Journal of Experimental Marine Biology and Ecology 442 (2013) 88–95

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

The use of ^{13}C and ^{15}N isotope labeling techniques to assess heterotrophy of corals

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ARTICLE INFO

Article history:
Received 25 September 2012
Received in revised form 5 December 2012
Accepted 7 January 2013
Available online 3 April 2013

Keywords:
Heterotrophy
Isotope labeling
Plankton
Trophic plasticity
$\delta^{13}C$
$\delta^{15}N$

ABSTRACT

This study was conducted in Bocas del Toro, Almirante bay, which is a reef environment strongly influenced from terrestrial and river run off, sedimentation from dredging and port activities, and eutrophication from waste water and agricultural effluences. The two most abundant coral species, *Porites furcata* and *Agaricia tenuifolia* were used to investigate differences in feeding strategies. An ex situ isotope ^{13}C and ^{15}N pulse chase labeling experiment with phytoplankton and zooplankton was developed to be able to determine differences in the corals competence to use them for their heterotrophic nutrition. These tracers were furthermore used to follow exchange rates between the coral hosts and symbionts. By conducting labeling experiments with phyto- and zooplankton, natural plankton cultures and corals, this study developed an optimized labeling technique to follow a coral food chain. Feeding experiments under low light conditions could furthermore elucidate qualitative differences in the heterotrophy.

P. furcata was found to have a stronger heterotrophic competence than *A. tenuifolia*, which was more dependent on photosynthetic nutrition, seen in the lower $\delta^{13}C$ and $\delta^{15}N$ ratios of *A. tenuifolia*. Furthermore, *P. furcata* demonstrated the ability to regulate their symbiotic interaction, to a strong exchange between host and symbiont and at the same time suppressing the autotrophic activity, seen in a strong overlap of $\delta^{13}C$ and $\delta^{15}N$ ratios. Also, *P. furcata* was able to decouple their symbiosis, seen in host and symbiont isotope values with distances of a whole trophic step. *A. tenuifolia* was found to increase its photosynthesis, using the host's waste products. This was seen in the accumulation of heavy isotopes within the zooxanthellae during the feeding experiments. Zooxanthellae of *A. tenuifolia* reached values significantly higher than those of *P. furcata*, due to their increased effort to maintain photosynthesis. Host waste products were incorporated into the symbiotic metabolism, thus a heavy isotope fractioning took place within the endodermic zooxanthellae metabolism. The methodological approach using ^{13}C as a food chain tracer was not successful when passing three food chain steps (phytoplankton-zooplankton-coral), whereby ^{15}N can be considered as a reliable tracer which could elucidate heterotrophic pathways up to the coral host and symbiont. Phytoplankton incorporated most heavy isotopes under constant light conditions after 24 h, zooplankton after 48 h feeding and corals after 4 days feeding. These findings will help to optimize experimental approaches to assess heterotrophic competences and thus to quantitatively valuate coral reefs as a natural filter system.

1. Introduction

Hermatypic corals are able to use a variety of food and energy sources. With an optimized symbiosis with algae (zooxanthellae), hard corals have the ability to conduct photosynthesis in addition to ingesting food heterotrophically. There are numerous studies about the trophic plasticity of corals (proportion between autotrophic and heterotrophic nutrition). During the day zooxanthellae are able to supply the coral host with nutrients and organic compounds, which can provide 100% of a corals energy requirements (Muscatine et al., 1981). Heterotrophy is considered most important at night (Muscatine and Porter, 1977; Porter, 1976) and increases in high turbidity and low light conditions (Anthony, 2000; Anthony and Fabricius, 2000). However, it is difficult to provide quantitative estimates of how heterotrophy is changing in a fluctuating environment and how corals fulfil a role as natural filter and water cleaning systems by capturing plankton, sediments, seston and organic particles from the water column. This information is necessary to understand mechanisms and capacities of corals to survive increasing anthropogenic impacts that result in high sedimentation and nutrification. Furthermore, knowledge regarding a corals heterotrophic competence is important for predicting the resilience of corals during bleaching events (NOAA, 2010), which results in a temporal restriction of photosynthesis and an increased importance of heterotrophy (Hoegh-Guldberg, 1999; Palardy et al., 2008).

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0022-0981/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jembe.2013.01.004

Carbon $\delta^{13}C$ and $\delta^{15}N$ isotope ratios are useful tools to follow metabolic pathways and food sources, outlining marine cycles, food chains and impact factors (Capone, 2008; Fry and Sherr, 1984; Sharp, 2007). They help to distinguish whether carbon fixation was received from the autotrophic or heterotrophic metabolism (Fry and Sherr, 1984). Differences in isotope ratios are due to a discrimination of heavy isotopes in the photosynthetic pathway and the enrichment of heavy isotopes from prey to predator (Fry, 1988; Minagawa and Wada, 1984). Also, natural $\delta^{13}C$ and $\delta^{15}N$ isotope ratios can track fertilizer, terrestrial or sewage run offs into the aquatic environment (Fry, 2006; Sharp, 2007).

Previous studies primarily described methods for ^{13}C and ^{15}N isotope labeling techniques for phytoplankton (Middleburg et al., 2000; Moodley et al., 2000) and coral algae symbionts (Grover et al., 2003; Tanaka et al., 2006) as primary producers. These methods followed photosynthetic pathways and the translocation of metabolic products from the symbiont to the host, however not vice versa. Studies conducted by Widdig and Schlichter (2001) and Szmant-Froelich (1981) used ^{14}C to label phytoplankton and zooplankton, respectively, to investigate heterotrophic uptake rates of soft and hard corals. In Naumann et al. (2010) a ^{15}N coral mucus labeling technique was applied to investigate mucus as a food source for reef associates. However, there is a knowledge gap in the methodology to trace consumers such as zooplankton as well as the coral hosts.

This study developed a reliable pulse-chase ^{13}C and ^{15}N labeling technique for phytoplankton, which were fed to zooplankton and subsequently to hard corals. This method contributes a new approach to isotope labeling techniques that will increase our understanding of qualitative and quantitative food cycling processes within a coral reef as well as heterotrophic feeding, thus filter capacities of different coral species inhabiting reefs with varying environmental conditions.

2. Material and methods

Experiments were conducted in 2010/2011 at the BGG Aquaculture facilities of the Aquadom Berlin, Germany and at the facilities of the Smithsonian Tropical Research Institute in Bocas del Toro, Panama.

