP of 2.0. Interestingly, a large variation in the TP of the Octocorallia examined in the present study suggests that Octocorallia may belong to multiple evolutionary stages, including the initial (e.g., TP = 2.2 for fluorescent green specimen), the middle (e.g., TP = 1.4 for long polyp white specimen), and the final (e.g., TP = 0.9 for center light specimen) stages, even within a single species. These shifts of evolutionary stages in Cnidarian species may be supported by endosymbiotic gene transfer (EGT), in which the size of genome of algal symbionts is reduced because of moving it to the host species, toward single species (e.g., Hackett and Bhattacharya 2006; LaJeunesse et al. 2005). Based on the TP of Cnidarian species in the present study, we conclude that important ecological and evolutionary aspects of autotroph-heterotroph symbiosis are the high efficiency of energetic balance to biomass (i.e., TP = 2 to 1) from diet to consumer species within the symbiosis, particularly that endosymbiosis (the final stage in Fig. 2-1-5(b)) can achieve 100% for the efficiency within the symbiosis. Hence, we predict that (1) a significant increase in the size of biomass at the base of food webs in the modern ocean as well as in Earth's history (e.g., McCarthy and Lehman 2013) is potentially partially explained by the achievement of endosymbiosis (i.e., an increase of the efficiency of trophic transfer in the evolution of photosynthetic eukaryotes), and (2) the TP of symbiotic species estimated based on the isotope ratios of AAs can quantify the energetic balance to biomass and identify the evolutionary stage of photosynthetic eukaryotes for many autotroph-heterotroph symbiotic species not only for Cnidarian-algal

symbiosis but also other symbiotic species including jellyfish, clams, and foraminiferans.

5. Conclusion

The high energetic balance to biomass of photosynthates from algal symbionts to animal hosts in their symbiosis was measured based on the compound-specific isotope analysis of amino acids for several species including sea anemones (n = 3), stony corals (n = 3), zoanthids (n = 5), and soft corals (n = 11), that were bred under an autotrophic condition in a laboratory aquarium. The energetic balance to biomass quantified is 56% (as median value, mean: 58%, 1σ deviation: 25%) for the utilization of algal products in animal hosts, in which the energy transfer in the symbiosis increases by approximately twice of that when non-symbiotic herbivorous species feed on primary producers. These results imply that the highly effective energy transfer driven by autotroph-heterotroph symbiosis is a critical factor controlling the development of highly productive and biologically diverse ecosystems in the modern ocean (e.g., coral reef ecosystems even in the oligotrophic environment) as well as throughout Earth's history.

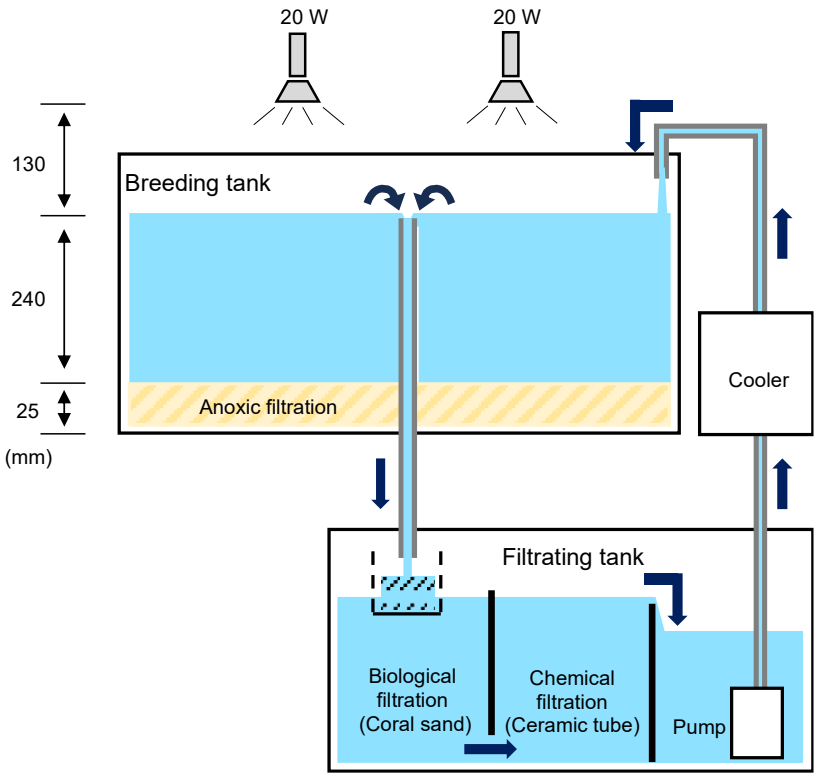

Fig. 2-1-1. The aquarium used in the present study.

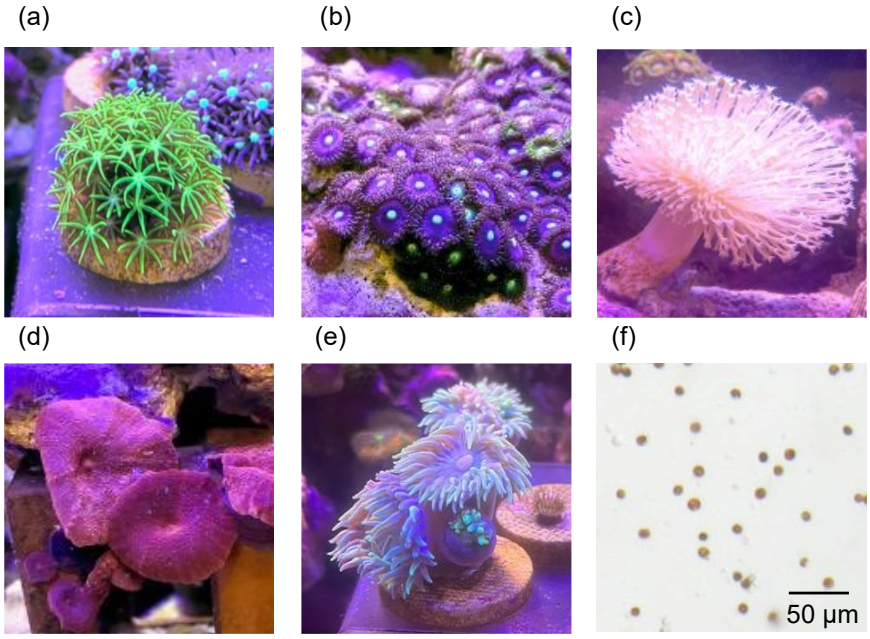

Fig. 2-1-2. Photos of representative Cnidaria specimens and algal symbionts purified from Cnidaria: (a) *Pachyclavularia violacea* sp. (b) *Zoanthus* sp. (c) *Sarcophyton* sp. (d) *Discosoma* sp. (e) *Duncanopsammia axifuga* sp, and (f) algal symbionts.

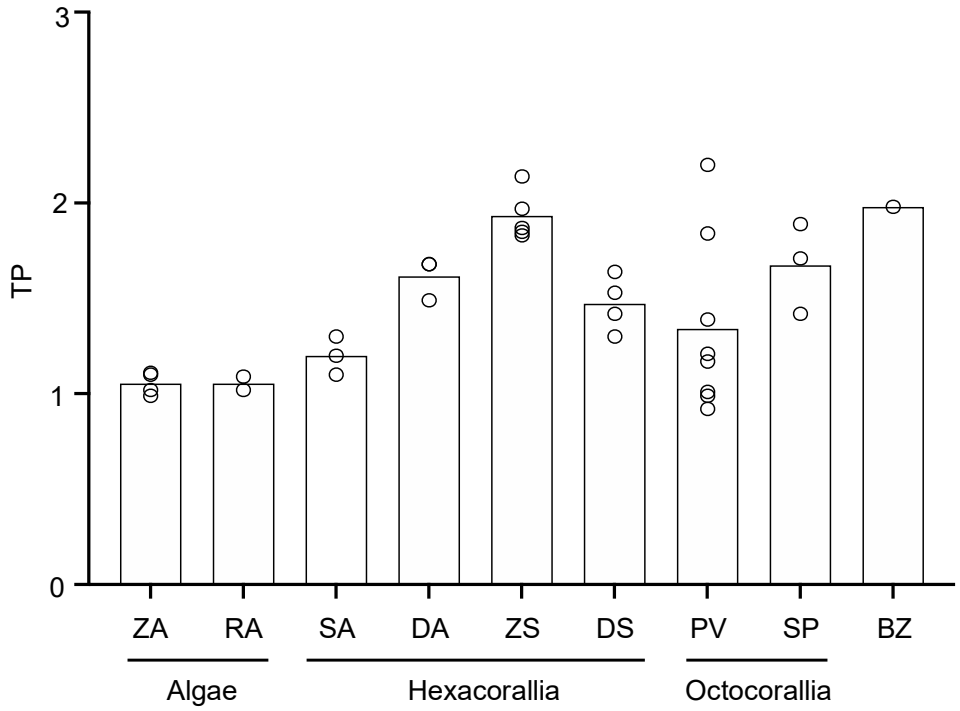

Fig.2-1-3. Trophic position (TP) estimated from the $\Delta\delta^{15}N$ value of amino acids for the algal symbionts (n = 5), reference algae (n = 2), Cnidaria (n = 26), and a bleached Zoanthid (n = 1, Supplementary information 1). Each dot represents an individual measurement, and the bar indicates the average TP value of that group. ZA: Zooxanthellae, RA: Reference algae, SA: Sea anemones, DA: *Duncanopsammia axifuga*, ZS: *Zoanthus*, DS: *Discosoma*, PV: *Pachyclavularia violacea*, SP: *Sarcophyton*, BZ: Bleached Zoanthid.

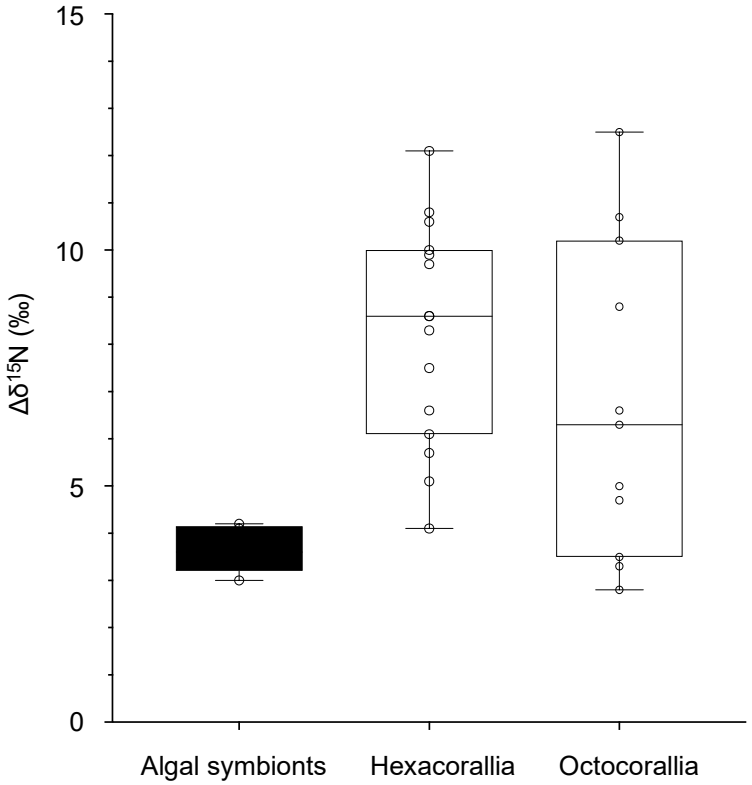

Fig. 2-1-4. Box plot of the $\Delta\delta^{15}N$ values in algal symbionts (n = 5) and Cnidaria (n = 15 for Hexacorallia and 11 for Octocorallia).

