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Quantifying the transfer of prey $\delta^{15}N$ signatures into coral holobiont nitrogen pools

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ABSTRACT: Nitrogen stable isotope ($\delta^{15}N$) signatures of coral and skeletal tissues are commonly used to identify spatial and temporal patterns in the source and supply of nitrogen to coral reefs. A ^{15}N labelled particulate food source (rotifers) was used to quantify the incorporation of prey nitrogen into the nitrogen pools (coral, algal tissues and skeletal organic matter) of *Porites lutea*, and to estimate the time taken for the $\delta^{15}N$ signature of source nitrogen to be reflected in the different tissue fractions of the coral holobiont. Neither coral nor algal fractions displayed the full expression of the food source $\delta^{15}N$ over a 60 d experimental period. The response of the skeletal $\delta^{15}N$ value to the food $\delta^{15}N$ was slower than the coral tissue, but this may have been caused by coarse sampling resolution coupled with a short experimental period. Using a mass-balance model, we determined that the corals must have been augmenting their rotifer diets by up to 50% with dissolved nitrogen from the water column. Using the $\delta^{15}N$ of the combined food source (i.e. dissolved and rotifer nitrogen), we calculated tissue turnover rates of 87 d for the coral tissue and 111 d for the algal symbionts. These values dictate that the duration of any change in the $\delta^{15}N$ of a coral's N source needs to be greater than 3 mo to register its full magnitude in the tissue and skeletal nitrogen pools. This has implications for studies in which the host, symbiont and skeletal $\delta^{15}N$ are used as a proxy for temporal changes in the source of nitrogen to coral reefs. Our results also support the notion of a bidirectional exchange of N between the coral and algae fractions, and provide estimations of the assimilation and excretion of N during heterotrophic feeding.

KEY WORDS: Coral nutrient recycling · Nitrogen isotopes · Palaeo-oceanographic reconstructions · *Porites* · Great Barrier Reef

1. INTRODUCTION

Nitrogen (N) is a fundamental dietary requirement for corals and their endosymbiont algae (Tanaka et al. 2015, Tremblay et al. 2015). Both the coral animal and the endosymbiont can take up dissolved N, and the coral is also an active consumer of particulate N (Grover et al. 2008). During assimilation, the nitrogen isotopic signature ($\delta^{15}N$) of the corals' N source is expected to be imprinted within the holobiont N pools, including coral tissue, endosymbiont algae and organic material trapped within the skeletal lattice during biomineralisation (Muscatine et al. 2005, O'Neil & Capone 2008). However, the incorporation of the $\delta^{15}N$ signature of the N source into the holobiont N pools is poorly understood (Sammarco et al. 1999).

Stable isotope analysis provides clues to the relative importance of auto-heterotrophy, since it is used to identify food sources (Peterson & Fry 1987) and measure fluxes between different trophic levels (Minagawa

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& Wada 1984, Rau et al. 1992). In particular, $\delta^{15}N$ values have been used in trophic ecology and biogeochemistry to deduce the sources of N for an organism (Heikoop et al. 2000, Wang et al. 2015, Yamazaki et al. 2015). Nevertheless, N turnover has a crucial influence on the rate of change of $\delta^{15}N$ in the organism, causing a time lag with respect to variations in the N sources (Tanaka et al. 2018). Autotroph–heterotroph symbioses, such as the coral–endosymbiont algal system, are complex, and the quantification of N turnover is important to understand the survival and adaptive strategies of organisms under N-limited environments (Tanaka et al. 2018).