2.1. Plankton labeling time series

Phyto- and zooplankton cultures were labeled prior to coral feeding experiments to assess labeling uptake rates and labeling peaks over distinct time intervals. Phytoplankton (*Nannochloropsis* spp.) were cultured in an artificial seawater medium at 23 °C (±0.3), pH of 8.7 (±0.2) and salinity of 34.1 ppm (±0.1). The culture was exposed to artificial light (Giesemann lamp, 8 watts) and was fed once at time 0 (pulse-chase-labeling) with 100 mg l^{-1} ammonium nitrate^{15}N (20% ^{15}N enriched NH_4NO_3, Cambridge Isotope Laboratories).

Preliminary results from the phytoplankton time serious revealed a ^{15}N peak after 24 h. Thus, the labeled phytoplankton culture (dry weight 84 ± 20 mg l^{-1}) was fed to the zooplankton culture (*Brachionus* spp., 150-250 μm, dry weight 31 ± 14 mg l^{-1}) after 24 h. The zooplankton culture was kept at 22 °C (±0.4), pH of 8.1 (±0.1), salinity of 33.6 ppm (±0.6) and under permanent aeration (86% oxygen saturation).

2.2. Feeding experiments

The two most abundant shallow species from Almirante bay were used for all experiments (Guzmán and Guevara, 1998): The finger coral *Porites furcata* (Lamarck 1816) and the lettuce coral *Agaricia tenuifolia* (Dana 1848).

To obtain the natural $\delta^{13}C$ and $\delta^{15}N$ isotope signatures, coral fragments from both species (n = 5) were directly sampled from the reef flat at a depth of 1-4 m (*initials*) at five different sites: Almirante (AL), Pastores (PA), Casa Blanca (CB), Juan Point (JP) and Salt Creek (SC) (Fig. 1). Fragments were carefully removed with pliers from the middle portion of different colonies. The average fragment size was approximately 10 cm^2 (5–10 cm length).

Additional fragments were fixed with cable ties to 9–12 fiberglass aquaria racks at each site (each rack had fragments from both species (n = 5), except for the site AL, which only contained *P. furcata*). Racks were left to heal at each respective site for 10 days prior to the commencement of the experiment. Subsequently, the racks were transported in water filled tanks to the aquaria at the Smithsonian research station. One rack from each site was transferred into 1 of 9 glass aquaria (30 l). Three additional racks from SC were put into 3 seperate aquaria that served as controls to evaluate the accuracy of the isotope labeling procedure. Aquaria were supplied with filtered seawater (charcoal filter) with a salinity of 34.1 ppm (±0.1) and temperatures of 27.5 °C (±0.3) under low light conditions (PAR <30 μmol m^{-2} s^{-1}). Vigorous aeration from aquarium pumps and air stones kept the water well mixed and provided water circulation (oxygen saturation 70 ± 2 %). During the 2-day aquaria acclimation period, corals were kept in a flow through system.

2.2.1. Experiment [a]

Prior to the experiment, 12 l of mixed plankton (dense phyto- and zooplankton mixture >2–250 μm) was sampled from in front of the field station (9°20 40.20 N, 82°15 47.96 W) using a plankton net at 1 m depth (SEA-GEAR Model 9000 Plankton Net, 2 μm mesh size, 50 cm opening diameter). Samples were divided and placed into two separate aquaria. The composition of plankton consisted of mainly nanoplankton (2–20 μm, up to 97 %) within the phytoplankton fraction (Hallegraeff and Jeffrey, 1984), while the zooplankton fraction was dominated by copepods (up to 84 %) (Michel et al., 1976; Moore and Sander, 1976).

Plankton was fertilized with ammonium nitrate and sodium carbonate in a C:N ratio of 106:16 (Redfield ratio) (Atkinson and Smith, 1983) (Table 1). One of the two plankton-aquaria was fertilized with ^{13}C and ^{15}N enriched nutrients (98 % ^{13}C-enriched $NaH^{13}CO_3$ and 99 % ^{15}N-enriched $Na^{15}NO_3$, Cambridge Isotope Laboratories). Both aquaria were incubated with an additional light source and physical conditions of 24 °C (±0.3), salinity of 31.6 ppm (±0.2) and an oxygen supply of 75 % (±4). After 24 h, 2 l of the plankton (dry weight 390 ± 124 mg l^{-1}) was added to the aquaria to feed the corals. Corals in three aquaria were fed with ^{13}C and ^{15}N labeled plankton and corals in an additional three aquaria were fed with unlabeled *control* plankton. This procedure was repeated three times every four days using freshly sampled plankton. Plankton cultures were sampled before and after the pulse-chase labeling every 4 h for 24 h and filtered through a Whatman GF/F filter (0.7 μm pore size) and rinsed with seawater.

2.2.2. Experiment [b]

For the second experiment, a phytoplankton culture (*Nannochloropsis* spp.) and a culture of freshly hatched *Artemia* sp. eggs were raised. The phytoplankton culture was also fertilized with ^{13}C enriched sodium carbonate and ^{15}N enriched ammonium nitrate (Table 1). Aquaria conditions were the same as was described in experiment [a]. After 24 h, 1 l of the labeled phytoplankton culture was added to the zooplankton culture. To obtain data on the labeling success, both phyto- and zooplankton were sampled before labeling (*initial*) and after labeling for 72 h at 12h intervals. Phytoplankton were filtered through a Whatman GF/F filter. Zooplankton were sampled with a sieve (150 μm). All samples were rinsed with seawater.

24 h after the labeling corals in six aquaria were fed with 1 l of the labeled phytoplankton culture (density: $6.25 \pm 1.32 \times 10^{-9}$ cells l^{-1}; dry weight 320 ± 4 mg l^{-1}). 24 h after zooplankton were labeled with labeled phytoplankton, corals in an additional six aquaria were fed with 2 g of zooplankton (2 g wet weight equals 0.16 ± 0.0001 g dry weight).