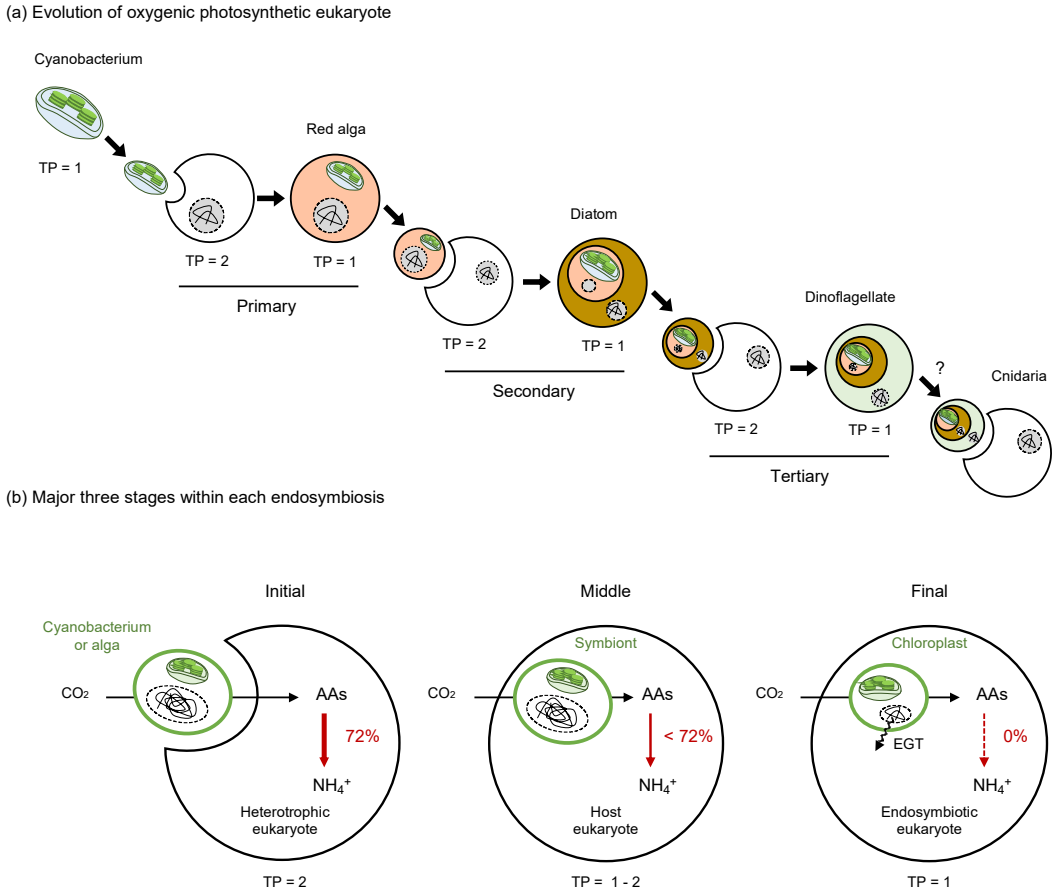

Fig. 2-1-5. Schematic view of the endosymbiotic events in oxygenic photosynthetic eukaryotes and of further possibility in the Cnidarian-algal symbiosis. (a) Evolution of oxygenic photosynthetic eukaryote: between cyanobacteria and heterotrophic eukaryote in the primary endosymbiosis; between the primary endosymbiotic eukaryote (e.g., Rhodophyta) and another heterotrophic eukaryote in the secondary endosymbiosis; between the secondary endosymbiotic eukaryote (e.g., Haptophyta) and another heterotrophic eukaryote in the tertiary endosymbiosis; and between the tertiary endosymbiotic eukaryote (i.e., Dinoflagellata) and Cnidaria in the middle stages of the quaternary endosymbiosis. (b) Three major stages within each endosymbiosis: 72 % of diet-derived amino acids is degraded to NH_4^+ and the other

28 % is used for biomass growth (i.e., $F = 0.28$) for the heterotrophic eukaryote in the initial stage; the effective trophic transfer results in the degradation percentage between 72 % and 0 % for the eukaryotic host cell in the middle stage; and endosymbiosis achieves no degradation of chloroplast-supplied amino acids for the endosymbiotic eukaryote in the final stage.

Samples	$\delta^{15}N$						$\Delta\delta^{15}N$	TP	*F*
	Ala	Gly	Glu	Val	Phe	Pro			
Hexacorallia									
Actiniaria									
Sea anemones									
Radianthus sp.	11.0	3.5	7.1	10.9	3.0	15.9	4.1	1.1	0.900
Entacmaea sp.	12.9	1.6	10.9	11.6	5.8	15.1	5.1	1.2	0.800
Entacmaea sp.	11.7	2.5	10.5	11.5	4.5	17.4	6.0	1.3	0.700
Duncanopsammia axifuga									
Whisker coral									
White	5.0	5.2	8.2	9.0	-0.3	n.d.	8.6	1.7	0.400
Green	11.0	7.9	9.7	12.5	1.1	n.d.	8.6	1.7	0.400
Whole green	6.0	9.2	13.0	9.5	2.4	2.4	10.6	1.5	0.555
***Zoanthus* sp.**									
Zoas									
Red	10.6	8.2	11.0	9.6	1.1	n.d.	10.0	1.9	0.333
Green	15.1	12.7	13.9	13.7	4.2	n.d.	9.7	1.8	0.376
Centre red	12.4	14.8	16.6	10.2	4.6	n.d.	12.1	2.1	0.261
Orange	13.2	10.9	14.6	14.7	3.8	n.d.	10.8	2.0	0.300
Purple	11.7	6.7	12.1	12.9	2.2	n.d.	9.9	1.9	0.333
***Discosoma* sp.**									
Disc coral									
Red	9.5	5.0	9.4	9.6	1.1	n.d.	8.3	1.6	0.500
Orange	7.8	1.7	9.6	5.5	2.2	n.d.	7.5	1.5	0.555
Green	7.7	1.6	7.1	5.3	0.5	n.d.	6.6	1.4	0.600
Light green	6.3	3.0	7.9	9.8	2.2	n.d.	5.7	1.3	0.700
Octocorallia									
Pachyclavularia violacea									
Star polyp									
Green	5.2	3.0	8.0	7.4	4.6	n.d.	3.3	1.0	1.000
Centre light	9.9	9.0	8.4	11.9	5.6	n.d.	2.8	0.9	1.130
Fluorescenet green	12.7	13.9	18.0	9.6	5.5	n.d.	12.5	2.2	0.230
long green	8.2	2.3	9.6	9.2	4.9	15.2	4.7	1.2	0.800
Metalic green	2.4	0.1	4.5	4.7	1.0	5.0	3.5	1.0	1.000
Long polyp white	2.0	1.8	5.1	0.9	-1.2	n.d.	6.3	1.4	0.600
Short polyp white	5.0	3.8	5.1	6.7	0.1	n.d.	5.0	1.2	0.800
Centre white	2.3	2.6	7.8	6.9	-2.9	n.d.	10.7	1.8	0.376
***Sarcophyton* sp.**									
Toadstool leather coral									
White	10.8	11.7	14.0	10.5	3.8	n.d.	10.2	1.9	0.333
Green	8.8	9.9	13.8	13.0	5.0	n.d.	8.8	1.7	0.400
Yellow	1.6	0.0	4.9	-1.1	-1.7	n.d.	6.6	1.4	0.600
Bleached Zoanthis									
***Zoanthus* sp.** **Zoas**	14.9	12.9	14.9	n.d.	4.1	n.d.	10.8	2.0	

Reference algae								
Caulerpa racemose	4.8	-2.3	7.6	5.9	3.6	n.d.	4.0	1.1
Green algae								
Meristotheca papulose	3.1	-3.1	5.2	3.1	1.6	n.d.	3.6	1.0
Red algae								
Endosymbiotic algae (*Zooxanthellae*)								
***Zoanthus* sp.**								
Zoas								
Red	3.0	5.9	4.9	4.0	0.8	7.2	4.1	1.1
Green	4.9	7.6	7.4	6.0	4.0	7.9	3.4	1.0
***Discosoma* sp.**								
Disc coral								
Red	4.8	4.2	5.2	6.1	1.0	7.2	4.2	1.1
Pachyclavularia violacea								
Star polyp								
Centre light	3.2	3.9	8.4	6.5	4.8	7.5	3.6	1.0
***Sarcophyton* sp.**								
Toadstool leather coral								
Yellow	-1.0	0.4	1.5	-2.5	-1.5	3.2	3.0	0.9

Table 2-1-1. The $\delta^{15}N$ values of amino acids, the difference in the $\delta^{15}N$ value between glutamic acid and phenylalanine ($\Delta\delta^{15}N$), the TP for Cnidarian holobionts, algal symbionts, a bleached coral, and reference algae, and the *F* for Cnidarians.

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2-2 Isolation of symbiotic algae from corals for measuring stable isotope ratios of amino acids in the trophic food web studies

Abstract

Over the last two decades, compound-specific isotope analysis (CSIA) of amino acids has been increasingly used in many studies for understanding the trophic interaction among organisms in food webs. It is expected that this CSIA application to host and symbiont species within a symbiotic partnership (e.g., coral and its algal symbiont, respectively) allows us to evaluate how the symbiotic partnership between host and symbiont species is constructed. Therefore, in the present study, we established a method for the isolation of algal symbionts (i.e., zooxanthellae) from coral hosts, for minimizing the contamination of coral tissues and the breakage of algal cells, in the preparation of CSIA. Applying the isolation procedure based on multiple filtration and a weak centrifuge, we successfully measure the stable nitrogen isotope ratios of amino acids in algal symbionts, and can illustrate the trophic interaction between host and symbiont species in corals. Indeed, the isotope ratios of glutamic acid (7.4‰) and phenylamine (4.0‰) shows that the trophic identity of algal symbionts is primary producer (i.e., trophic position = 1.0), which is far from that of coral hosts (i.e., primary consumer, trophic position = 2.0). We predict that the isolation method established in the present study will be useful to evaluate the trophic interaction between algal symbionts and host species for not only corals but also other species such as giant clams, jellyfish, and anemones.

1. Introduction

Many marine animals, including corals, jellyfish, giant clams, sea slugs, and sponges, have a symbiotic partnership with photosynthetic algae (zooxanthellae), that are generally called algae-eukaryote symbiosis (Yellowlees et al., 2008). These zooxanthellae fix the solar energy into organic products via the photosynthesis, while animal hosts receive the photosynthates from algal symbionts (Davy et al., 2012). These partnerships can be found not only in the modern ocean but also throughout Earth's history, as proved by that the endosymbiosis is derived from the symbiotic partnership of cyanobacteria with an ancestral eukaryotic cell, resulting in the formation of the chloroplasts in eukaryotic photoautotrophs (e.g., green and red algae) (Archibald, 2015). Coral is one of the representative species among such 'algae-eukaryote symbiosis' found in the modern ocean, by which the symbiotic relationship with algae supplies photosynthates into food webs and therefore has built coral reef ecosystems (that show high productivity and biological-diversity, Tremblay et al. 2012; Tremblay et al. 2016; Yellowlees et al. 2008) even in oligotrophic tropical ocean. Zooxanthellae in corals are unicellular dinoflagellates of the genus *Symbiodinium*, and indeed provide photosynthates (e.g., protein, lipids, and carbohydrates) to their coral hosts (Muscatine and Porter, 1977; Yellowlees et al., 2008; Trembaly et al., 2012). Thus it is hypothesized that the high productivity and biological-diversity in coral reef ecosystems are supported by the symbiotic partnership between algal symbionts and coral hosts at the bases of food webs. However, despite the ecological and evolutional importance of the symbiotic

partnership between algal symbionts and eukaryote hosts, the trophic interaction (e.g., flux of energy and nutrients) between these species within the symbiosis is poorly understood. In particular, the first key question is to clarify whether the trophic function of algal symbionts and coral hosts is simply primary producers and primary consumers, respectively in coral reef ecosystems. This question is highly required for understanding how energy and nutrients are fixed, moved, stored, and consumed within the algae-eukaryote symbiosis, such as the symbiotic partnership between zooxanthellae and corals at the bases of food webs in coral reef ecosystems.

Over the past decade, compound-specific isotope analysis (CSIA) of amino acids has been employed as a powerful tool for estimating the trophic position (TP) of organisms in food webs (e.g., Chikaraishi et al., 2009). This TP estimate is based on the experimental observation on the change in the isotope ratio (i.e., $\delta^{15}N$ value) during trophic transfer from diet to consumer species, such that the trophic amino acids in consumer species exhibit a large elevation in the $\delta^{15}N$ values (e.g., by ~ 8‰ for glutamic acid) while the source amino acids exhibit little elevation in the $\delta^{15}N$ value (e.g., by ~ 0‰ for phenylalanine) from the values of respective amino acids in diet species (Chikaraishi et al., 2007; McCarthy et al., 2007; Popp et al., 2007). Indeed, the TP can be estimated with the following equations (1) (Chikaraishi et al. 2009):

$$TP = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4) / 7.6] + 1 \quad (1)$$

where $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ are the $\delta^{15}N$ values of glutamic acid and phenylalanine,

respectively, found in consumer species. Moreover, the applicability of CSIA-AAs has recently been extended to diverse studies for not only the estimation of TP of organisms but also the illustration of nutrient and energy cycle of organisms that have a specific life strategy for adapting to their habitats, such as autophagy (Takizawa et al., 2017), symbiosis (Li et al., 2025), and cannibalism (Lurette et al., 2023).

However, the applicability of CSIA to the symbiotic system (i.e., algae-eukaryote symbiosis) is still limited, particularly because a suitable method is not established for the purification of algal symbionts from eukaryote hosts. The complex nutrient-sharing system in the symbiosis (e.g., the possible use of mixotrophic nutrition mode in algae, and complex nutrient cycles within this symbiosis e.g., Dales 1957; Addicott and Freedman 1984) also causes a strong limitation in the study based on the CSIA of whole samples (i.e., admixture samples of algal symbionts and eukaryote hosts). Thus, the isotope ratios of algal symbionts and eukaryote hosts (e.g., zooxanthellae and coral itself in the case of corals) should be analyzed independently, to illustrate what type of trophic transfer is found in the symbiotic system and to evaluate how the algae-eukaryote symbiosis is constructed.

To address these issues, we established a method for the isolation of algal symbionts (i.e., zooxanthellae) from coral hosts, for minimizing the contamination of coral tissues and the breakage of algal cells, in the preparation of CSIA. This method combines multiple filtration and a weak centrifuge, to remove coral tissue fragments and to prevent the breakage of algal cells, respectively. The use of artificial seawater is effective to maintain sample stability during the isolation. The isolation method

established in the present study will be useful to evaluate the trophic interaction between algal symbionts and host species in the algae-eukaryote symbiosis.

2. Materials and Methods

2.1 Coral species used for algae isolation

One coral species, zoanthid *Zoanthus* sp. (n = 1), was used in the present study. For this coral sample, some parts of its colony were accidently bleached, resulting in the escape of zooxanthellae from the coral tissue. The colony subsequently recovered within a couple of weeks as zooxanthellae returned to the coral tissue. The absence and presence of zooxanthellae in the bleached coral was confirmed with a microscope during bleaching and after recovery (Fig 2-2-1). Because there were no algae inside the coral polyps during bleaching, this coral sample allows us to know the real TP values of the coral itself, the zooxanthellae, and the coral host. Moreover, these TP values enable us to quantify the relative mass contribution of algal symbionts to the whole coral holobionts using a mass balance calculation method.