The $\delta^{15}N$ of the intra-crystalline coral skeleton organic material (CS-$\delta^{15}N$) is becoming increasingly popular as a means of reconstructing both spatial and temporal patterns of N supply to coral reefs (Yamazaki et al. 2013, Erler et al. 2016). However, a better comprehension of the isotopic transfer process is required if coral N pools, particularly the skeletal organic N fraction, are to be used for palaeoreconstructions of oceanic N cycling. The skeletal technique relies on the fact that N sources to coral reefs often have different $\delta^{15}N$ signatures; for instance, N derived from nutrient upwelling is enriched in ^{15}N relative to that derived from N_2 fixation (Heikoop et al. 2000, Montoya et al. 2002). However, in addition to the source of N, the partitioning of N within the coral holobiont and the availability of N also influences the coral $\delta^{15}N$ recorded in tissues (Erler et al. 2015, Wang et al. 2015). As such, cycling of N between the coral and its endosymbiont algae has important implications for the use of coral skeletons to reconstruct histories of N cycling in coral reef environments.

Information is required on the magnitude of change in the external $\delta^{15}N$ pool required to elicit the observed response in skeletal $\delta^{15}N$. Furthermore, tissue turnover rate, which regulates how quickly an external ^{15}N signal is expressed within the holobiont, remains largely unknown for many *Porites* coral species, which are often used for these types of palaeoreconstructions. While some field-based studies have shown that the ^{15}N of coral tissue is correlated with the amount of external $\delta^{15}N$ of N available to the coral, few experimental studies have quantified the exposure time required for coral/algal tissues and skeletal organic material to fully incorporate the $\delta^{15}N$ signature of the N source. In particular, Tanaka et al. (2018) showed that for whole tissue (algae + coral), it takes about 370 d for N to be replaced with new N from the seawater, including uptake from both autotrophic and heterotrophic sources. Other studies have also found long N tissue turnovers in corals (Tanaka et al. 2006).

Of the studies that have looked at the uptake of ^{15}N into coral tissues, most have been focussed on inorganic N uptake (Pernice et al. 2012, Kopp et al. 2013, Tanaka et al. 2015), and only a few have looked at the fate of heterotrophically derived N sources within the coral–algae system (Rodrigues & Grottoli 2006). Furthermore, some of these studies have been based exclusively on short period (48 h) pulse–chase experiments in which it is difficult to calculate reliable tissue turnover rates (Piniak & Lipschultz 2004, Tremblay et al. 2011). In addition, these short labelling experiments do not provide an opportunity to investigate the partitioning of N resources between the coral and the endosymbiont algae. In contrast to N, the effects of heterotrophy on carbon (C) dynamics in the skeletal and tissue fractions of corals have been studied with ^{13}C-labelled tracers, showing evidence of large and bidirectional flow of C between the algae and coral host (Hughes et al. 2010, Levas et al. 2013, Baumann et al. 2014).

Efficient mechanisms for the recycling and conservation of essential nutrients are found in the coral–algae symbiosis, and N has been the focus of attention in this regard (Davy et al. 2012). However, as the endosymbiont lives within the host coral tissue, it remains challenging to unravel the internal recycling of N, and distinguish this N from external sources. Additionally, the flux of N within the coral–algae symbiosis has been shown to be highly variable and dependent on the specificity of the host–symbiont relationship, clade-specific metabolic demands, food source and irradiance levels (Tanaka et al. 2015, Tremblay et al. 2014, 2015).

The objective of this study was to understand the response of coral holobiont N pools, including the N within the skeletal organic matrix, to changes in the $\delta^{15}N$ of a heterotrophic food source. Specifically, we aimed to (1) quantify the time taken for the coral/algal tissue to respond to changes in the availability and $\delta^{15}N$ of a heterotrophic food source (i.e. calculate tissue turnover rates under high and low feeding regimes); (2) confirm that skeletal $\delta^{15}N$ also responds to changes in the heterotrophic food source $\delta^{15}N$; and (3) shed light on the assimilation and excretion of N by the coral–algae symbiosis during heterotrophic feeding. To answer these questions, we fed *Porites lutea* colonies with different amounts of an isotopically enriched heterotrophic food source (rotifers with $\delta^{15}N$ of −3, 5 and 10‰; see Section 2.2 for details) for 60 d and followed the ^{15}N into both the tissue and skeletal N compartments. *P. lutea* was chosen for

study as it is one of the most commonly used coral species for palaeo-biogeochemical reconstructions of oceanic $\delta^{15}N$.