An additional aquarium was used to feed corals with a mixture of labeled phytoplankton (0.5 l) and labeled zooplankton (2 g) in order

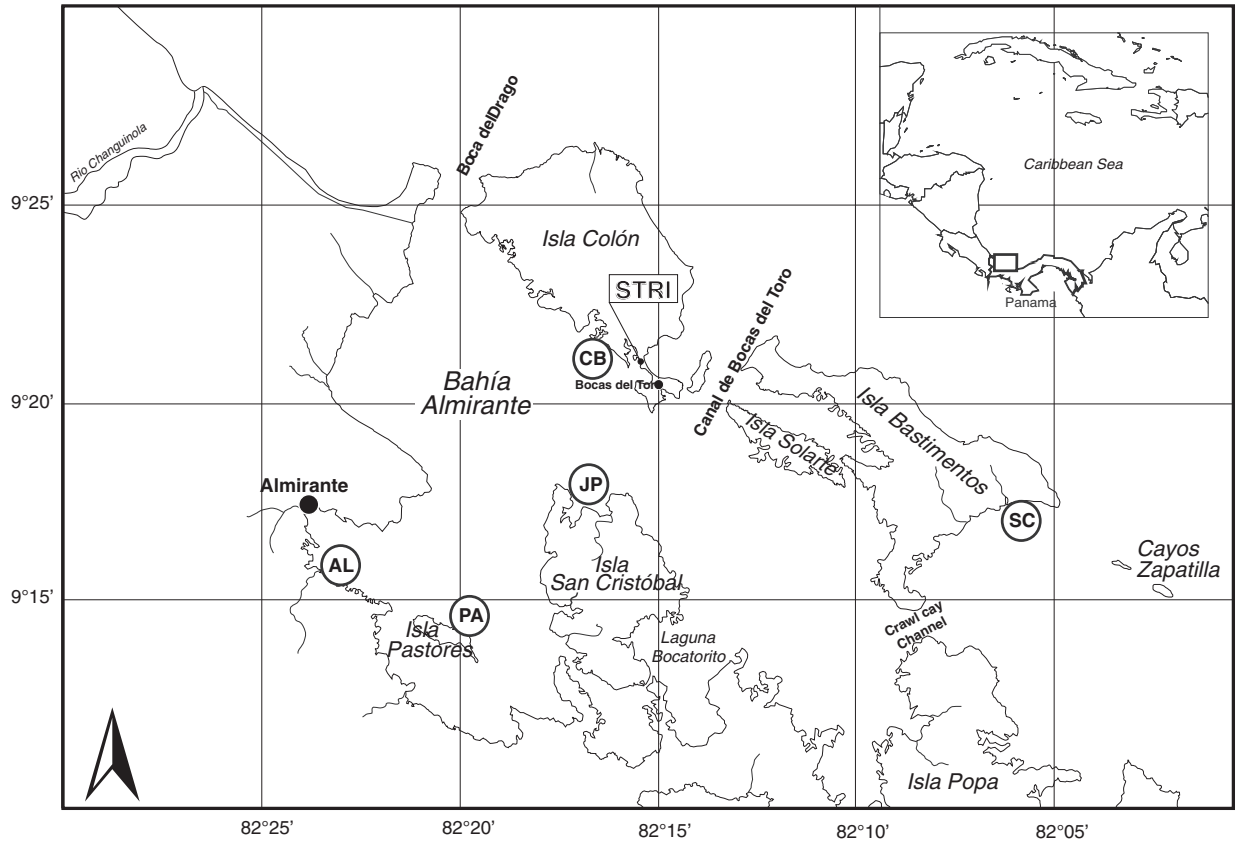

Fig. 1. Sampling sites: 4 sampling sites were located within the bay, Almirante (AL), Pastores (PA), Casa Blanca (CB), Juan Point (JP), and one outside the bay, Salt Creek (SC).

to detect the highest tracing signal in the coral fragments within a time series of 7 days (*coral-time-series*).

2.3. Sample processing

Based on results from Middleburg et al. (2000) and Szmant-Froelich (1981), which found the isotope uptake peak of heterotrophs after 2–3 days, corals were sampled before labeling and 72 h after feeding on labeled plankton.

Coral tissue was removed from the skeleton with an airgun (<1 cm distance from coral) and approximately 10 ml of filtered seawater. The tissue slurry was homogenized with an Ultra Turrax (Johannes and Wiebe, 1970) and separated into coral host tissue and zooxanthellae via centrifugation (4000 ×*g*; 5 min, Fisher Scientific Speed). The supernatant (coral host tissue) was filtered onto a Whatman GF/F filter. The zooxanthellae pellet was re-suspended in filtered seawater and centrifuged again two times for purification (Papina et al., 2003; Treignier et al., 2008). Both, the filter and zooxanthellae pellet were oven dried for 24 h (60 °C).

All samples (plankton, coral host tissue, zooxanthellae) were analyzed for $\delta^{13}C$ and $\delta^{15}N$ using stable isotope mass spectrometry (IRMS, DELTA V Advantage) with an elemental analyzer (Thermo Flash EA 1112). Plankton samples from the plankton labeling time series experiment were only analyzed for $\delta^{15}N$.

Table 1
Weight of nutrient powders (sodium carbonate and ammonium nitrate) were calculated based on the Redfield ratio, the molecular weight [M] of the compound fed, divided through the number of atoms of the target component (N or C).

Nutrients	Sodium Carbonate^{13}C	Ammonium Nitrate^{15}N
Redfield ratio	106	16
Molecular weight [M]	106	80
No of atoms C and N, resp.	1	2
Weight of nutrient powder (ratio∗molecular weight/ number of atoms)	11.24 mg	0.64 mg
Fertilization		
2.1 Plankton time series	n.a.	100 mg l^{-1} (20%)
2.2.1 Experiment [a]: Natural Plankton mix	175.3 mg l^{-1} (98%)	10 mg l^{-1} (98%)
2.2.2 Experiment [b]: Phytoplankton culture	70.8 mg l^{-1} (98%)	4 mg l^{-1} (98%)

2.4. Statistical analyses

Differences between treatments, sites, species and time were tested by comparing means with a t-test. ANOVA was applied when differences in species and treatments were tested combining all sites and times. The software JMP 9.0.2 (SAS Institute) was used for all analyses and results and presented as means ± SE.

3. Results

3.1. Plankton labeling time series

The enrichment of ^{15}N in phytoplankton showed a clear peak after 24 h (Fig. 2). Zooplankton fed with these phytoplankton showed an immediate uptake of the ^{15}N enriched phytoplankton, with the highest peak after 17 and 42 h. The maximum $\delta^{15}N$ for phytoplankton reached 127‰ and the average $\delta^{15}N$ values for zooplankton were 1/3 the phytoplankton values (35 ± 4‰).