2.2 Isolation of zooxanthellae from coral host

Zooxanthellae were isolated from coral hosts with an established method including multiple filtrations and a weak centrifuge to ensure sample purity and algal cell integrity before isotope analysis. Fig. 2-2-2. shows a schematic pathway outlining the isolation process. In brief, coral polyps were put into a petri dish that was filled with artificial seawater, and carefully cut into several pieces to release zooxanthellae (approximately to 10 μm) that inhabited inside cells of coral polyps. For filtration,

first, the mixture of coral polyps and zooxanthellae was passed through 25 μm (Polypropylene Net Filters, Merck Millipore Ltd) pore-sized filters, where larger coral tissues were retained on the filter while zooxanthellae passed through and remained in the filtrates. This process was repeated for two times to remove large coral tissues. Subsequently, the filtrates containing zooxanthellae and small coral tissues were passed through 12 μm (Nuclepor^{TM} Track-etch Membrane, Whatman^{TM}) filters, where small coral tissues were retained on the filter while zooxanthellae remained in the filtrates. This process was repeated for 2-3 times to eliminate small coral tissues. For centrifuge, the filtrates were concentrated with a hand-controlled centrifuge under a low speed at 1200 rpm for 10 seconds to avoid breaking the cell of algae. After the filtration and centrifuge, the integrity of algae was confirmed with a microscope (SMZ1270 Microscope, Digital Sight 1000 Camera, Nikon).

2.3 Isotopic analysis

The coral host collected and zooxanthellae purified were prepared for the analysis of stable isotope ratios ($\delta^{15}N$, ‰ relative to AIR) of amino acids according to Chikaraishi et al. (2009). In brief, the samples were hydrolyzed with 12 N HCl at 110°C overnight to break down proteins into individual amino acids. The hydrolysate was washed with n-hexane/dichloromethane (3/2, v/v) to remove hydrophobic compounds. Amino acids were then derivatized sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4). The $\delta^{15}N$ values of the *N*-pivaloyl/isopropyl (Pv/OiPr) esters of amino acids were determined with a gas chromatograph - isotope ratio mass spectrometer (GC-IRMS) using a 7890B GC

(Agilent Technologies) coupled to a DeltaV IRMS through combustion (950°C) and reduction (550°C) furnaces via a GC-C/TC III interface (Thermo Fisher Scientific) (e.g., Takizawa and Chikaraishi 2021). The derivatives of AAs were separated on an Agilent Ultra-2 capillary column (50 m length, 320 μm i.d., 0.5 μm film thickness). The carrier gas (He) flow rate was controlled with a constant flow mode at 1.4 mL/min. The $\delta^{15}N$ values were expressed relative to the isotope ratio of AIR, on scales normalized to known $\delta^{15}N$ values of the reference AAs (Indiana University; Shoko Science Co.). The 1σ standard deviation for the reference amino acid analysis was 0.4‰ – 0.7‰.

3. Results and discussions

In the present study, we found a significant reduction of coral tissues in the purified algae after the isolation under a microscope (Fig. 2-2-3). Also, reduction in the breakage of algal cells was observed when we use a mild speed centrifuge for the purification (Fig. 2-2-4(b)), compared to the use of a strong and long-time centrifuge for purification (Fig. 2-2-4(a)). These results from the observation demonstrate that the isolation method established in the present study is useful for purifying the intact algal symbionts from coral polyps. The usefulness of this method was also supported by isotope analysis. The $\delta^{15}N$ values of glutamic acid and phenylamine are (7.4‰) and (4.0‰), respectively, for zooxanthellae, which indicates the trophic identity of algal symbionts is primary producer (i.e., TP = 1.0). For bleached coral, the $\delta^{15}N$ values of glutamic acid (14.9‰) and phenylamine (4.1‰) corresponds to a TP of 2.0, reflecting its trophic identity as a primary consumer. On the other hand, for coral host,

the $\delta^{15}N$ values of glutamic acid and phenylamine are (13.9‰) and (4.2‰), respectively and has a TP of 1.8 (Fig. 2-2-5 (b)). According to the mass balance calculation from these TP values, we estimated that zoanthid holobionts are composed of 16% zooxanthellae and 84% for coral itself (Fig. 2-2-5 (c)). We thus expect that isotopically contribution of algal symbionts within Cnidaria holobionts is substantially small, which is consistent to the microscopic observations.

4. Conclusion

Because this isolation method is tested to be effective for clarifying the trophic function of algal symbionts and coral hosts, respectively in coral reef ecosystems. We thus think this method is applicable to evaluate the trophic interaction between algal symbionts and host species for not only corals but also other Cnidarian species such as giant clams, jellyfish, and anemones. We suggest that our understanding of trophic interactions within algae-eukaryote symbiosis can be advanced with a combination use of this isolation method with compound specific analysis of amino acids in the future.

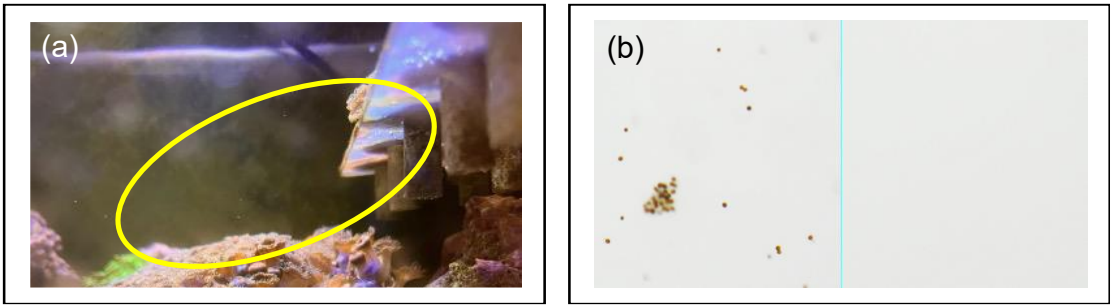

Fig. 2-2-1. (a) A photo of bleached zoanthids (*Zoanthus* sp. (Green)), with the yellow circle indicating the bleached area; (b) No algae were observed in *Zoanthus* sp. (Green) under the microscope after bleaching.

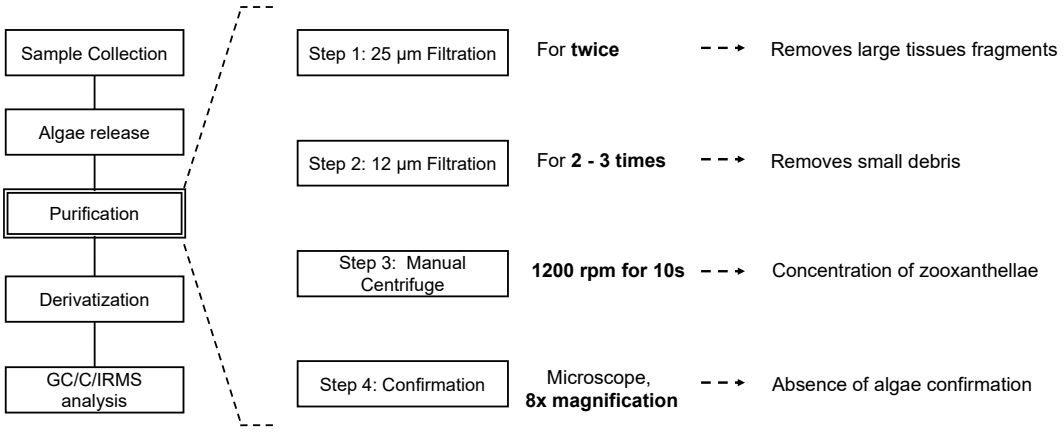

Fig. 2-2-2. Schematic pathway outlining the purification process of symbiotic algae from coral. tissues.

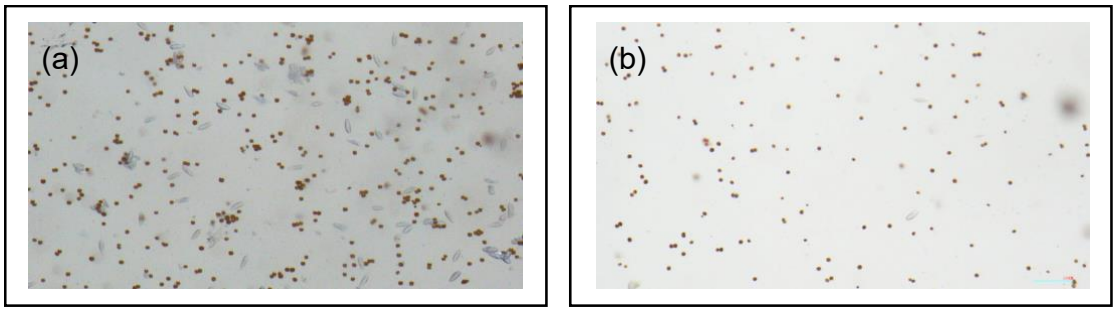

Fig. 2-2-3. (a) Mixture of coral tissues and symbiotic algae before isolation. (b) Significant reduction of coral tissues in purified algae after isolation. The colorless, transparent structures indicate coral tissues, while the yellow-brownish cells are symbiotic algae (zooxanthellae).

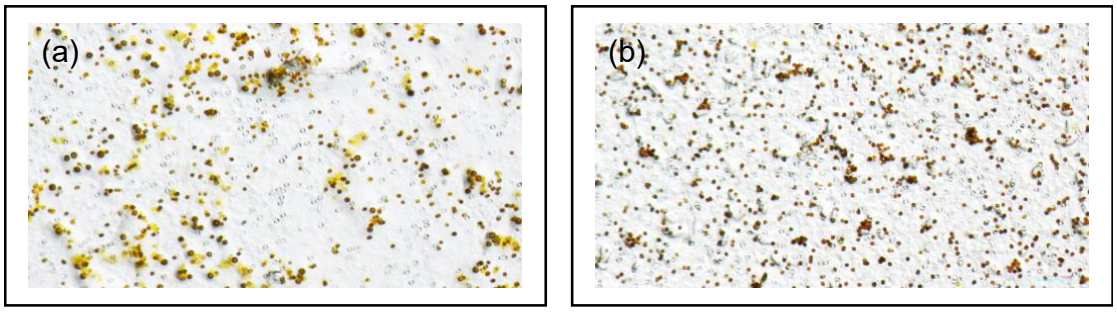

Fig. 2-2-4. (a) A large number of algal cells are broken with strong and long-time centrifuge. Yellow regions represent the cell contents leakage. (b) A small number of algal cells are broken, while the remaining cells are intact and undamaged with a weak centrifuge.

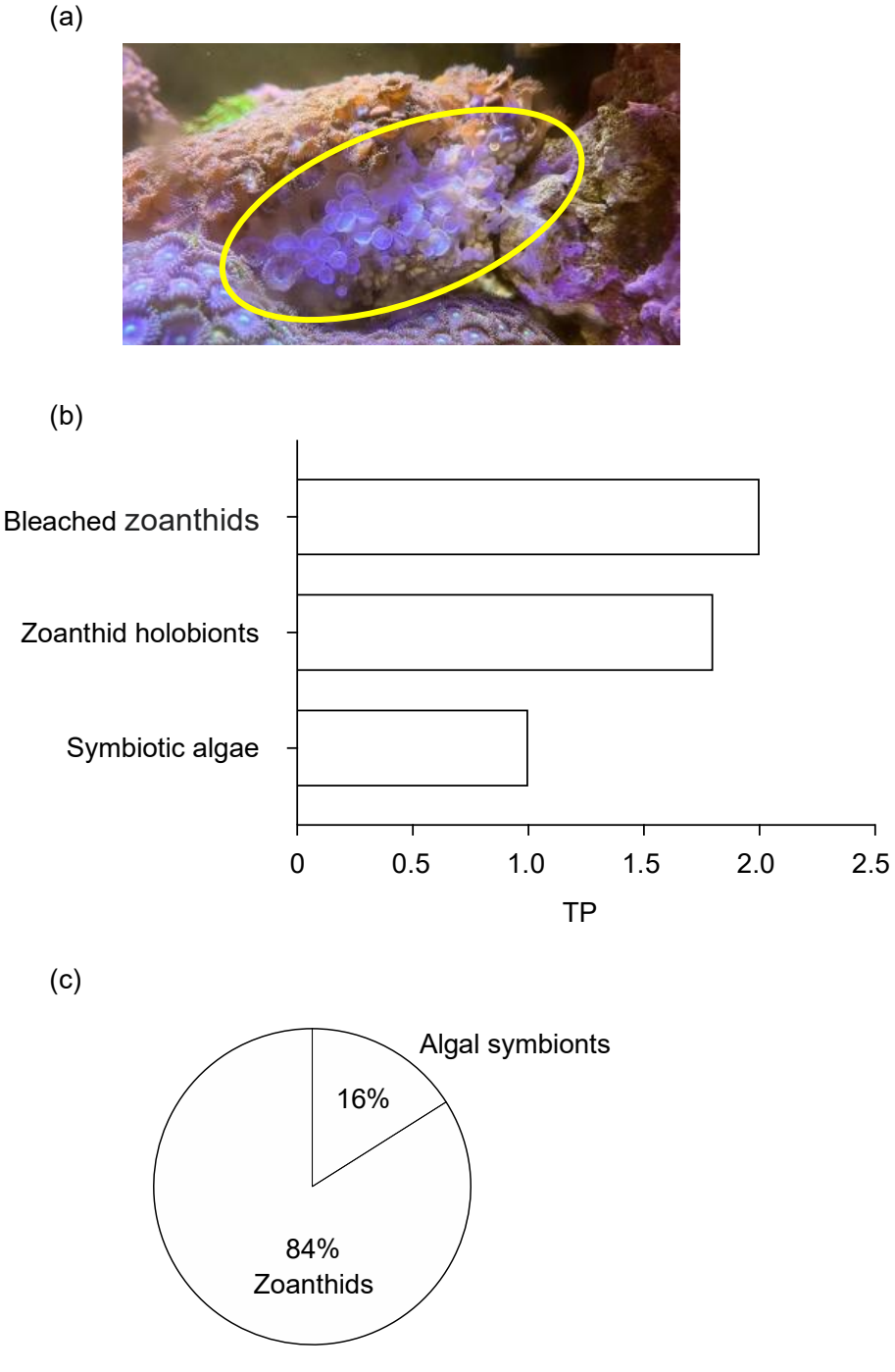

Fig. 2-2-5. (a) A photo of bleached zoanthids (*Zoanthus* sp. (Green)), (b) the comparison of TP of algal symbionts, zoanthid, and a bleached zoanthids, and (c) contribution of algal symbionts within zoanthid.