2. MATERIALS AND METHODS

2.1. Coral maintenance

A total of 15 healthy coral colonies of *Porites lutea* from the Great Barrier Reef (QLD, Australia) were sourced from a commercial supplier (Cairns Marine), transported and maintained at the National Marine Science Centre (Southern Cross University). Colonies were fragmented into 5–7 cm diameter pieces and held for a 2 mo acclimation/healing period. During this period, the corals were maintained in a 1200 l outdoor tank system with flow-through seawater pumped from the adjacent beach (30° 16′ 0.91″ S, 153° 8′ 25.27″ E), which was filtered to remove particulate material using an in-sump protein skimmer (JNS; model SK-6), a zeolite media filter (JNS; model FR-2E), an active carbon media filter (JNS; model FR-2E) and a cartridge particle filter (EMAUX®; model CF25, <15 µm). The system was maintained at 26°C using a heat pump (EVO-F5, 2100 l h^{-1}). During this period corals were fed twice a week with hatchery-reared rotifers.

Salinity, temperature and pH were constantly monitored using probes located in the tanks via an Apex Controller (Neptune Systems). Ammonia (mean ± SE: 0.43 ± 0.30 µM), phosphates (0.18 ± 0.07 µM), calcium (3.84 ± 0.20 mM of $CaCO_3$) and alkalinity (78.88 ± 4.47 mg of $CaCO_3$ l^{-1}) were measured once a week with a bench-top photometer system (Palintest®; model 7100). Superficial photon flux density (photosynthetically active radiation [PAR]) was obtained using a light-meter (LI-COR; model 250A) and measured 4 times a day during the experimental period (07:00, 11:00, 15:00 and 19:00 h), with an average of 164.20 ± 215.30 µmol photons m^{-2} s^{-1} (min. = 0 µmol photons m^{-2} s^{-1}, max. = 1071.10 µmol photons m^{-2} s^{-1}; n = 720 measurements). Over the course of the experiment, water temperature was 25.20 ± 0.81°C (n = 720 measurements) on average. An average pH of 8.09 ± 0.09 was recorded.

2.2. Heterotrophic food source maintenance

The heterotrophic food source selected for this study was the rotifer *Brachionus plicatilis* (L-type), as its body size (~0.24 mm; Lavens & Sorgeloos 1996) relative to the coral's polyp size (~1–1.50 mm; Hoeksema 2015) makes it a food type easy to capture, and because it is able to provide N, phosphorus and essential amino acids to corals (Rainuzzo et al. 1997, Houlbrèque et al. 2004). The rotifers were enriched with ^{15}N by feeding on previously ^{15}N-enriched green algae *Nannochloropsis oculata*. The algal cultures were maintained in 3 l bottles. The culture medium was prepared adding 0.65 mM of NH_4SO_4, 0.13 mM of $NaHPO_4$, a supplement of vitamins and trace elements to filtered sterilized seawater. Depending on the level of isotopic enrichment required per treatment, a mixture of isotopically labelled and unlabelled N (in the form of NH_4Cl) was added to the algal cultures. The concentration and enrichment level required to reach –3, 5 and 10‰ enrichment of rotifer N was determined in a pilot experiment. These 3 different levels of enrichment were chosen for our experiment in order to identify gradual increases in the $\delta^{15}N$ values recorded in all tissue fractions, and considering a reasonable number of coral replicates according to our culture setting (Fig. 1A). When the algal density reached around 30 million cells ml^{-1}, an aliquot of concentrated rotifer culture (~1650 rotifers ml^{-1}) was added into each algal bottle. After 2 d of growth with the algae, rotifers were harvested by filtering the cultures with a 45 µm mesh, thoroughly washed with filtered seawater, and resuspended in a minimal volume (~200–500 ml) of 0.22 µm filtered seawater. The isotopically enriched rotifers (which we refer to as ^{15}N-enriched rotifers) were used to feed the coral fragments according to the regime described in the experimental design (see Section 2.3). Aliquots of the enriched rotifer cultures used for each feeding session were dried into tin capsules and wrapped for further $\delta^{15}N$ analysis; the actual $\delta^{15}N$ values along the entire experimental period were (mean ± SE) –3.10 ± 0.96, 4.60 ± 0.61 and 10.20 ± 1.27‰, for simplification purposes referred to here as –3, 5 and 10‰ respectively.