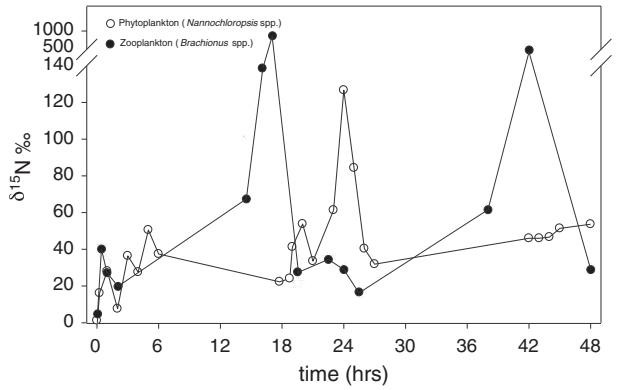

Fig. 2. Time series for a ^{15}N enrichment experiment in phytoplankton fertilized with 20 % ammonium nitrate-^{15}N and zooplankton fed with 24 h ^{15}N incubated phytoplankton. The maximum $\delta^{15}N$ for phytoplankton reached 127‰ and average $\delta^{15}N$ values for zooplankton were at 35 ± 4‰.

3.2. Feeding experiments

Initial $\delta^{13}C$ values of *P. furcata* and *A. tenuifolia* sampled directly from the field varied between −14 to −16‰ and $\delta^{15}N$ were between 1 and 5‰. Zooxanthellae had lower values than the corresponding host tissue for both species. $\delta^{13}C$ values did not differ significantly between species (for both host and zooxanthellae). $\delta^{15}N$ were significantly higher in the host tissue of *P. furcata* (3.9 ± 0.1‰) than for *A. tenuifolia* (3.2 ± 0.1) (ANOVA, F = 4, P = 0.05, $n_{P.\ furcata} = 32$, $n_{A.\ tenuifolia} = 24$). $\delta^{15}N$ zooxanthellae values were also higher in *P. furcata* (2.45 ± 0.37‰) compared to *A. tenuifolia* (1.97 ± 0.18‰), however not significantly higher. *P. furcata* fragments from the site AL, closest to the port, showed similar $\delta^{15}N$ values between the host tissue and zooxanthellae (4.0 ± 0.1 and 4.1 ± 0.5‰, resp.), while the other sites showed more variability between the host-zooxanthellae $\delta^{15}N$ ratios. The largest difference between host and symbiont was exhibited in *P. furcata* from PA (2.7‰). *A. tenuifolia* showed less difference in the δ ratios between host and symbiont than *P. furcata*, however at SC (outside the bay, Fig. 1) the difference in the $\delta^{13}C$ ratio between host and zooxanthellae was highest in *A. tenuifolia* 1.1‰.

3.2.1. Experiment [a]

The *control* plankton had $\delta^{13}C$ values between −7 and −28‰, while the ^{13}C plankton labeling experiment (three replicates) revealed values of 3022 ± 52 (1.), 1000 ± 48 (2.) and 5666 ± 91 (3.)‰ after 24 h labeling time (Fig. 3A). $\delta^{15}N$ values of *control* plankton revealed values between 3 and 21‰, while the labeled plankton reached values up to 7319 ± 635 (1.), 1690 ± 225 (2.) and 2170 ± 158 (3.)‰ after a 24 h labeling period. The ^{15}N signal was detected after 4 h (Fig. 3B).

^{13}C and ^{15}N uptake rates were generally higher in *A. tenuifolia* (Table 2). Both species had the highest $\delta^{13}C$ and $\delta^{15}N$ ratios at PA (Fig. 1), with significantly higher $\delta^{15}N$ values in *P. furcata* from this site (t-test). $\delta^{13}C$ and $\delta^{15}N$ ratios of *A. tenuifolia* were always lowest at SC (outside the bay). Additionally, the differences between mean zooxanthellae and host tissue values of $\delta^{13}C$ and $\delta^{15}N$ mean values were higher in *A. tenuifolia* than in *P. furcata* (Table 2). Values of *control* fragments fed with unlabeled plankton remained in the range found for the *initial* values.

3.2.2. Experiment [b]

Initial phytoplankton values were −18‰ for $\delta^{13}C$ and 14‰ for $\delta^{15}N$ (Fig. 4). Initial $\delta^{13}C$ and $\delta^{15}N$ values of the zooplankton were −7‰ and 15‰, respectively. The labeling revealed a continuous increase in the heavy ^{15}N isotope signal, reaching $\delta^{15}N$ values of 36,000‰ after 72 h for phytoplankton and 370‰ after 48 h for zooplankton (Fig. 4). ^{13}C labeling reached constant $\delta^{13}C$ mean values of

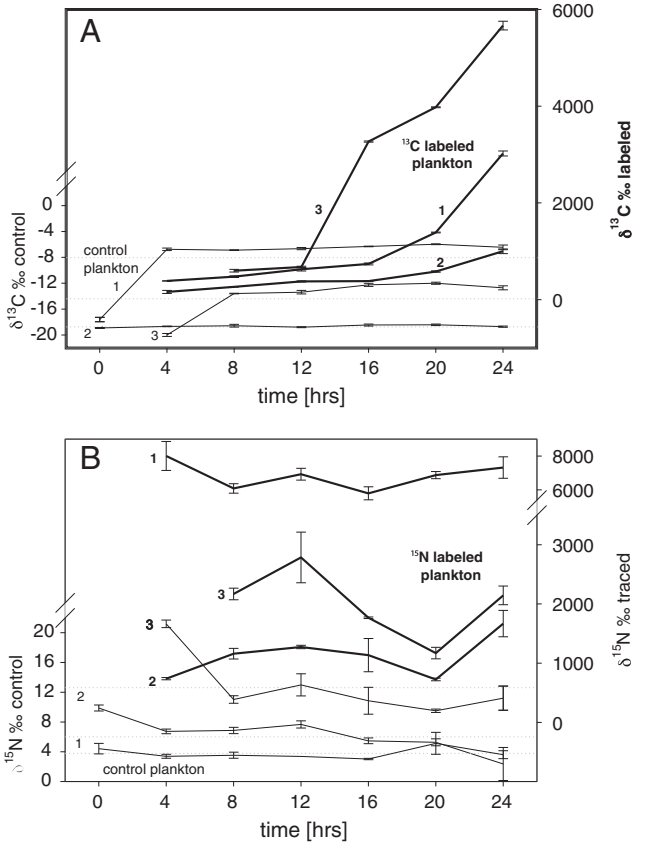

Fig. 3. Experiment [a]: Labeling of natural mix plankton >2-250 μm (phyto- and zooplankton mixture) for 24 h. Numbers (1–3) indicate replicated time series used for experiment [a], which was replicated three times. Values at time 0 are start values also for labeled plankton. Horizontal dashed lines indicate the mean value of the *control* plankton. a) $\delta^{13}C$ labeling signal (bold black line) was highest after 24 h (n = 3). b) $\delta^{15}N$ labeling showed a peak after 4 h and an increase after 24 h. Plankton was fed to the corals after a 24 h labeling period.