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CHAPTER III.

Mixotrophy-Like Characteristics in Cnidarian-Algal Symbiosis, as a Factor to Understand Nutrient Flow of Coral Reef Ecosystems

Abstract

A mixotroph is an organism that can obtain nutrients from multiple nutritional modes, such as autotrophy and heterotrophy. In coral reefs, many autotroph-heterotrophic symbiotic species, such as Cnidarian-algal symbiosis, exhibit mixotrophy-like characteristics, obtaining autotrophic photosynthates from zooxanthellae symbionts and heterotrophic food from eukaryotic hosts, and this allows them to adapt to different environments. However, the nutrient flux within Cnidarian-algal symbiosis remains poorly understood, particularly in (1) the balance between autotrophic and heterotrophic contributions to Cnidarian-algal symbiosis and (2) the potential incorporation of heterotrophically acquired nutrients into symbiotic algae. Both factors are essential for understanding energy flow in coral reef ecosystems. In the present study, we measured stable nitrogen isotope ratios of amino acids for several Cnidarian species that bred under a mixotrophic condition in a laboratory aquarium. Based on the isotope analysis, we quantified the relative contributions of autotrophy and heterotrophy as 65.4 ± 27.2% and 34.6 ± 27.2%, respectively. Furthermore, we found no direct transfer of heterotrophically derived nutrients from Cnidarian hosts to their symbiotic algae (i.e., 0%) in the Cnidarian-algal symbiosis investigated. These results highlight that while the heterotrophy plays an important role in Cnidarian nutrition, it does not (at least directly) support the nutritional requirements for algal symbionts. This finding suggests that the Cnidarian-algal symbiosis can incorporate allochthonous nutrients into food webs, and this incorporation may indirectly support the high productivity in coral reef ecosystems.

Overall, our findings provide insights into the adaptive strategies of Cnidarians, highlighting their ability to switch between autotrophy and heterotrophy in response to varying environmental conditions, such as fluctuations in light availability and nutrient levels.

Keywords

Nitrogen isotopic composition; amino acids; autotrophy; heterotrophy; coral; anemone; zooxanthellae; food web

1. Introduction

Mixotrophy, the ability to combine two metabolic modes: autotrophy and heterotrophy, within a single organism, is a highly flexible form of metabolism that allows organisms to thrive under various growth-limiting conditions (Ward and Follows, 2016). This strategy is widespread across diverse species, particularly among many dinoflagellates in marine environments (Cohen, 2022). Through mixotrophy, organisms can produce organic matter via photosynthesis from inorganic nutrients and light energy (i.e., autotrophy) while simultaneously acquiring nutrients by consuming organic prey (e.g., zooplankton) as nutrients (i.e., heterotrophy), particularly when photosynthesis is not feasible (Edwards, 2019). By combining these two different nutritional modes (i.e., autotrophy and heterotrophy), mixotrophs blur the traditional boundary between primary producers and consumers, allowing them to access

multiple energy and biomass sources and occupy multiple trophic levels within the food web (Mitra et al., 2014; Ward and Follows, 2016). Indeed, the nutritional strategy of mixotrophs may be particularly advantageous in nutrient-poor, oligotrophic waters, where increased ocean stratification due to global warming limits nutrient availability. Under such conditions, mixotrophs rely more on autotrophically derived nutrients to meet their metabolic demands. Conversely, in nutrient-rich, eutrophic waters, which are often caused by increased nutrient runoff from anthropogenic activities (i.e., agriculture and urbanization), mixotrophs can switch to or supplement their diets through heterotrophy, utilizing the increased availability of organic matter. Given these dynamic adaptations, we thus have common interests in how these mixotrophs adjust their trophic strategies across diverse ecosystems in marine environments.

Coral reefs are one of such ecosystems that host a wide variety of mixotrophs (e.g., dinoflagellate) and mixotroph-like organisms (e.g., stony and soft corals, foraminiferans, sponges, giant clams, and anemones). The latter organisms can acquire nutrients from both independent nutrition through autotrophically by the photosynthesis of symbiotic dinoflagellates (which are called zooxanthellae) and heterotrophically by the capture of foods (e.g., zooplankton and particulate organic matter) (Muscatine and Porter, 1977), which may be switching depending on environmental conditions (i.e., light availability and nutrient levels). For example, it can be simply expected that autotrophy is dominant in shallow waters with sufficient light but low nutrient availability, whereas heterotrophy is dominant in deep waters

with low light but high nutrient availability. Mixotrophs and mixotroph-like organisms thus have a flexibility in the nutrient and energy cycles, which may contribute the ecosystem resilience in the complex food web characteristics of coral reef ecosystems (Palardy et al., 2005; Ward and Follows, 2016; Houlbrèque and Ferrier-Pagès, 2009). For illustrating the energy flow of ecosystems where these mixotrophs and mixotroph-like organisms live, quantitative data on the flexibility in the nutrient and energy cycles are required, particularly for (1) the balance between autotrophy and heterotrophy in these organisms and (2) the incorporation of nutrients to symbiotic algae through heterotrophy. In a previous study, Houlbrèque and Ferrier-Pagès (2009) well demonstrated the balance between autotrophy and heterotrophy in tropical scleractinian corals, such that heterotrophy accounts for between 15 and 35% of daily metabolic requirements in unbleached corals but for up to 100% in bleached corals. However, it is expected that the balance between autotrophy and heterotrophy in corals varies based on species, environmental conditions, and food availability (Falkowski et al., 1984; Muscatine and Porter, 1977; Grottoli et al., 2004; Palardy et al., 2008; Houlbrèque and Ferrier-Pagès, 2009). Nevertheless, at this moment, little is available in the quantitative data enough to start illustrating the energy flow in coral reef ecosystems.

One of the useful tools to evaluate the relative contributions of autotrophy and heterotrophy in mixotrophs is the comparison of stable isotope ratios of nitrogen ($^{15}N/^{14}N$) of amino acids between two species within a trophic interaction (e.g., Goto et al. 2018) or, more simply, the comparison of the trophic position (TP, which is

sometimes called the energetic position of organisms in food webs, as TP = 1 for primary producer, TP = 2 for primary consumer, and TP = 3 for secondary consumer in the traditional knowledge) that are calculated from the isotope ratios of two types of amino acids (i.e., trophic and source) within a single species (e.g., Chikaraishi et al. 2007; McCarthy et al. 2007; Popp et al. 2007). For instance, the trophic interaction results in a variable enrichment in ^{15}N for AAs of organisms in food webs, such that glutamic acid (a trophic amino acid) has a large enrichment in ^{15}N whereas phenylalanine (a source amino acid) has a little enrichment in ^{15}N from diet to consumer species (e.g., the enrichment is approximately +8.0‰ and +0.4‰ as the $\delta^{15}N$ values, respectively) (e.g., Chikaraishi et al. 2009).

The size of enrichment in ^{15}N is primarily controlled with (1) the types of AAs (e.g., Chikaraishi et al. 2009) and (2) the flux of deamination (e.g., Takizawa et al. 2020) and its isotopic fractionation factor (α) specific for individual AAs (e.g., α = 0.9938 for glutamic acid) (e.g., Goto et al. 2018). Based on this knowledge, the isotope ratios of AAs allow us to assess the relative contribution of heterotrophy in mixotrophs. Based on these knowledge, this method provides insights into the structure of the food web pyramid (e.g., biomass size for each of trophic steps) in coral reef ecosystems. By enhancing our understanding of these processes, we can better appreciate the role of mixotrophs in coral reef ecosystems and their impact on nutrient and energy dynamics. In the present study, we determined the TP based on the isotope ratios ($\delta^{15}N$, ‰ relative to AIR) of glutamic acid and phenylalanine for six Cnidarian species including one Hexacorallia (two zoanthid, and four discosoma

specimens) and two Octocorallia (two star polyp, and one leather coral specimens) classes that were bred under both autotrophic and mixotrophic conditions in a laboratory aquarium. The TP was estimated with the following equations (1) and (2) (e.g., Chikaraishi et al. 2009) :

$$TP = [(\Delta\delta^{15}N - 3.4) / 7.6] + 1 \quad (1)$$

$$\Delta\delta^{15}N = \delta^{15}N_{Glu} - \delta^{15}N_{Phe} \quad (2)$$

Based on the estimated TP, we quantified the relative contribution of autotrophy (A) and heterotrophy (H) in mixotrophic Cnidarians. To achieve this, we applied a two-end-member mass balance calculation with the following equations:

$$TP_{\text{Mixotrophic condition}} = TP_{\text{Autotrophic condition}} \times A + 4.1 \times H \quad (3)$$

$$A + H = 100\% \quad (4)$$

where, $TP_{\text{Mixotrophic condition}}$ is the TP observed in Cnidarians under mixotrophic conditions; $TP_{\text{Autotrophic condition}}$ represents the TP of Cnidarian-algal symbiosis when nutrition is derived from symbiotic algae; A and H represent the relative contributions (%) of autotrophy and heterotrophy, respectively, and constrained by A + H = 100%. Notably, the TP of commercial coral food (i.e., containing zooplankton with body size ranging from 0.2 mm to 2.0 mm) is calculated as 3.1, according to the conventional definition of TP ($TP_{consumer} = TP_{diet} + 1$; Chikaraishi et al., 2009), if Cnidarians exclusively relying on this food source, the TP would reach 4.1. Therefore, this value was used as the reference for 100% heterotrophy in our model.

2. Methods/Experiments

2.1 Aquarium for breeding Cnidarian species

An aquarium used in the present study consists of two “breeding tank” (Fig 3-1-1) Cnidarians were growing in two breeding tanks under different two different nutrient conditions. One tank was set under autotrophic conditions, where Cnidarians rely solely on photosynthates produced by their symbiotic zooxanthellae. The second tank is maintained under mixotrophic conditions, where Cnidarians are fed commercial coral food containing zooplankton (0.2–2.0 mm body size), which means both algal-derived photosynthates and zooplankton are food sources for Cnidarian growth. To investigate the effects of different amount of zooplankton on Cnidarians, the feeding experiments are conducted in two stages: in the first stage (H1), Cnidarians are fed once per week for three months, while in the second stage (H2), the feeding rate is increased to three times per week for two months. Seawater is circulated between breeding tanks and filtrating tanks as explained in Chapter II. In brief, wastewater is overflowed and is fallen into the filtrating tank, while filtrated water is pumped up to the breeding tank. In the filtrating tank, waste (NH_3) in the water is oxidized to nitrate (NO_3^-) with ammonia-oxidizing bacteria and nitrate bacteria, and the nitrate produced and ammonia remaining are absorbed with chemical absorbents. In the breeding tank, the nitrate remaining is denitrified to dinitrogen gas (N_2) at the anoxic layer of sands. The seawater is thus kept clean (no detection of ammonia and nitrate) for the experiment term. The temperature of the seawater is set at 26ºC (±0.2ºC). The light was provided with two UV-LED lamps (Lightening Master 24 DC Sapphire blue, 20

W, B.R.S-Best Reef Systems) at 215 mm upper from water surface in the breeding tank. The lighting condition is set as an 8 h light: 16 h dark cycle in a day. Here, I would like to note that to keep seawater clean during feeding experiments, the water volume used for circulation in the filtrating tank is 5.6 times larger than in the previous systems (Li et al. 2025).

2.2 Cnidarian species

Two Hexacorallia including the zoanthid *Zoanthus* sp. (n = 2), and three Octocorallia including the star polyp *Pachyclavularia violacea* sp. (n = 2) and the leather corals *Sarcophyton* sp. (n = 1) were used in the present study (Fig 3-1-2). Notably, in this study, because TP estimation based on compound-specific isotope analysis of amino acids has high analytical precision, with measurement error of ± 0.1– 0.2 units (Chikaraishi et al., 2009; Nielsen et al., 2015), ensuring the reliability of single-sample estimates. Also, most coral colonies used were clonally propagated in the aquarium, meaning neighboring individuals were genetically the same due to asexual reproduction. For these reasons, we did not perform technical or biological replicates for individual Cnidarian samples, including symbiotic algae and Cnidarian hosts. The purification of symbiotic algae from Cnidarians follows the procedure described in Li et al. (2025).

2.3 Isotope analysis

The Cnidaria collected and symbiotic algae purified were prepared for the analysis of stable isotope ratios ($\delta^{15}N$, ‰ relative to AIR) of amino acids, following the

procedure described in the previous study (Chikaraishi et al. 2009). In brief, the samples were hydrolyzed with 12 N HCl at 110°C overnight (>12 h). The hydrolysate was washed with *n*-hexane/dichloromethane (3/2, v/v) to remove hydrophobic constituents. Derivatizations were then performed sequentially with thionyl chloride/2-propanol (1/4) and pivaloyl chloride/dichloromethane (1/4). The $\delta^{15}N$ values of the *N*-pivaloyl/isopropyl (Pv/OiPr) esters of amino acids were determined with a gas chromatograph - isotope ratio mass spectrometer (GC-IRMS) using a 7890B GC (Agilent Technologies) coupled to a DeltaV IRMS through combustion (950°C) and reduction (550°C) furnaces via a GC-C/TC III interface (Thermo Fisher Scientific) (e.g., Takizawa and Chikaraishi 2021). The derivatives of amino acids were separated on an Agilent Ultra-2 capillary column (50 m length, 320 μm i.d., 0.5 μm film thickness). The carrier gas (He) flow rate was controlled using a constant flow mode at 1.4 mL/min. The $\delta^{15}N$ values were expressed relative to the isotope ratio of AIR, on scales normalized to known $\delta^{15}N$ values of the reference amino acids (Indiana University; Shoko Science Co.). The 1σ standard deviation for the reference amino acid analysis was 0.4 – 0.7‰. The TP was estimated with the equations (1) and (2), and the energetic balance to biomass was estimated with the equations (3) and (4), with an assumption that the isotopically contribution of algal symbionts within Cnidaria is substantially small.