For feeding purposes, an average optimal concentration of 60 rotifers ml^{-1} was calculated for *P. lutea*, by analysing the coral feeding rate (FR):

$$FR = \frac{(\text{Initial} - \text{Final density of rotifers})}{(\text{Number of polyps} \cdot \text{Time})};$$ (Hii et al. 2009)

at 6 different densities of rotifers, using a nonlinear least squares regression with the Michaelis-Menten model (Anthony 1999). This fixed rotifer concentration was used later during all feeding episodes.

It is important to note that rotifer concentrations used in this experiment were substantially higher than the mean density of zooplankton reported in many coral reefs from the Indo-Pacific (Yahel et al.

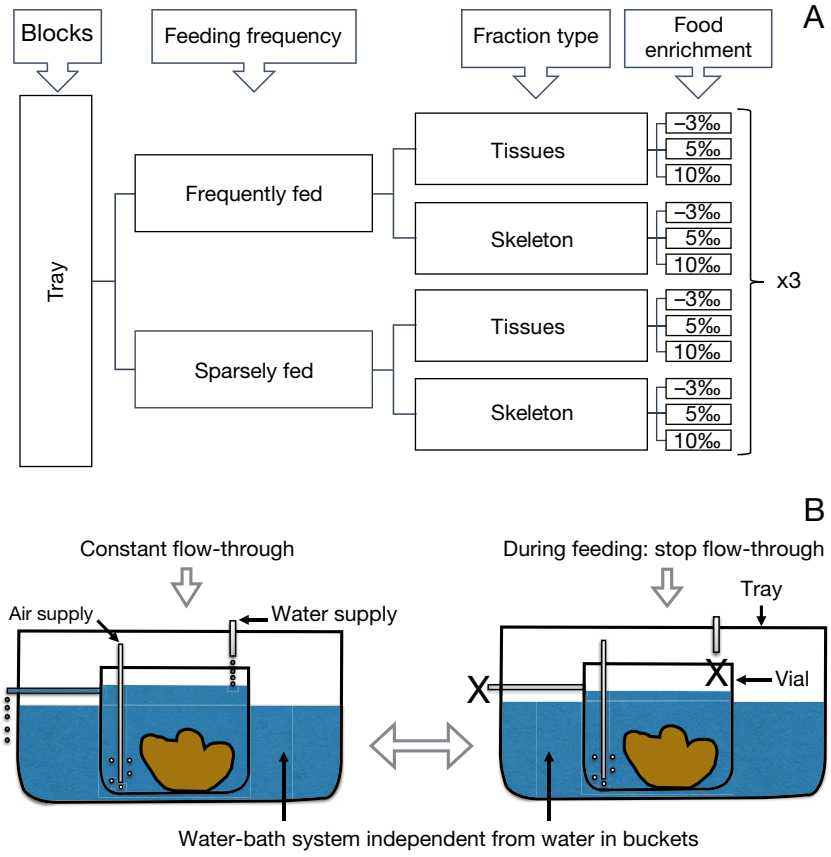

Fig. 1. Flowchart of the (A) experimental design and (B) flow-through system to test the effects of ^{15}N-enriched heterotrophic feeding on the tissue and skeletal δ^{15}N signatures of *Porites lutea*

2005), but within the same order of magnitude as that previously reported in some Caribbean reefs, with zooplankton densities between 23 and 92 ml^{-1} found during the day in Barrie Bow Cay, Belize (Ambler et al. 1991, Heidelberg et al. 2004). As coral feeding rates are known to increase with food availability (Palardy et al. 2006, 2008, Tagliafico et al. 2018a), it could be assumed that under the experimental conditions of this experiment, the input of N via heterotrophic feeding would be substantially higher than under natural reef conditions.