6300 ± 700‰ for phytoplankton and 56 ± 10‰ for zooplankton. After 24 h (when plankton were fed to the corals) phytoplankton had $\delta^{13}C$ mean values of 5700 ± 1500‰ and $\delta^{15}N$ of 12,400 ± 4100‰, whereby zooplankton had values of $\delta^{13}C$ 56 ± 18‰ and $\delta^{15}N$ 145 ± 57‰, which represents a labeling success from phytoplankton to zooplankton of approx. 1 %.

The feeding with ^{13}C labeled zooplankton was not detectable within the corals tissues ($\delta^{13}C$ values ranged between −11 and −18‰, Fig. 6B). Corals fed with ^{15}N labeled zooplankton revealed 10-12% of the zooplankton signal (plankton $\delta^{15}N$ 145 ± 57‰, host $\delta^{15}N$ 18 ± 2, zooxanthellae $\delta^{15}N$ 15 ± 2‰; Fig. 6D). These values were also similar to field *initial* values of $\delta^{15}N$ 5‰ (host) and $\delta^{15}N$ 6‰ (zooxanthellae). Phytoplankton labeling of coral tissue using ^{13}C tracers was approximately 0.2%, which shows insufficient labeling success. $\delta^{13}C$ in zooxanthellae revealed higher labeling values than the coral host ($\delta^{13}C$ tissue 2 ± 6, zooxanthellae 14 ± 10‰) (Fig. 6A). $\delta^{15}N$ values in the corals fed with phytoplankton exhibited 1.7 % of the phytoplankton signal (12,400 ± 4100‰) in the coral tissue (210 ± 17‰), and 2.8 % in the zooxanthellae (348 ± 74‰) (Fig. 6C).

In both set ups, feeding with labeled phyto- and zooplankton, *P. furcata* had a stronger similarity between host and zooxanthellae $\delta^{13}C$ and $\delta^{15}N$ values than *A. tenuifolia* (Fig. 6). Feeding with labeled phytoplankton revealed higher $\delta^{13}C$ and $\delta^{15}N$ values in zooxanthellae than the host. Zooplankton feedings revealed lower zooxanthellae values than tissue values, however not for $\delta^{15}N$ in *A. tenuifolia*.

Significant differences between sites were not found due to the inherent noise of this methodology, however, $\delta^{13}C$ and $\delta^{15}N$ values

Table 2
Experiment [a]: $\delta^{13}C$ and $\delta^{15}N$ tracing signals of the corals *P. furcata* and *A. tenuifolia* (host tissue and zooxanthellae) fed with ^{13}C and ^{15}N labeled mix-plankton from the field. The experiment was repeated three times.

		Mix plankton	*P. furcata*		*A. tenuifolia*	
			Host tissue	Zooxanthellae	Host tissue	Zooxanthellae
No		(n = 3)	(n = 20)	(n = 20)	(n = 14)	(n = 14)
1. replicate	$\delta^{13}C$ [‰]	3022 ± 52	54 ± 10	96 ± 18	66 ± 16	187 ± 45
	$\delta^{15}N$ [‰]	7319 ± 225	1302 ± 185	2132 ± 206	1283 ± 153	3484 ± 518
2. replicate	$\delta^{13}C$ [‰]	1000 ± 48	81 ± 16	138 ± 26	88 ± 22	219 ± 51
	$\delta^{15}N$ [‰]	1690 ± 225	2226 ± 233	3949 ± 512	2158 ± 200	5260 ± 366
3. replicate	$\delta^{13}C$ [‰]	5666 ± 91	114 ± 25	163 ± 33	159 ± 33	254 ± 50
	$\delta^{15}N$ [‰]	2170 ± 158	3495 ± 361	4352 ± 375	3342 ± 369	5586 ± 421
Tracing signal in corals	$\delta^{13}C$ [%]	100	4 ± 2	7 ± 4	5 ± 2	11 ± 6
	$\delta^{15}N$ [%]	100	104 ± 44	154 ± 63	100 ± 42	205 ± 80

from fragments sampled outside the bay (SC) were lower compared to those sampled inside the bay (Fig. 6).

The *coral-time-series* revealed a ^{15}N labeling signal within the coral tissue after 1 day, however was most distinct after 4 days (Fig. 5). Zooxanthellae values (342 ± 65‰) were significantly higher than coral host values (66 ± 8‰, t-test). The ^{13}C labeling was not detectable and remained similar to the initial values (between −14 and -17‰) for both zooxanthellae and tissues.

4. Discussion

The field survey indicated that there were site-specific differences in the corals trophic plasticity. Corals at the site PA, which is exposed to the Almirante port and the Boca del Drago inlet showed a $\delta^{15}N$ between host and zooxanthellae in *P. furcata* and 3‰ presents one trophic step (Peterson and Fry, 1987). Thus, a strong decoupling between host and zooxanthellae was observed (Leinfelder et al., 2012). These differences in values showed that the metabolic exchange rates between zooxanthellae and host were low at this site. However, similar $\delta^{15}N$ zooxanthellae and host values in *P. furcata* at the site closest to the port (AL) indicated that this species has a strong metabolic connectivity and exchange between host and symbiont. The generally higher $\delta^{15}N$ values (>4.0‰) at this site indicated that the supply of nitrogen was most distinct from the coral animal to the zooxanthellae. Thus, the nutrition was on a higher trophic level than at the other sites probably due to the higher turbidity and eutrophication. This finding is contrary to other studies, which claim that zooxanthellae mainly provide metabolites for the host (Hoegh-Guldberg and Smith, 1989; Muscatine et al., 1981, 1989). This relationship between zooxanthellae and host allowed corals to grow and survive in a strongly sedimented and eutrophied area that contains high abundances of plankton, by using their heterotrophic competence for nutrition (Borell and Bischof, 2008). *A. tenuifolia* had a lower competence for heterotrophy, seen in lower $\delta^{13}C$ and $\delta^{15}N$ values under natural conditions. Also, the coupling of symbionts and hosts, which was observed in generally lower differences in δ values, were more distinct in *A. tenuifolia*, compared to *P. furcata*.