3. Results

3.1 Nitrogen isotope ratios and TP of Cnidarians and symbiotic algae

Differences in the $\delta^{15}N$ value between glutamic acid and phenylalanine ($\Delta\delta^{15}N$) correlate with the TP of organisms. Typically, $\Delta\delta^{15}N$ values of 3.4‰, 11.0‰, and 18.6‰ correspond to primary producers (TP = 1, photoautotrophs), primary consumers (TP = 2, herbivores), and secondary consumers (TP = 3), respectively, with a 7.6‰ elevation from diet to consumer species (e.g., Chikaraishi et al. 2009). In this study, after feeding Cnidarians with food (i.e., zooplankton), we observed significant variations in the $\Delta\delta^{15}N$ value (Fig 3-1-3). This change reflects the contribution of heterotrophy on Cnidarian growth. For example, the Zoas Green, star polyp long polyp (SP LP), and Toadstool leather coral (UK) show considerable elevation in the $\Delta\delta^{15}N$ value, ranging from 6.3‰ to 18.5‰, from 6.6‰ to 17.2‰, and from 6.4‰ to 17.6‰, respectively, after the second stage of heterotrophic feeding. These observed elevations indicate a strong reliance on heterotrophically derived nutrients in these species. Conversely, Zoas Red exhibit a slight change, with $\Delta\delta^{15}N$ value ranging from 10.9‰ to 13.6‰, suggesting a small reliance on heterotrophic feeding. Interestingly, the $\Delta\delta^{15}N$ values of star polyp metallic green (SP MG), ranged from 4.6‰ to 5.9‰, closely matching the trophic identity of the symbiotic algae, which consistently has the TP of 1.0, implying a stronger reliance on autotrophy. Thus, with the exception of star polyp metallic green (SP MG), heterotrophic feeding significantly influenced the nitrogen assimilation and trophic identity of Cnidarians, and such influence can be attributed to using distinct nutritional strategies among different Cnidarian species.

However, under the influence of heterotrophy, Cnidarians exhibit diverse responses to different food availability. During the first stage of the feeding experiment (H1; feeding once per week), the $\Delta\delta^{15}N$ values of Cnidarians range from 3.7‰ to 21.8‰, corresponding to TPs of 1.0 to 3.4. In the second stage of the feeding experiment (H2; feeding three times per week), the $\Delta\delta^{15}N$ values of Cnidarians range from 2.7‰ to 18.5‰, corresponding to TPs of 0.9 to 3.0. To test whether increased feeding frequency led to a significant difference in TP, we performed an unpaired t-test between the five Cnidarian samples under H1 (one feeding/week) and H2 (three feedings/week), and each Cnidarian sample has only one individual specimen. No substantial difference in the TP between H1 (TP = 2.3) and H2 (TP = 2.5) is observed (t-test: $t = 0.2267$, $df = 8$, $p > 0.1$) (Fig 3-1-4). This suggests that higher heterotrophic feeding rates do not lead to a substantial increase in TP.

Although there was no substantial difference in mean TP between different feeding rates, Cnidarians show both inter- and intra-specific diversities in the assimilation balance between autotrophic and heterotrophic nutrition, as well as in their response to increased feeding rates (Fig 3-1-5). For instance, the TP of Zoanthids (green) increases significantly from 2.0 to 3.0 with the increase of feeding rates from one-time feeding to three-time feeding, whereas Zoanthids (Red) slightly increases from 2.2 to 2.3, suggesting a different degree of reliance on heterotrophy within Zoanthids species. Interestingly, some cnidarians exhibited a decrease in TP with increased feeding rates. For instance, the TP of star polyp (*Pachyclavularia* sp., long polyp) declined from 3.4 to 2.8, while the TP of leather coral (*Sarcophyton* sp.) decreased

from 3.1 to 2.9 as the feeding rates increased.

3.2 Assimilation balance between autotrophy and heterotrophy in Cnidarians

Based on the relative contribution of autotrophy and heterotrophy estimated with equations (3) and (4), the relative contribution of heterotrophy to Cnidarians in a mixotrophic condition ranges from 1% to 61%, with an average of 34.6% ± 27.2 (Fig 3-1-6). Notably, the estimations in this study were under the assumption that Cnidarians 100% assimilate the nitrogen from their food sources, which means that the TP used to represent "pure heterotrophy" reflects a maximum value. In reality, assimilation efficiency is often lower, so the actual heterotrophic contribution may change to some extent.

Although the exact ratio of contribution between autotrophy and heterotrophy varies, our results indicate that the nutrients provided by zooxanthellae through autotrophy are generally dominant for Cnidarians. Also, while our study focused on investigating the trophic interaction between Cnidarian hosts and algal symbionts based on $\delta^{15}N$ values of amino acids, it did not include other potential nitrogen and phosphorus sources such as nitrate, ammonia, urea, or phosphate. These nutrients are thought to be primarily derived from surrounding seawater, particularly in inorganic forms (D'Elia et al., 1983; Yellowlees et al., 2008), further research is thus needed to illustrate these pathways within Cnidarian-algal symbiosis under oligotrophic conditions. Moreover, while Cnidarians can utilize heterotrophic food, it is not a major resource for their growth. These findings imply that the additional assimilation

of nutrients from heterotrophy by Cnidarians is another critical factor to explain the high productivity of coral reefs, even in oligotrophic oceans.

4. Discussions

4.1 Food sources to Cnidarians affected by environmental conditions

In this study, although no substantial difference in the TP of Cnidarians between different feeding rates was observed, we confirmed that there are species-specific variations among Cnidarians, even within the same species. This reflects the flexible nutritional strategies among Cnidarians (see Section 3.1). For example, some species such as Zoas Green and Zoas Red exhibited remarkable increases in TP under higher feeding frequency, while other Cnidarian specimens like SP MG showed consistently low TP values, suggesting a stronger reliance on autotrophy. These inter- and intra-specific differences highlight that the assimilation balance between autotrophic and heterotrophic nutrients is distinct across Cnidarians. The assimilation balance between autotrophic and heterotrophic nutrients to Cnidarians is influenced by multiple environmental and physiological factors. Although there was no substantial difference observed in the TP of Cnidarians between different feeding rates under laboratory-controlled condition, food sources potentially remain the most crucial factors affecting assimilation balance in Cnidarians in the wild environments. Such food sources vary greatly in natural coral reefs, depending on environmental conditions such as nutrient levels, turbidity, and water flow in a reef region. More generally explaining, the waters with high nutrient levels promote zooplankton growth,

providing more food to Cnidarians for heterotrophic feeding, whereas low nutrient conditions make Cnidarians rely more on autotrophy (Houlbrèque & Ferrier-Pagès, 2009). Also, some reef regions are near river mouths and experienced high sedimentation, where suspended particles (e.g., sand, slit, and organic matter) accumulated river runoff (Fabricuou, 2005). Such sedimentation may increase water turbidity and reduce light penetration through the water column, impairing the photosynthesis of zooxanthellae and necessitating a greater reliance on heterotrophy for Cnidarians (Anthony and Fabricius, 2000). Additionally, the reef regions with lower water flow may limit food availability to Cnidarians, while higher flow may increase difficulty for them to capture their prey (Sebens and Johnson, 1991). Importantly, it is thought that heterotrophy is more energetically costly than autotrophy because it requires energy for foraging, capturing, and digesting the prey. In contrast, autotrophy allows for the direct uptake of photosynthates from symbionts to Cnidarians with minimal energy cost. Thus, the relative contribution of autotrophy and heterotrophy in Cnidarians is highly dynamic, influenced by both environmental conditions and energy budget within Cnidarian-algal symbiosis.

4.2 Influence of Cnidarian morphology on nutrient assimilation

In addition to food availability, the morphology of Cnidarians, which enables them to adopt a range of trophic strategies, particularly in heterotrophic feeding, may also play a significant role in nutrient assimilation. This is primarily because the shape and structure of each Cnidarian species is thought to affect both prey capture efficiency and the types of prey they consume (Palardy et al., 2005). In this study, Cnidarian

species with different morphologies exhibited varying trophic responses to feeding. For example, Zoas Green and Zoas Red (Toadstool leather coral), which possess large, fleshy polyps with well-developed tentacles, showed considerable increases in TP after feeding, suggesting effective heterotrophic nutrient acquisition. In contrast, star poly metallic green (SP MG) maintained a low TP regardless of feeding frequency, which indicates a limited capacity for prey capture, potentially linked to morphological constraints. Such species-specific differences may be attributed to their distinct physical forms. For instance, branching corals (e.g., Acropora spp.) have a large surface area relative to their volume, allowing for a greater number of polyps and tentacles to capture prey. Also, the complex structure of these corals can enhance water flow through the branches, bringing more food into contact with the tentacles. Plate and table corals (e.g., Montipora spp., Acropora spp.) have large, horizontal surfaces that intercept plankton and allow them to settle, facilitating heterotrophic feeding. This plate morphology not only facilitates heterotrophic feeding by capturing prey but also maximizes light capture for the autotrophy of zooxanthellae, thereby complementing heterotrophic nutrition in Cnidarians (Houlbrèque & Ferrier-Pagès, 2009). Other Cnidarians, such as free-living corals (*Fungia* spp. and *Scolymia* spp.), possess well-developed polyps with extensive tentacles for prey capture. Unlike most other Cnidarians, which are almost motionless, these organisms can move across the substrate, positioning themselves in optimal locations for prey capture. These morphological adaptations allow Cnidarians to obtain heterotrophic nutrients effectively with various trophic strategies and to maintain a flexible balance between

autotrophy and heterotrophy. Such adaptations likely contribute to their ability to thrive in diverse marine environments (Palardy et al., 2005).

4.3 Contribution of heterotrophy to symbiotic algae zooxanthellae

Traditionally, it has been widely believed that zooxanthellae live symbiotically with Cnidarian hosts, providing hosts with photosynthetic products such as glucose, glycerol, and amino acids, which serve as nutrient sources for Cnidarian growth and metabolism (Muscatine and Porter, 1977). In return, Cnidarians supply metabolic products, including ammonia (NH_3), carbon dioxide (CO_2), which zooxanthellae require for their metabolism (i.e., ammonia for growth; carbon dioxide and water for photosynthesis) (Wang and Douglas, 1999). However, some studies reported that zooxanthellae may obtain additional organic nutrients from Cnidarian hosts, potentially leading to a TP exceeding 1.0, which indicates a partial reliance on heterotrophic-derived food (Fox et al., 2019). In addition, some studies have identified the gene clusters in zooxanthellae associated with the uptake and metabolism of dissolved organic compounds, including amino acids and small peptides (Shoguchi et al., 2013), implying that zooxanthellae might have the genetic capacity to assimilate host-derived organic matter. However, our study consistently observed that the TP of zooxanthellae is always close to 1, confirming zooxanthellae primarily rely on metabolic byproducts from their hosts rather than directly assimilating zooplankton-derived protein. This can be simply explained by the fact that although there are related genes, it does not mean they are actively transcribed and translocated into functional proteins in an organism's genome under normal

conditions. Such genes are only expressed under specific environmental stressors, such as symbiosis breakdown or extreme nutrient limitation (Shoguchi et al., 2013). In the present study, heterotrophic feeding by Cnidarians primarily serves to meet the nutritional demands of the host itself, rather than being transferred to the symbionts. This finding supports the idea that heterotrophy in Cnidarians does not significantly contribute to symbiont nutrition but rather sustains host metabolism and growth (Houlbrèque & Ferrier-Pagès, 2009; Fox et al., 2019).

5. Implication

5.1 Contribution of this study to advance coral reef ecosystems

Over the past few decades, numerous studies have focused on understanding the molecular and cellular processes that facilitate Cnidarian-algal symbiosis. For example, a molecule called LePin was found in Cnidarian-algal symbiosis, which enables the uptake of symbiotic algae by Cnidarians from the ambient environments, thus promoting the formation of the symbiotic partnership (Hu et al., 2023). However, obtaining a clear understanding of the interaction between the environment and this symbiosis remains challenging due to the complex cellular processes and metabolic exchanges involved within symbiosis under different environments. If this is the case, stable isotope analysis can be one potential tool for evaluating these metabolic exchanges in the trophic interactions of coral reef food webs. By analyzing the stable isotope ratios in amino acids, we can trace the flow of nutrients between the symbiotic partners and gain insights into the energetic balance to biomass and the metabolic

functions within the symbiosis (Ohkouchi et al., 2017). This method allows for the differentiation of nitrogen sources utilized by both Cnidarian hosts and algal symbionts, illustrating their metabolic interactions. Additionally, CSIA provides a deeper understanding of trophic dynamics between consumers and prey based on the fractionation of nitrogen isotopes in different amino acids that can reveal patterns of assimilation and energetic balance to biomass (Chikaraishi et al., 2007). Thus, integrating molecular biology with stable isotope analysis can significantly enhance our understanding of the biology and metabolic functioning of this symbiosis. In the present study, stable isotope analysis of amino acids was applied for the first time to investigate the metabolic functions such as the energetic balance to biomass from symbiont-derived photosynthates to Cnidarians, within Cnidarian-algal symbiosis. Also, the breeding technique is significantly improved in the present study by using well-filtrated aquariums, which allows for the maintenance of Cnidarians in controlled conditions for several months to years. This breeding technique addressed the issue of short maintenance periods reported in the previous studies and ensured a stable and healthy environment for the Cnidarians, which is crucial for long-term research and the accurate quantification of metabolic functions and symbiotic relationships.