2.3. Experimental design and feeding experiment

The experimental design was based on a generalized randomized block design, with one random factor: blocks (3 trays containing 12 vials each with the coral fragments); and 3 fixed factors: (1) fraction type (levels: coral/endosymbiont algal tissues and skeletons); (2) rotifers ^{15}N-enrichment (levels: –3‰ [non-enriched], 5‰ and 10‰); and (3) feeding frequency (levels: frequently fed and sparsely fed). A total of 3 replicates treatment^{-1} were used, giving a total of 36 coral fragments for all tissue and skeletal analyses (Fig. 1A). Each coral was randomly placed in an individual 500 ml vial with independent air supply, within a tray containing water at constant temperature. A flow-through system was connected (Fig. 1B), with water entering each vial at an average rate of 33 ml min^{-1} and the overflow water being discharged to waste via a separate outlet. Vials were cleaned and corals inspected once a week to remove overgrowing algae.

Prior to the initiation of the ^{15}N-enriched rotifers addition, each coral fragment randomly designated to be part of the skeletal fraction type was incubated with Alizarin to produce a coloured mark in their skeletons in order to identify the starting point of feeding treatments. Fragments were incubated for 6 h with 15 mg l^{-1} of Alizarin.

The ^{15}N-enriched rotifers (60 rotifers ml^{-1}) were added every 2 d to the frequently fed (FF) corals, whereas the sparsely fed (SF) fragments were fed every 2 wk over a total of 60 d. Each feeding episode lasted 2 h, during which time the water flow was stopped. To determine the degree of isotopic change over the course of the feeding trial, the coral and algal tissue from 3 fragments were sampled for their N content and isotopic composition (see below) prior to the initiation of the feeding trial.

Coral health was constantly monitored by measuring colour brightness using the coral health monitor-

ing chart (Siebeck et al. 2006). Additionally, water samples from each coral vial and the supply tank were collected 3 times during the experiment (every 20 d) and 24 h after a feeding session to measure total dissolved nitrogen (TDN) and NO_x concentrations. These samples were filtered (0.45 µm) and frozen (–20°C) for later analysis of ^{15}N in NO_3^- and in the TDN pool.

After 2 mo of feeding, coral replicates intended for skeletal analysis were placed in an aquarium tank (maintaining the same water conditions) for 8 mo to allow the corals to grow and avoid coral tissue contamination in the sections of skeletons used for analysis of CS-$\delta^{15}N$. The remaining coral fragments selected for coral and endosymbiont algal tissue isolation were directly processed after the 60 d period. Tissue samples were separated from the skeletons using an airbrush connected to an air compressor. A total volume of 3 ml of 0.22 µm filtered seawater was used to rinse every coral fragment during air-brush processing to create a tissue slurry and allow the maximal possible tissue extraction. The extracted tissue was collected in individual Ziploc bags, and then poured into plastic centrifuge tubes using a spatula to recover all tissue from the bags. An aliquot of ~500 µl of tissue was separately preserved in 2 ml Eppendorf tubes for determination of symbiont density, as well as chlorophyll and protein concentrations. All samples were immediately placed in liquid nitrogen and then maintained at –80°C until processing. The combined tissue–algal material was homogenised using an OMNI Tissue Master 125; then stirred by using a vortex mixer and centrifuged to isolate endosymbiont algal cells from the coral tissue following Erler et al. (2015). Coral tissues (pooled supernatant from 3 consecutive centrifugations) were vacuum-filtered onto pre-combusted GF/F papers (Whatman). The endosymbiont algal pellets remaining after centrifugation of every sample were freeze-dried, weighed into tin capsules and wrapped for further total N content and $\delta^{15}N$ measurement (as described in Section 2.4). The algal symbiont N content was measured directly from the pellets. This N value was then subtracted from the homogenate to give the remaining N, which is assumed to be from the coral animal. We measured $\delta^{15}N$ on the coral solid material only and therefore assumed that any dissolved N from the animal had the same $\delta^{15}N$ as that caught on the filters.