The experiments [a] and [b] revealed a similar pattern as the results from the field, which might be due to species specific competences for heterotrophic feeding and phototrophic activity under low light conditions. This was indicated through the labeling signal from plankton uptake and the ability of the corals to maintain autotrophy, also by using host waste products. The comparison between phyto- and zooplankton feeding in experiment [b] showed a higher labeling success using phytoplankton instead of zooplankton (Fig. 7, seen in the tracing signal). However, both are considered an important food source for the investigated species, in particular for building up energy reserves (lipids) (Seemann et al., 2012).

While host and zooxanthellae $\delta^{13}C$ and $\delta^{15}N$ ratios of *P. furcata* were similar in both feeding experiments, *A. tenuifolia* showed dissimilar zooxanthellae and host δ ratios. This indicated a lower exchange of metabolites in *A. tenuifolia* between the symbionts and hosts, which resulted in poor health and bleaching. However, high $\delta^{13}C$ and $\delta^{15}N$ zooxanthellae ratios in *A. tenuifolia* indicated a strong effort to conduct

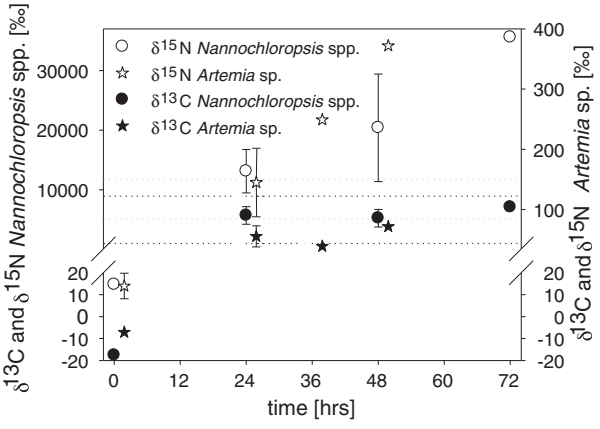

Fig. 4. Experiment [b]: ^{13}C and ^{15}N pulse-chase labeling (at time 0) of a phytoplankton (*Nannochloropsis* spp.) and zooplankton (*Artemia* sp.) culture. Zooplankton were fed with the labeled phytoplankton culture. Dashed lines represent overall mean values of ^{13}C (black) and ^{15}N (gray) labeled plankton (n = 1–3).

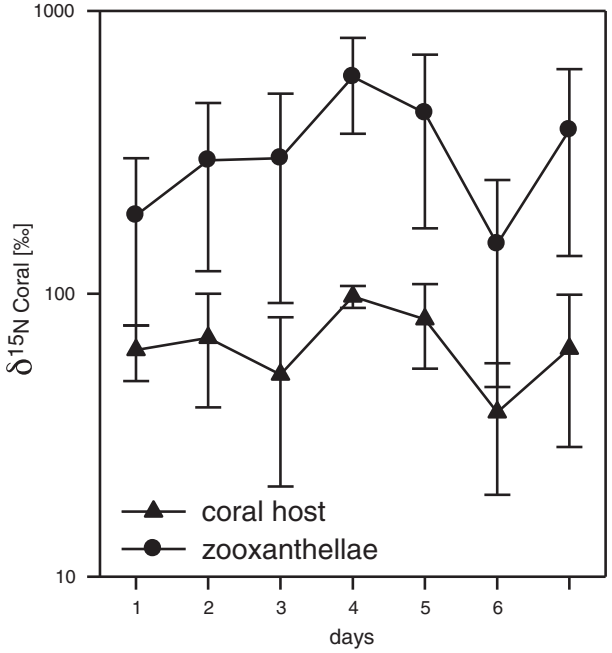

Fig. 5. Seven day time series of corals (*P. furcata*) after being fed with a mixture of 24 h ^{13}C and ^{15}N labeled phyto- and zooplankton cultures. The peak was most distinct at day 4 (n = 3).