5.2 Comparison between natural and lab samples

In the present study, Cnidarian samples are generally bred in laboratory aquariums. Although using both natural and laboratory samples could provide a comprehensive understanding of the Cnidarian-algal symbiosis under varying conditions, obtaining

natural Cnidarian samples for research purposes has many challenges. For instance, many coral reefs are located within marine protected areas where sampling is strictly regulated to preserve the biodiversity of coral reefs and ecosystem health, and thus requires permits for sampling. Additionally, once samples are collected, they must be carefully preserved and transported to maintain a stable physiological condition. Previous studies have reported the TPs of natural Cnidarians range from 0.8 to 1.4, using limited Cnidarian species (Fox et al., 2019). In contrast, the present study indicates that the TPs of laboratory Cnidarians across several species range from 1.0 to 3.4. This large variation suggests a possible overestimation of TPs in the present study, which may be related to the fact that laboratory conditions cannot fully replicate the complex and dynamic nature of natural coral reef environments. In natural reefs, factors such as water movement, light availability, and interactions with other marine organisms, including inter- and intra-species competition, particularly in diet availability, makes it difficult to mimic accurately in laboratories (Houlbrèque & Ferrier-Pagès, 2009). For instance, in this study, Cnidarians were provided with a controlled diet to meet their basic nutritional requirements, which consists of dead plankton with body sizes ranging from 0.2 mm to 2.0 mm. While this feeding ensures a stable and consistent food supply, it lacks the influence from natural variability on food availability observed in nature. Also, laboratory conditions eliminate competition between species for prey, which can impact feeding rates and trophic dynamics in wild Cnidarians. Therefore, future research should integrate both field studies with laboratory research to provide a more comprehensive understanding of Cnidarian

biology and ecology, ensuring the results are relevant to the wild environments and can better inform the conservation efforts for coral reef ecosystems.

5.3 Correlation between calcification and assimilation balance

Although we focused on non-calcifying Cnidarians and did not consider the role of calcification in the present study, it is important to consider that, in reef-building corals, the calcification process may also play a critical role in shaping the energy allocation between autotrophy and heterotrophy. Calcification is the process by which Cnidarians, particularly reef-building corals, deposit calcium carbonate ($CaCO_3$) to form their skeletons. In coral reefs, the precipitation of $CaCO_3$ by Cnidarian hosts and the photosynthetic fixation of carbon dioxide by algal symbionts are thought to be tightly linked, both spatially (from the zooxanthellae cell to the coral reef ecosystem) and temporally (from day to night) (Allemand et al., 2011).

For example, some studies indicate that the calcification rate is, on average, three times higher in light than in darkness (Gattuso et al., 1999). This can be explained by that during photosynthesis, zooxanthellae reduce CO_2 levels within Cnidarian tissues, thereby increasing pH and CO_3^{2-} concentrations. This high availability of CO_3^{2-} promotes the $CaCO_3$ precipitation, enhancing the calcification process. It is thus thought that photosynthesis has a positive correlation with calcification. However, although calcification benefits from photosynthesis, for calcification process, it also demands significant energy and resources (e.g., ATP, nutrients) to supply and transfer the inorganic carbon and calcium ions to calcification sites. This high demand of

energy and resources for calcification can limit the availability of these nutrients for other metabolic processes, including those supporting photosynthesis and the growth of zooxanthellae, and thus in turn affect the photosynthetic efficiency of zooxanthellae. As a result, heterotrophy may serve as an additional resource to compensate for this energy demand (Houlbrèque & Ferrier-Pagès, 2009).

Thus, while our current results emphasize the autotrophy–heterotrophy balance in non-calcifying Cnidarians, we should carefully consider future investigations into how calcification might further affect this balance in reef-building corals. Understanding these metabolic interactions relationship (e.g., photosynthesis, calcification, and heterotrophic feeding) could be essential for evaluating energy budgets in Cnidarian-algal symbiosis under changing environmental conditions.

6. Conclusions

The relative contribution of heterotrophy to Cnidarians is 34.6% ± 27.2 based on the compound-specific isotope analysis of amino acids for several species, including zoanthids (n = 1), and soft corals (n = 4), that were bred under mixotrophic conditions in a laboratory aquarium. These results imply that the additional assimilation of nutrients from heterotrophy by Cnidarians is another significant factor contributing to the high productivity of coral reefs, even in oligotrophic oceans. This finding highlight the trophic flexibility of Cnidarians, which allows them to maintain their metabolic needs through both autotrophic and heterotrophic pathways and thus adapt to oligotrophic oceans.

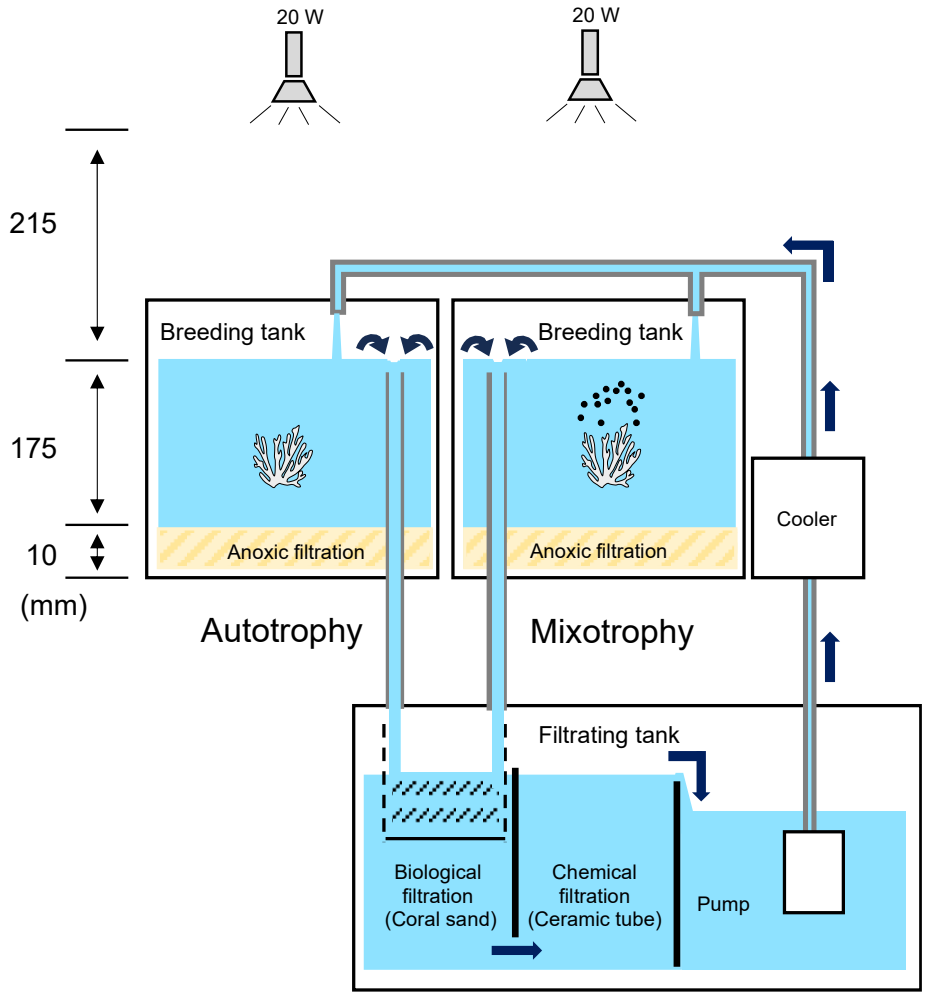

Fig. 3-1-1. The aquarium used in the present study.

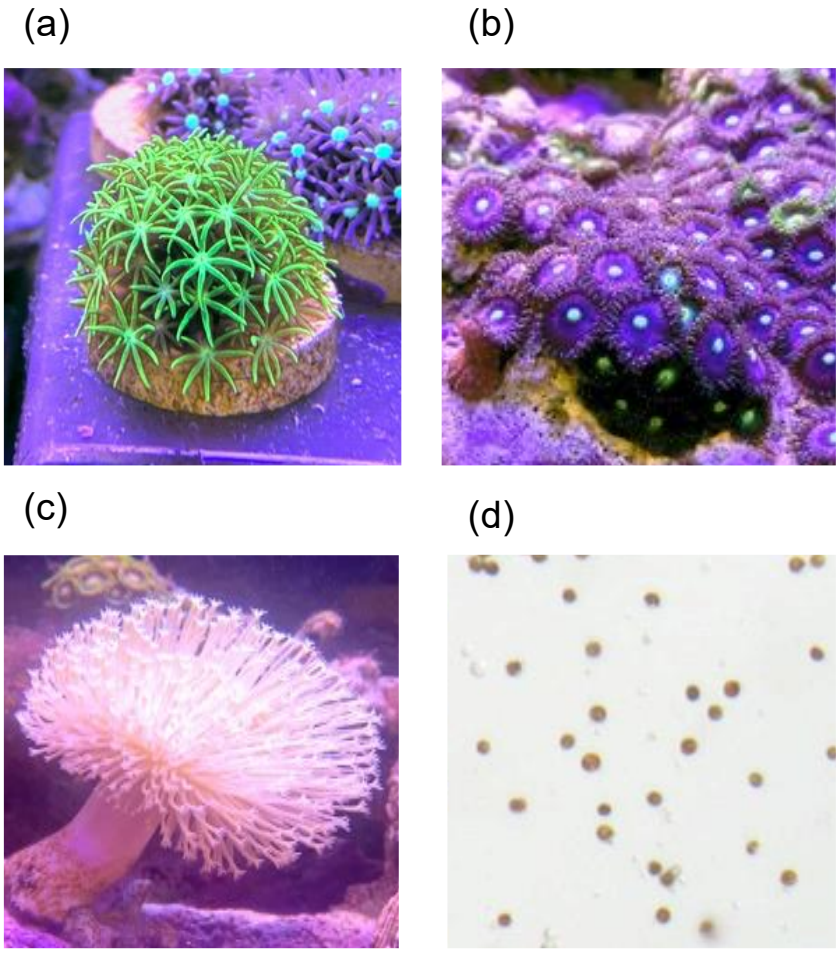

Fig. 3-1-2. Photos of representative Cnidaria specimens and algal symbionts purified from Cnidaria: (a) *Pachyclavularia violacea* sp. (b) *Zoanthus* sp. (c) *Sarcophyton* sp., and (d) algal symbionts.

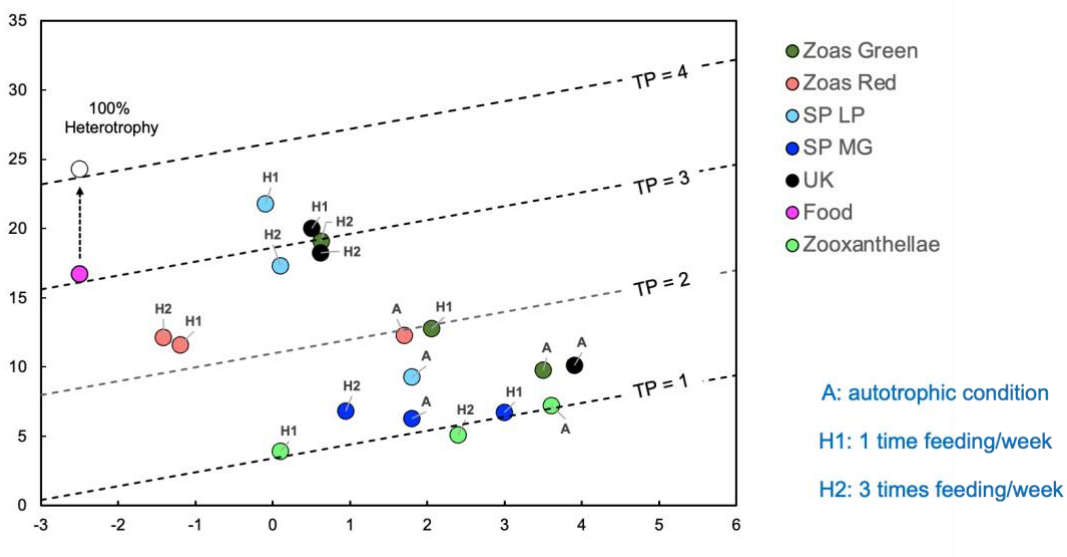

Fig. 3-1-3. Cross-plot of the $\delta^{15}N$ values of amino acids of Cnidarians from autotrophic condition and two mixotrophic conditions. Lines represent integer trophic positions (TP) from the primary producers to the tertiary consumers. The blue circle indicates the commercial coral food used in the feeding experiment has TP as 3.1, and the white circle represents the TP of Cnidarians if fed exclusively on this coral food (no reliance on algal-derived photosynthate), may have TP of 4.1. This value is used as the reference for 100% heterotrophy in our study. A is autotrophic condition (symbiont-derived photosynthate as the sole resource); H1 is mixotrophic condition with one-time feeding per week; and H2 is mixotrophic condition with three times feeding per week.