For the tissue aliquot designated for chlorophyll, symbiont density and host protein determinations, the same procedure (homogenisation followed by 3 sequential centrifugations) was performed. Supernatants were kept at –80°C for host soluble protein measurements (Bradford 1976). Endosymbiont algal tissues (pellets) were resuspended in 3 ml of filtered seawater; 1 ml was separated and used for symbiont counting, and the remaining volume was used for chlorophyll extraction following Gibbin et al. (2015). Chlorophyll *a* (chl *a*) and c_2 (chl c_2) were calculated using the formula for samples in 100% acetone specific for dinoflagellates (Jeffrey & Humphrey 1975). Symbiont density was calculated following the protocol provided by Hill & Scott (2012). Growth rate was calculated by measuring the buoyant weight (Herler & Dirnwöber 2011) at the beginning and end of the 60 d period with an analytical balance (0.001 g resolution). Coral surface area was determined using the aluminium foil method (Marsh 1970) and used to normalise parameters such as total N content, symbiont density, chlorophyll content, host soluble proteins and growth rates.

After 8 mo, coral fragments used for skeletal analyses were cleaned of tissues, rinsed with fresh water, sun-dried for 2 wk, and cut into ~1 cm thick slabs using an electric saw equipped with a diamond blade. Skeletal samples of ~300 µg were drilled from the regions immediately before and after the Alizarin band. The drill hole was ~2 mm, representing about 2 mo of growth (growth rate was based on the distance between the Alizarin band and the top/surface of the skeleton).

2.4. Sample analysis

Concentrations of NO_3^- in water samples from each coral vial and the inlet were measured colourimetrically via flow injection analysis (Wada & Hattori 1971, Patey et al. 2008). TDN was measured colourimetrically after persulphate oxidation of all dissolved N into NO_3^- (Hales et al. 2004, Erler et al. 2015). The concentrations of NO_3^- were too low (<1 µmol l^{-1}) to measure $\delta^{15}N$. The $\delta^{15}N$-TDN was measured following persulfate oxidation of TDN to NO_3^- (Knapp et al. 2005) and then its conversion to N_2O via the denitrifier method (Sigman et al. 2001, Erler et al. 2015). The analysis of the $\delta^{15}N$-N_2O produced was performed with a Thermo Delta V Plus IRMS as described later in this section.

Each of the $\delta^{15}N$ values presented correspond to the average of 3 coral replicates separately analysed via a Thermo Flash EA 1112 coupled to a Thermo Delta V Plus isotope ratio mass spectrometer (EA-IRMS). The $\delta^{15}N$ values for the samples were standardised against Urea and N_2, and the measurement precision was 0.20‰ (standard deviation of the mean

of standards), whereas the precision of repeated measurements of tissue samples was 0.30‰.

Skeletal powder was ground extensively using an agate mortar to destroy any remnants of the corallite structure and then cleaned with sodium hypochlorite (15% for 24 h) to avoid contamination of endoliths, fungi etc., repeatedly washed with ultrapure water and dried at 60°C. For CS-$\delta^{15}N$ analysis, ~15 mg of coral powder was dissolved by reaction with 6 N HCl, oxidised with potassium peroxydisulphate ($K_2S_2O_8$) to yield NO_3^-, and converted to N_2O via the denitrifier method for $\delta^{15}N$-N_2O measurements (Sigman et al. 2001, Erler et al. 2015, 2016, Wang et al. 2015). CS-$\delta^{15}N$ values reported correspond to the average of 3 replicates coral^{-1}.