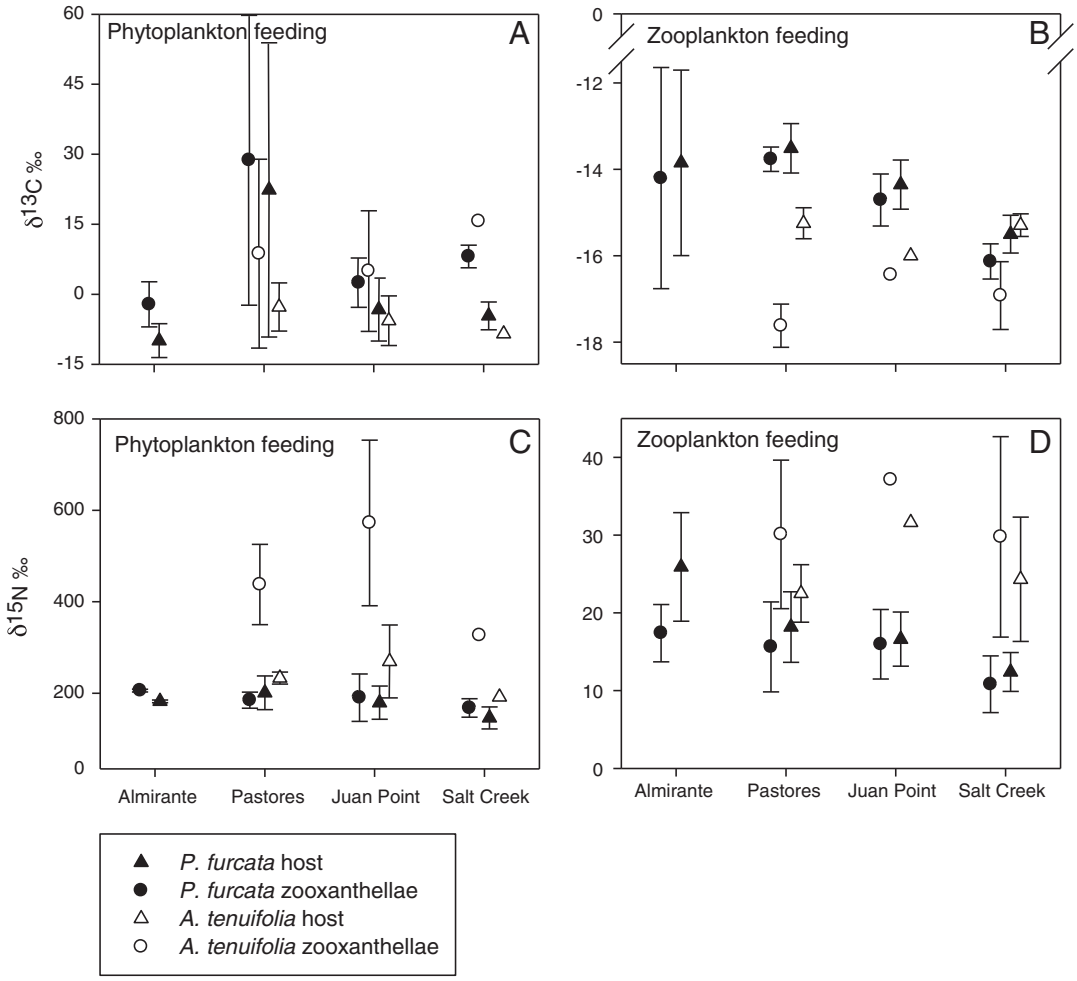

Fig. 6. The coral labeling signal at the different sampling sites was higher when corals were fed with phytoplankton (A and C) than when fed with zooplankton (B and D). The $\delta^{15}N$ signal (C and D) was more distinct than $\delta^{13}C$ (A and B).

photosynthesis. This species can compensate for low light conditions with increased photosynthetic activity (Seemann et al., 2012). Consequently, it can be assumed that zooxanthellae increased their photosynthetic activity. Even though light availability was limited, zooxanthellae still incorporated nutrients provided through the corals waste products, which were enriched with heavy ^{13}C and ^{15}N isotopes. The accumulation of heavy isotopes from plankton to host to zooxanthellae showed the typical isotope fractioning along a food chain (Fry, 1988), however not the typical discrimination of heavy isotopes within the photosynthetic metabolism, which is evidence for the direct use of host waste products through the endodermic zooxanthellae (Borell and Bischof, 2008; Sutton and Hoegh-Guldberg, 1990). Considering heterotrophic input, experiment [a] indicated that the site with the combined impact of the port and the Boca del Drago bay inlet [PA] had the highest heterotrophic competences seen in highest values for both species. Lower $\delta^{13}C$ and $\delta^{15}N$ ratios of host tissue from SC (outside the bay) coral fragments indicated a lower feeding competence than corals from inside the bay system, which was most distinct in *A. tenuifolia*. Opposite to what was found a decade ago, in which *A. tenuifolia* was considered the more dominant species, regular bleaching events have resulted in the expansion of *P. furcata*, which are more tolerant to bleaching events (Aronson et al., 2004). Due to its greater ability to acquire nutrients through heterotrophy, *P. furcata* showed a strong competence to survive in this turbid bay environment. *A. tenuifolia* is more dependent on nutrition through autotrophy, thus mortality is high during bleaching events. This was also indicated by a strong ability to increase zooxanthellae and chlorophyll *a* in low light conditions, possibly by using nutrients from river inputs and human waste water (Seemann et al., 2012).

4.1. Optimized labeling method

Tracing via ^{13}C can be considered a less efficient method compared to the ^{15}N labeling approach, in particular when being circulated through a trophic food web. The experiments found a consistently faster and higher incorporation of ^{15}N in both, phyto- and zooplankton. An additional factor for a successful incorporation of the ^{15}N signal is the strong limitation of nitrogen compartments under culturing conditions (McCarthy and Goldman, 1979). Since there were no alternative nitrogen sources available, there was a strong uptake of the ammonium nitrate ^{15}N (Needoba et al., 2003).

The fact that the uptake of ^{13}C labeled zooplankton was not detectable in corals (Fig. 7) could be due to an access of alternative carbon sources from dissolved CO_3^{2-} derivates (dilution of atmospheric CO_2 in water) with light isotope ratios, which is preferred to the heavy isotopes during the photosynthetic metabolism from phytoplankton (Korb et al., 1996; Michener and Lajtha, 1994).

In general, the labeling of plankton cultures is less efficient compared to the labeling of mixed plankton taken from the field. This can be explained by the selectivity of zooplankton in their nutrition and their feeding preferences for prey of about 16 µm (Fernández, 2001; Makridis and Vadstein, 1999). Feeding rates are probably much higher within a natural plankton food web compared to feeding zooplankton