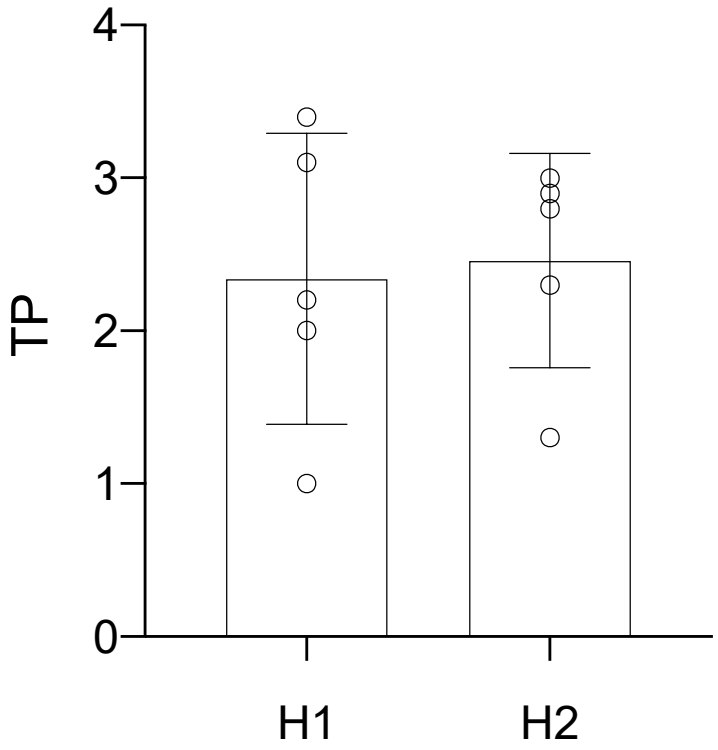

Fig. 3-1-4. Box plot of the TP of Cnidarians in first stage (H1) and second stage (H2).

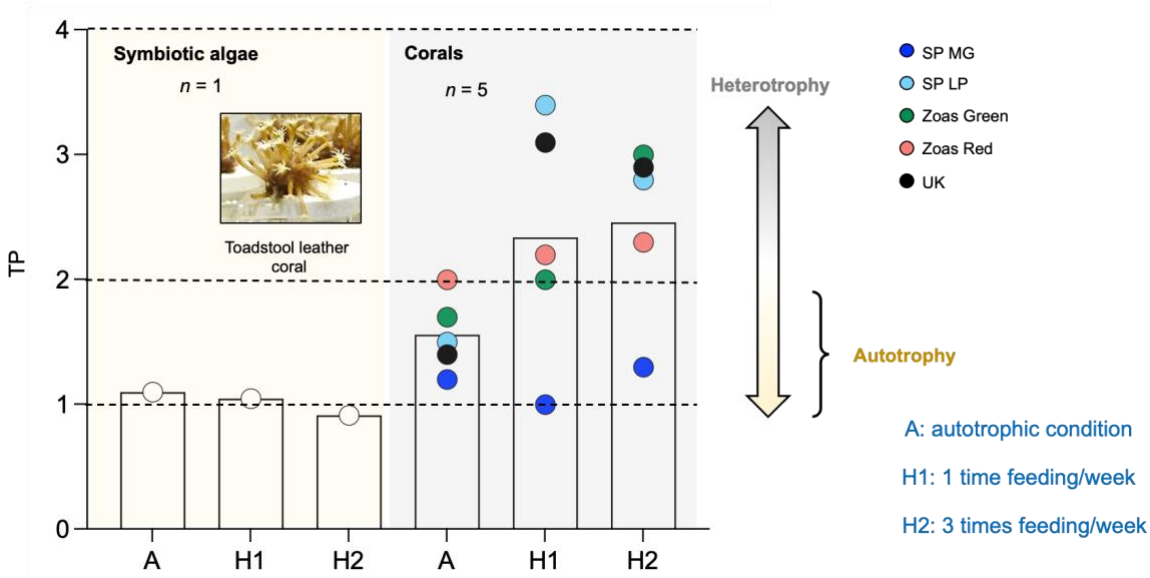

Fig. 3-1-5. Effect of heterotrophy on symbiotic algae and Cnidarians. A is autotrophic condition (symbiont-derived photosynthate as the sole resource); H1 is mixotrophic condition with one-time feeding per week; and H2 is mixotrophic condition with three times feeding per week. Arrow indicates the degree of autotrophy versus heterotrophy in the samples. Higher TP values suggest greater reliance on heterotrophy while lower TP values suggests a greater reliance on autotrophy.

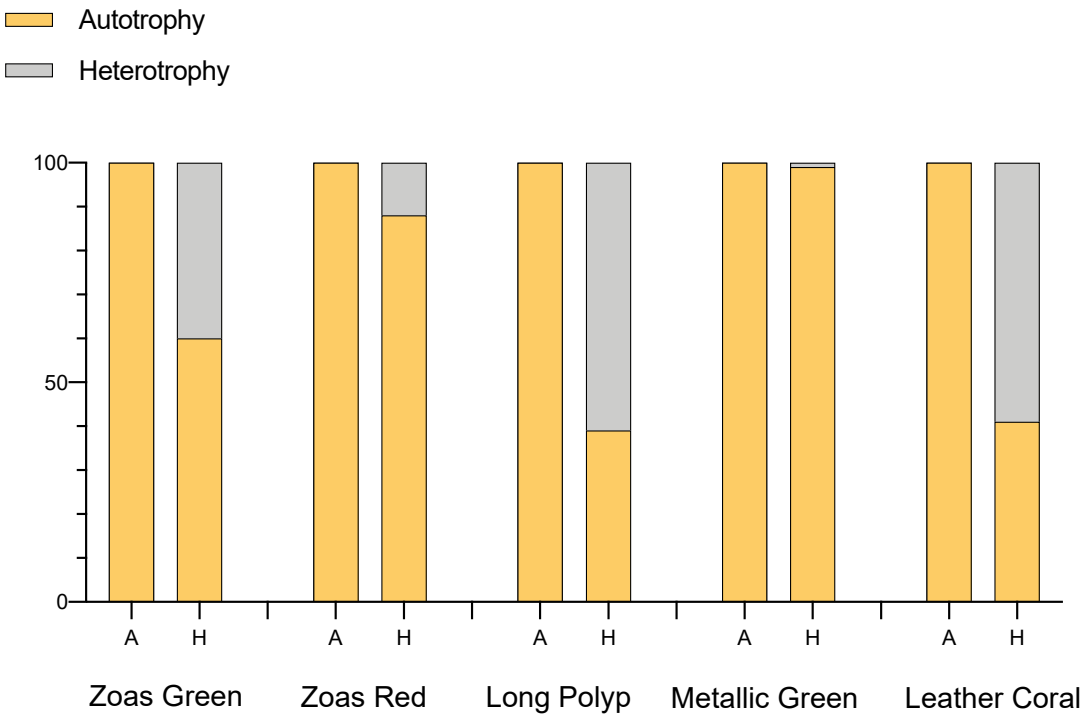

Fig. 3-1-6. Relative contribution of autotrophy and heterotrophy to Cnidarians under mixotrophic condition.

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CHAPTER IV.

Implications

1. Efficiency of trophic transfer (E_{troph}) in coral reef ecosystems

1.1 Definition and components of E_{troph}

The trophic transfer efficiency (E_{troph}) is commonly defined as the proportion of energy or biomass transferred from one trophic level to the next in a food web (e.g., Eddy et al. 2020). It reflects how effectively the energy flows between trophic levels and ultimately how much of energy is transferred to higher trophic levels for supporting top predators in the food web. However, such trophic transfer is not always 100% efficient from one trophic level to the next. For instance, when an animal grazes on other animals, only some of the consumed food is digested and assimilated, and then such assimilated part is converted into production parts (e.g., used for growth and reproduction) in animal bodies (Lindeman, 1942). Thus, only a relatively small fraction (generally <1–25%) of the food energy at one trophic level can be transferred to the next trophic level in a food chain (Eddy et al., 2021). Based on this concept, E_{troph} is broken down into three major components: the efficiencies of consumption (CE; which represents the fraction of available energy ingested by the organisms), assimilation (AE; which represents the fraction of ingested energy that is assimilated into the organism's body), and production (PE; which means the fraction of assimilated energy produced for growth and reproduction), respectively. These components affect the efficiency of trophic transfer (E_{troph}) together and such relationship is expressed with the formula (Chapin, 2011): $E_{troph} = E_{CE} \times E_{AE} \times E_{PE}$.

1.2 E_{troph} in Cnidarian-algal symbiosis

As describe in Chapter II, we measured the energetic balance between degradation and biomass growth (which may be directly related to PE), which is a major factor of the efficiency of trophic transfer. This measure confirmed the high productivity of coral reef ecosystems can be partly explained by a highly effective trophic transfer from symbiotic algae to corals, and such high trophic transfer within many eukaryotes-algae symbiosis may be propagated to the higher trophic levels and thus promotes the production in coral reefs (Muscatine and Porter, 1977). However, our understanding on high trophic transfer within symbiosis is still limited because mot only PE, but also the other factors, such as the CE and AE, must also be taken into account for determining E_{troph} (Ishikawa, 2018).

1.3 Potential factors affecting E_{troph}

E_{troph} is thought to be influenced by both biotic and abiotic factors, and such factors significantly affect the energy flow in food webs. The biotic factors include the trophic interactions among organisms from different species or the same species regarding the food availability and consumption (Odum and Odum, 1955). On the other hand, abiotic factors, such as temperature, can influence metabolic rates, with warmer conditions typically making organisms increase their energy demands and thus enhance nutrient cycling in food webs (Brown et al., 2004). Also, nutrient availability in a certain reef region affects the assimilation balance between autotrophic and heterotrophic nutrition; for instance, as discussed in Chapter II, tight nutrient recycling within Cnidarian-algal

symbiosis enhances trophic transfer efficiency in food webs, in oligotrophic environments. However, environmental stressors such as coral bleaching, pollution, and ocean acidification can disrupt such symbiotic interactions, destroying the metabolic functions of both hosts and symbionts and thus potentially decreasing E_{troph} in food webs (Muscatine and Porter, 1977). Based on such knowledge, we can see that quantifying E_{troph} in coral reef ecosystems remains challenging because of the complexity of trophic interactions, variability in environmental conditions, and difficulties in measuring direct energy flows between symbiotic partners (Ward and Follows, 2016). However, for predicting how coral reef ecosystems respond to environmental changes and for developing effective conservation strategies, it is necessary to understand such dynamics in coral reefs (Spalding et al., 2017).

1.4 Challenges in measuring E_{troph} and current approaches *for* E_{troph} measurement

In a typical food web, it is difficult to measure E_{troph} under the estimation of all three E_{troph} components (CE, AE, and PE), because these components are highly variable not only within individual organisms but also in relation to their trophic interactions in food webs. Given these difficulties, researchers often estimate E_{troph} indirectly by analyzing TP and production rates at different trophic levels (Pauly and Christensen, 1995). Traditional methods for estimating E_{troph} rely on dietary and biomass-based analysis, and each has its strengths and limitations. For instance, diet analysis, including gut content studies and stable nitrogen isotope analysis, offers us direct evidence of diet composition but only records a snapshot of diets information

because it only reflects recent feeding events and does not reflect differential digestion rates among prey. On the other hand, biomass-based production estimates, such as the Production-to-Biomass (P/B) Ratio Method and Biomass Size Spectra Method, provide indirect assessments of E_{troph} by relating the production or biomass in two successive trophic levels (Edwards, 2019). The P/B ratio method estimates E_{troph} by measuring organismal growth and mortality rates relative to biomass, making it useful for predicting the dynamic of some organisms with high turnover rates (Kinsey, 1985). Meanwhile, the Biomass Size Spectra Method analyzes biomass distribution of organisms across different size classes, assuming that E_{troph} follows predictable patterns based on body size and trophic structure (Brown et al., 2004). Although these methods provide insights into E_{troph}, they are often constrained by data availability and the need for ecosystem-specific calibrations during data analysis (Flynn et al., 2013). Thus, to increase data reliability and reduce high field work, so far, many researchers use model-based approaches to integrate information of TP and production data, simulate energy flow, and quantify E_{troph}. However, although these models provide a more integrative understanding of trophic transfer processes, their accuracy highly depends on the quality of input data and assumptions about trophic structures of certain food webs (Eddy et al., 2021). Thus, despite these advances, estimating E_{troph} still remains challenging.

1.5 Challenges in measuring E_{troph} in coral reefs

It is known that coral reefs are among the most productive ecosystems, with high nutrient recycling efficiency, microbial activity, and symbiotic interactions driving

their productivity (Hatcher, 1990). However, assessing E_{troph} in coral reefs presents unique challenges, especially in Cnidarian-algal symbiosis, where zooxanthellae live within coral cells, making it difficult to quantify energy flux between partners (Falkowski et al., 1984). In this study, PE was estimated based on nitrogen isotope fractionation in glutamic acid, as TDF is closely linked to PE (Ishikawa, 2018). However, CE and AE remain difficult to measure due to the lack of a distinct ingestion process, the high rates of nitrogen recycling within symbiosis, and the absence of clear fecal loss. Notably, recently, some scientists have thought AE is generally determined by food digestibility and microbial processing, both of which involve enzymatic reactions that may influence TDF (McCarthy et al., 2007). However, the relationship between AE and TDF remains unclear due to the complexity of nitrogen recycling and microbial activity in symbiosis. In this study, lower TDF values between glutamic acid and phenylalanine in five coral species under autotrophic conditions (ranging from -0.8‰ to 6.3‰, mean 3.8 ± 2.8‰) were observed, whereas higher TDF values were recorded under heterotrophic conditions. These results suggest that TDF may serve as a potential but imperfect proxy for AE. Future research integrating controlled feeding experiments and isotope mixing models may help clarify this relationship and thus determine whether or not TDF can estimate AE in coral-algal symbiosis.