Analysis of the $\delta^{15}N$-N_2O produced from the coral powders was performed on a Thermo Delta V Plus IRMS. Nitrous oxide was concentrated with a custom-built purge and trap system coupled to the IRMS via a Thermo GasBench II interface. Amino acid reference materials with known $\delta^{15}N$ (USGS 40, $\delta^{15}N$ = −4.52‰; and USGS 41, $\delta^{15}N$ = 47.55‰) were used in each batch of analyses to correct for the reagent and operational blanks. Also, for each batch of samples, 3 blanks with only persulfate reagent were used to determine the blank N concentration. Finally, an in-house coral standard (CBS-II, $\delta^{15}N$ = 6.25‰) provided a metric for reproducibility both within and across batches (Wang et al. 2015, 2016). Analytical precision was 0.50‰ (standard deviation of the mean of CBS-II).

2.5. Statistical analysis

Three-factor permutational ANOVAs (feeding frequency, enrichment and block) were performed using PRIMER v6 with PERMANOVA add-on software (Clarke & Gorley 2006, Anderson et al. 2008); however, in all cases 'block' was not statistically significant and did not interact with the other factors ($p > 0.05$). Two-factor univariate permutational ANOVAs were used to compare the total N content, $\delta^{15}N$ values of coral, endosymbiont algae and skeletal fractions, as well as symbiont density, chl a+c_2 content, host soluble proteins and growth rates between treatments. Data were fourth-root transformed, and Euclidian distance-based resemblance matrices were used to perform the analyses, using 9999 permutations, followed by a post hoc pair-wise test on significant factors and their interactions. For all analyses, statistically significant results were accepted at $p < 0.05$.

Flow rate, temperature and light levels did not show significant effects (all $p \geq 0.38$) when analysed as co-variables of the $\delta^{15}N$ values of the different tissue fractions, symbiont density, chlorophyll, host soluble proteins or growth rates. Similarly, NO_x and TDN content were evaluated as co-variables when total content of N was compared between samples and tissues, and they were not significant ($p \gg 0.05$). Therefore, all co-variables were not included in the final analyses.

2.6. N mass-balance model

Tissue turnover rates (plus growth) for the coral and endosymbiont algal tissues were estimated using the following equation (Matley et al. 2016):

$$\delta_t = (\delta_f + w) + [\delta_i - (\delta_f + w)]e^{(-vt)} \quad (1)$$

where δ_t is the $\delta^{15}N$ value of the correspondent tissue fraction analysed at time t, δ_i is the initial $\delta^{15}N$ value (in this case taken as the $\delta^{15}N$ of tissue in the corresponding control), δ_f is the asymptotic $\delta^{15}N$ at equilibrium (i.e. the isotopic value of the ^{15}N-enriched rotifers used as food), w the difference between the food $\delta^{15}N$ and the final tissue $\delta^{15}N$ value (i.e. the enrichment relative to the food source), and v is the fractional rate of isotopic incorporation into the tissue or tissue turnover. For this calculation, there are 2 unknowns: v and w. To determine these values, we generated 2 simultaneous equations using the $\delta^{15}N$ tissue values for 2 of the treatments. Assuming that v and w will be the same regardless of δ_f, their values were determined by solving the 2 equations. We used only the 10 vs. −3‰ treatment combination of rotifer enrichment to derive an average tissue turnover rate for the FF corals. Tissue turnover could not be calculated for the SF corals because their isotopic composition did not change relative to the control fragments over the feeding period.