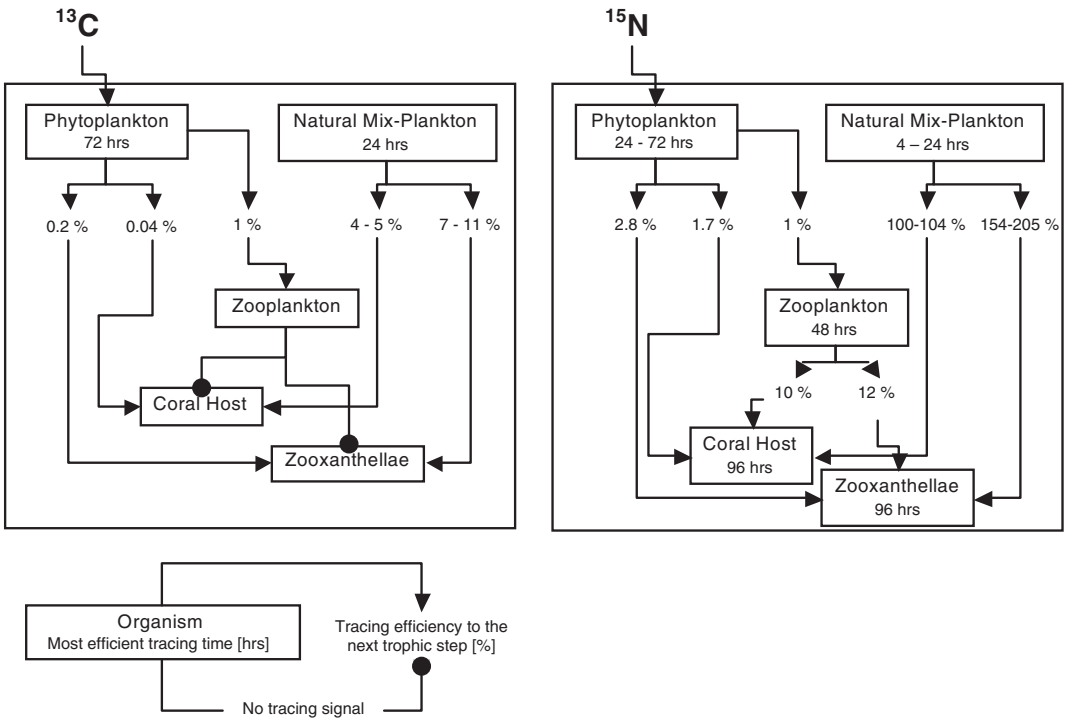

Fig. 7. Labeling success along a heterotrophic food chain using ^{13}C and ^{15}N pulse-chase labeled phytoplankton (*Nannochloropsis* spp.), zooplankton (*Artemia* sp.) and a natural plankton mix (>2-250 μm) from the field.

with only one phytoplankton culture. This might explain the low tracing success from phytoplankton to zooplankton cultures (1%) (Fig. 7).

The reason that the labeling peak of the phytoplankton culture revealed strong temporal differences was due to differences in the physical conditions of the different labeling experiments. Specifically differences in the division rates of the phytoplankton cells (Welschmeyer and Lorenzen, 1984). While the plankton labeling time series was conducted in an aquarium with a permanent artificial light penetration (Fig. 2), the plankton labeling for the aquarium experiment [a] and [b] was conducted in an outdoor aquaculture facilities with natural and artificial light penetration, thus a day-night light fluctuation.

In order to optimize the labeling success the following method is suggested:

Labeling should be conducted with 98% ammonium nitrate^{15}N. To reveal a constant cell division in phytoplankton, thus a doubling of the cells after 24 h, an artificial 8 watt aquarium light should be used. To achieve a high intake of phytoplankton by zooplankton, a phytoplankton culture with different species should be used that is similar to what is found within natural conditions for the particular zooplankton species being used. Phytoplankton should reach a tracing value of $\delta^{13}C<4000‰$ and $\delta^{15}N<10{,}000‰$, because values above these do not reveal reliable values using the isotope mass spectrometry technique. $\delta^{15}N$ labeling will lead to a minimum labeling success of 1 % within the zooplankton, whereby the plankton labeling time series from this study found a maximum labeling success of 33 % from phyto- to zooplankton. To obtain a high $\delta^{15}N$ signal the amount of nutrients used within this study in experiment [b] should be applied (1 mg l^{-1} of a 98 % ammonium nitrate^{15}N), however the cell density of about 1.5×10^{-9} cells l^{-1} can be increased to obtain $\delta^{15}N$ values <10,000‰. Zooplankton should be incubated for 48 h before being fed to the corals since they reached their highest labeling peaks after 48 h (Fig. 4). Corals should be sampled 4 days after phyto- or zooplankton feeding (Fig. 5). To investigate phytoplankton feeding in corals, the corals should be fed the phytoplankton culture after 24 h incubation time. To investigate the heterotrophic competence, natural mixed plankton is most useful due to its tracing success for both the incorporation of ^{13}C and ^{15}N. The labeling success from plankton to the coral was shown to be 4.5 – 9 % for ^{13}C and over 100 % for ^{15}N. Thus, initial values of the labeled plankton should have δ values of >1000‰ for both $^{13}C/^{12}C$ and $^{15}N/^{14}N$. However, the mixed-plankton tracing experiment showed that the tracing of natural plankton is strongly variable (Fig. 3), which is most likely due to the differences in plankton composition at each collection period (Glynn, 1973).

Light should be minimized to exclude autotrophic nutrition of the corals during the feeding period.

5. Conclusion

This study developed a new approach to trace the heterotrophic food sources of corals. The pulse-chase ^{13}C and ^{15}N labeling technique for phytoplankton is a reliable basis to indicate phyto- and zooplankton feeding of corals. ^{15}N was a more successful tracer than ^{13}C, particularly, when being used for three step labeling experiments (phytoplankton culture → zooplankton culture → coral).

P. furcata was found to have a stronger heterotrophic plasticity than *A. tenuifolia* thus a more distinct filter capacity. Additionally *P. furcata* decreased their photosynthetic activity in low light conditions, contrary to *A. tenuifolia*, which maintained it. These different strategies can be useful when exposed to fluctuating environments common within Almirante bay. *A. tenuifolia* can acclimatize to low light conditions, by supporting their zooxanthellae, while *P. furcata* has the ability to suppress their symbionts and thereby to reduce energy requirements. These strategies explain the ecosystem patterns observed in the field, in which *P. furcata* is dominant in areas most impacted by sedimentation, eutrophication and regular bleaching events, while *A. tenuifolia* dominates deeper areas (>3 m), where light is a more limiting factor (Seemann et al., 2012). Since *A. tenuifolia* are not able to compensate the loss of zooxanthellae and maintain their energy requirements, unlike *P. furcata*, which are able to switch to a heterotrophic nutrition, mass mortalities were observed in this species during recent bleaching events (NOAA, 2010).

Acknowledgement

I would like to thank the staff from the Smithsonian Tropical Research Institute (STRI) Bocas del Toro, in particular Eric Brown, Plinio Gondola, Gabriel Jacome and Rachel Collin for their invaluable help, great support in organization and space acquisition at the field station. Thanks to Cindy T. González and Rodrigo E. Carballo for their big help with the aquarium and lab work, to Ewgenija Kuhl and Ulrich Struck from the Museum für Naturkunde, Berlin for their help with the isotope analysis. Further thanks to Kathryn Berry for her English revision. My gratitude also goes to Reinhold Leinfelder, Georg Heiss and Claudio Richter and their helpful input, discussions and advices.

This project was funded by the DAAD (Deutscher Akademischer Ausstauschdienst), the von Pawel-Rammingen Foundation and the Humboldt Innovation. **[SS]**

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