2. Applying energetic balance of degradation to biomass (*F*) to estimate primary production in coral reefs

Coral reefs are known for their remarkably high primary productivity although they are generally found in oligotrophic waters. Some studies have reported that coral reefs exhibit net primary productivity levels, which is comparable to tropical rainforests, and measured in the scale grams of carbon per square meter per year. However, it is important to distinguish net primary productivity from gross primary productivity, because the reported values in such studies do not clarify whether they represent only primary producers (e.g., symbiotic algae, macroalgae, phytoplankton) or the entire reef community. Historically, primary productivity in coral reefs has been estimated using oxygen flux measurements, laboratory experiments, and ecosystem modeling, all of which have its technique limitations and may limit the accuracy of estimates. Currently, no effective method provides a direct and reliable measurement of primary production or primary productivity in coral reefs. Although this is not directly related to the main topic of my study, I propose a potential method for estimating primary production in coral reefs using the high *F* values estimated in Chapter II. It is known that primary production at a given trophic level can be estimated if we know the production of a higher trophic level and the *F* between them. The reason for why we cannot estimate the primary production in coral reefs is primarily because we have no idea about the *F* from primary producers to primary consumers in coral reef food webs. Based on my findings, if we quantify the biomass and TP of top predators (e.g., sharks) and combine it with known *F* (28% at most

trophic levels and 56% from symbiotic algae to corals), we can estimate production at each TL, from sharks to herbivorous fish, corals, and ultimately to primary producers (symbiotic algae, zooxanthellae). Although this estimation assumes a simplified trophic structure with constant transfer efficiency, we acknowledge that omnivory and some trophic generalists in reef ecosystems may introduce uncertainty into such models (e.g., Trebilco et al., 2013). Nonetheless, this approach provides a useful first-order approximation of energy flow across trophic levels.

3. Potential nitrogen sources for Cnidarian-algal symbiosis in the laboratory aquarium

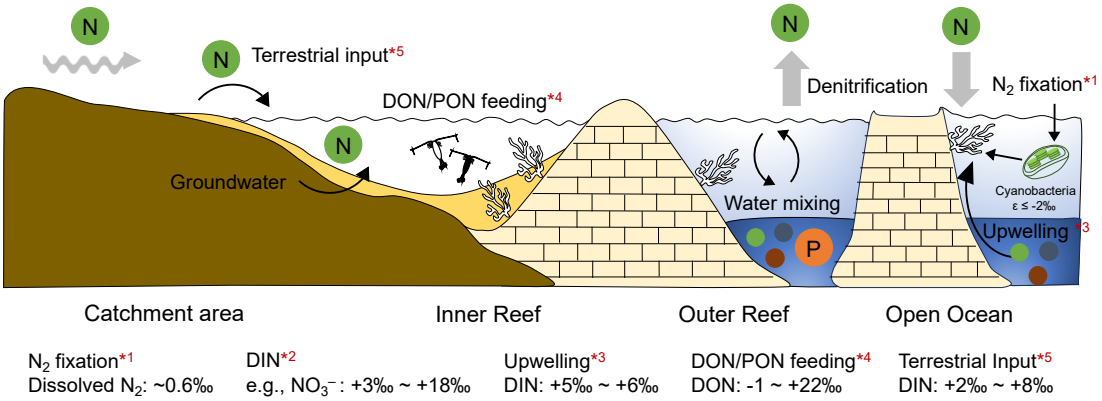
As illustrated in the method part of Chapter II, because Cnidarians have adapted to low nutrient environments, the nitrate (NO_3^-) and ammonium (NH_4^+) in the aquarium are filtrated and ultimately converted to dinitrogen gas (N_2) in the anoxic sand layer of the breeding tank, we thus initially hypothesized that N_2 fixation would be the primary nitrogen source for Cnidarian growth. If this were the case, we expected $\delta^{15}N$ values of phenylalanine in Cnidarians to fall between -3.6‰ and -1.6‰, as suggested by previous studies on nitrogen-fixing systems (Yamazaki et al., 2019). However, our results indicate that $\delta^{15}N$ values of phenylalanine in Cnidarian holobionts and their symbiotic algae range from -1.7‰ to 5.6‰, with an average of 1.9 ± 2.6‰. This range suggests that N_2 fixation is not the dominant nitrogen source as initially expected. Given the observed $\delta^{15}N$ values in this study, it is likely that Cnidarian-algal symbiosis relies on a combination of these nitrogen sources in the laboratory aquarium rather than a single dominant source. However, the specific nitrogen sources

and related nitrogen cycling remain unclear. I also expect the function of symbiotic microbes associated with Cnidarians should take into accounts. Further investigations, such as using stable isotope tracing techniques and microbial DNA sequencing (16S rRNA) may help us determine the relative importance or contributions from different nitrogen sources and identify the role of microbial communities for nitrogen transformation within Cnidarian-algal symbiosis.

4. $\delta^{15}N$ variations and nitrogen cycling in coral reef ecosystems

In contrast to the controlled conditions of laboratory aquariums, coral reef ecosystems exhibit a more complex nitrogen cycling, potentially influenced by diverse nitrogen sources and dynamic biogeochemical processes there (Fig 4-1-1). In general, coral reefs generally are divided into four major zones, each with distinct nitrogen inputs. For instance, coastal waters near catchment areas receive groundwater discharge, while inner reefs experience nitrogen cycling influenced by land-derived nutrients, biological nutrient cycling (e.g., zooplankton dynamics, metabolism of Cnidarians), and microbial activity. On the other hand, outer reefs and open ocean regions rely more on biological nitrogen fixation and deep-sea denitrification processes (Sigman et al., 2009). These regions are influenced by strong wave action and currents, which enhance water mixing and nutrient exchange. Additionally, upwelling events induced by typhoons, may be another nitrogen source for coral reefs. The $\delta^{15}N$ values of those nitrogen sources in coral reefs, as reported in several studies using bulk isotope methods, can vary widely depending on the reef zone and environmental conditions. For instance, deep ocean nitrate, that is

introduced via upwelling typically has $\delta^{15}N$ values between +4‰ and +6‰ (Knapp et al., 2018). Another key nitrogen source is biological nitrogen fixation, primarily conducted by benthic microalgae and diazotrophic cyanobacteria, which convert atmospheric N_2 into dissolved and particulate organic nitrogen. The $\delta^{15}N$ signature of nitrogen fixation is typically close to 0‰, much lighter than deep ocean nitrate (Sigman et al., 2009). Besides natural processes, anthropogenic nitrogen inputs have increasingly affected nitrogen budgets in coral reefs. For instance, agricultural runoff and sewage discharge introduce high-$\delta^{15}N$ nitrogen (+2‰ to +8‰) due to multiple isotopic fractionation processes in soil and wastewater systems, which significantly elevate the $\delta^{15}N$ values in Cnidarian tissues. On the other hand, apart from the external nitrogen sources, internal nitrogen recycling within Cnidarian-algal symbiosis is historically thought to be important in sustaining reef organisms, in which NH_4^+ and dissolved organic nitrogen (DON) excreted by coral hosts can be rapidly taken up by symbiotic algae, promoting for their photosynthetic productivity (Casciotti et al., 2002). The $\delta^{15}N$ values of such recycled nitrogen have large variations, depending on microbial processing and the degree of isotopic fractionation.



*1: Sigman et al. (2009); *2: Liu et al. (1996), Knapp et al. (2008); *3: Casciotti et al. (2008); *4: Saino and Hattori (1987)

Fig 4-1-1 Potential nitrogen sources and their bulk isotope ratios in natural coral reefs.

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CHAPTER V.

General Conclusions

In the Ph.D. thesis, the main purpose is developing the scientific tools and skills to quantify the energetic balance to biomass (i.e., a major factor of trophic transfer efficiency) within Cnidarian-algal symbiosis in coral reef ecosystems. For this purpose, we conducted several studies over the past three years. In the initial studies, we quantified the energetic balance to biomass of photosynthate from symbiotic algae to Cnidarians within symbiosis under an autotrophic condition. These studies include three parts: (1) determining the trophic position (TP) of Cnidarian holobionts (sum of Cnidarian and symbiotic algae) where symbiont-derived photosynthate is a solo resource for corals, (2) determining the TP of symbiotic algae (zooxanthellae), and (3) illustrating the effects of symbiosis on the TP for Cnidarians (i.e., consumers) and zooxanthellae (i.e., diets) in holobionts. Based on the $\delta^{15}N$ values of amino acids of Cnidarians bred in the laboratory aquarium, we illuminated that:

1. **Diversity in TP:** There is a large inter- and intra-diversity in the TP of Cnidarian holobionts, ranging from 0.9 to 2.2 (mean ± 1σ: 1.5 ± 0.4). This suggests that their trophic identity is more closely with mixotrophy-like organisms, rather than herbivorous.

2. **Zooxanthellae as Primary Producers:** The zooxanthellae, purified from Cnidarian holobionts, have a TP close to 1.0, indicating that their trophic identity is that of primary producer in coral reef ecosystems.

3. **Variation in TP between and within Cnidarians:** Compared to general herbivores (TP = 2), the TP of Cnidarians declined by 0-1, showing large variation

between and within species. This variation may be related to multiple strategies or evolutional stages in the use of algal products by Cnidarians.

Our results demonstrate that the analysis of stable isotope ratios of nitrogen ($^{15}N/^{14}N$) of amino acids (AAs) is a useful tool for evaluating the efficiency of trophic transfer in coral reef food webs. After completing my Ph.D. course, I plan to continue research related to my thesis because coral reefs are crucial ecosystems for ocean health, including the sustainability of food supply and human economies but are increasingly threatened by climate change and ocean acidification. The studies focus on understanding the mechanisms for high productivity in coral reefs, can promote conservation efforts to protect reef habitats. My study quantified the size of trophic transfer efficiency in coral reef food webs, providing crucial information for understanding primary production there. However, there are still many unsolved problems. For instance, It is known that chlorophyll-a is widely used as a conventional indicator of photosynthetic activity and primary productivity of primary producers. However, chlorophyll-a is highly sensitive to light exposure and oxidative degradation, leading to substantial losses during extraction and measurement processes. This may significantly decrease the accuracy and reliability of quantification in many ecological and physiological studies. To address this issue, I expect the use of phytol, the hydrophobic side chain of chlorophyll molecules, as an alternative indicator because it is chemically resistant to light and oxidation, and thus can serve as a proxy for chlorophyll-a concentration. For further studies, at this moment, I have three ideas to estimate the primary production in coral reefs: (1)

Developing a new chemical method to extract phytol from chlorophyll; (2) Addressing the issue of equilibrium fractionation for the application of the analysis of stable isotope ratios of carbon ($^{13}C/^{12}C$) in marine environments; (3) Applying the analysis of stable isotope ratios of carbon ($^{13}C/^{12}C$) to measure the photosynthetic activity and algal biomass of zooxanthellae, thus estimating primary production in coral reefs.

Moreover, to evaluate the effect of heterotrophy on the energy transfer in Cnidarian-algal symbiosis, we did several studies over the past three years. For these studies, we bred five soft coral species under a mixotrophic condition (i.e., both symbiont-derived photosynthate and commercial coral foods are resources for corals) and measured their changes in the isotope ratio. Based on the measured $\delta^{15}N$ values, we illuminated that:

(1) Elevated TPs: The TP of five Cnidarians is elevated by an average range from -0.1 to 1.8, suggesting that they feed on the zooplankton as food resources. The large inter- and intra-diversities of TP observed in Cnidarians indicate significant diversity in the use of heterotrophic assimilation.

(2) Zooxanthellae as Primary Producers: The TP of zooxanthellae remained close to 1.0, suggesting they do not access the zooplankton-derived protein.

(3) Contribution of heterotrophy: The relative contribution of heterotrophy to Cnidarian holobionts is 34.6% ± 27.2 under a mixotrophic condition.

Based on these results, we conclude that the additional assimilation of nutrients

from heterotrophy of Cnidarians is another factor contributing to the high productivity of coral reefs, even in oligotrophic oceans. However, there are still many unclear points to explore. Thus, after completing my Ph.D. course, I plan to continue this research through the following two studies: (1) Developing a novel method to trace assimilation efficiency within Cnidarian-algal symbiosis, with the goal of achieving a more accurate estimation of the trophic transfer within Cnidarian-algal symbiosis in coral reef food webs; (2) Applying this research to natural coral reef ecosystems, in order to evaluate trophic strategies and nutrient assimilation in Cnidarian-algal symbiosis under real-world environmental variations. This may improve our understanding of nutrient cycling and energy flow in natural coral reef systems, and contribute to a more comprehensive view of coral reef functioning in the face of environmental changes.

Acknowledgements

It is difficult to find the right words to thank all the people who have supported me throughout this Ph.D. journey. This thesis is not just the result of my individual efforts, but a product of the encouragement, guidance, and love I have received along the way.

First and foremost, I would like to express my deepest gratitude to my supervisor, Prof. Yoshito Chikaraishi. Thank you for your patience, insightful feedback, and for always believing in me, especially during the most difficult times of my doctoral journey. Your guidance has shaped not only my scientific thinking but also my perspective on research and life. I am also sincerely thankful to Prof. Yuko Takizawa for her kind support and encouragement, which gave me great strength at key moments.

I am grateful to all the professors and committee members who supported my thesis and research progress. Special thanks to Prof. Jun Nishioka, Prof. Tomohisa Irino, and Dr. Naoto F. Ishikawa for your advice and valuable comments.

I also thank all the members of the IsoPEG laboratory, especially those who assisted me with discussions, including Drs. Shibuya, Choi, Park, and Simu, Mrs. Nasuhara, Ms. Lurette, Mr. Funahashi, Ms. Ohno. Without your help, this work would not have been completed.

To my parents, Quan Li, Kunlian Du, Mingyong TU—thank you for giving me life, and for always standing behind me with unconditional love and support. You made it

possible for me to pursue my dreams freely. I owe you everything.

And finally, to Satoru Inoue—my husband, also my partner in future life. Thank you for growing with me, supporting me through every emotional wave, and being my safest harbor. I could not have made it here without your calm, strength, and endless encouragement.

This work was partly supported by JST SPRING Grant Number JPMJSP2119, and by Silicone Industry Association of Japan. I deeply appreciate their financial and academic support.