The mass balance was based on the change in the ^{15}N content of the different holobiont pools. The premise of the model is that the change in coral tissue N content is equivalent to the amount of N consumed (FN), minus the change in N content of embedded endosymbiont algae and the N excreted from the holobiont to the water column (given as ExN). Each N pool has an associated $\delta^{15}N$ value and therefore 2 equations can be developed (Table 1; Eqs. 2 and 3) and solved to isolate the unknown N transfer pathways, namely the amount of N consumed and the amount of N excreted. The $\delta^{15}N$ value of the excreted N ($\delta ExN = \left[\frac{(\delta CN_a - \delta CN_b)}{2}\right] - x$) is taken to be the same

as that excreted by the coral animal and estimated as the average of the coral tissue $\delta^{15}N$ value over the feeding experiment, minus the difference between the food source and tissue $\delta^{15}N$ calculated earlier in the estimate of tissue turnover (i.e. 3.40‰). The model was run for the –3 and 10‰ treatments. Similar to the tissue turnover, the 5‰ treatment was not used, as the tissue and food source $\delta^{15}N$ values were already close. According to the model there was a negative excretion of N, which is not possible. Further examination of the model equations suggested that the $\delta^{15}N$ of the food source was somehow erroneous. The most likely reason is that, in addition to rotifer N, the holobiont was assimilating TDN with a $\delta^{15}N$ of 6.50‰ (see Section 4 for a full interrogation of this idea). To implement this into the model, we adjusted the food source $\delta^{15}N$ [i.e. δFN = Rotifer $\delta^{15}N \times y$ + TDN × (1 – y)] by accounting for the potential contribution of N from the rotifers and the external TDN pool. We adjusted the fraction of consumed N derived from the rotifers (parameter y) until the FN and ExN values for both the –3 and 10‰ treatments agreed. Both treatments converged on a value of up to 50% contribution of water column TDN to the N content of the holobiont (Fig. 2). Next, we adjusted the $\delta^{15}N$ of the food source values used in the calculation of tissue turnover to estimate a new rate that accounted for the uptake of TDN by the holobiont. Finally, as multiple treatments were used to calculate tissue turnovers, propagation of errors was estimated by decomposing Eq. (1) into smaller components, which were derived following the general rules of subtraction, division and natural logarithm (Rouaud 2013).

Table 1. Symbols and equations used in the mass-balance model of nitrogen movement within the coral–algae symbiosis under heterotrophic feeding. See Fig. 6 for full description of all symbols used in the model equations. FF: frequently fed corals; TDN: total dissolved nitrogen

Symbol	Description	
CN_a, CN_b	Coral N biomass for control and FF colonies (µg of N cm^{-2})	
δCN_a, δCN_b	$\delta^{15}N$ of coral fractions for control and FF colonies (‰)	
ZN_a, ZN_b	Endosymbiont algae N biomass for control and FF colonies (µg of N cm^{-2})	
δZN_a, δZN_b	$\delta^{15}N$ of algae fractions for control and FF colonies (‰)	
y	Fraction of assimilated N coming from the rotifer N pool	
ExN	N excreted by the coral	
δExN	$\delta^{15}N$ of excreted N	
FN	N consumed (rotifers + TDN)	
δFN	Average $\delta^{15}N$ consumed	
x	Tissue turnover + growth	
Equations	$(CN_b - CN_a) = FN - (ZN_b - ZN_a) - ExN$	(2)
	$[(CN_b \times \delta CN_b) - (CN_a \times \delta CN_a)] = (FN \times \delta FN) - [(ZN_b \times \delta ZN_b - ZN_a \times \delta ZN_a)] - (ExN \times \delta ExN)$	(3)

To further illustrate the degree of enrichment of the tissues relative to the food source, we divided Δcoral-$\delta^{15}N$ and Δalgae-$\delta^{15}N$ by the level of food source enrichment. The food source enrichment was normalised by subtracting the starting tissue $\delta^{15}N$ values from the enrichment level of the rotifers. The calculation was also performed using the combined $\delta^{15}N$ of the rotifers and the TDN being consumed by the corals.

3. RESULTS

3.1. Tissue parameters, total N content and $\delta^{15}N$

Univariate and multivariate analysis showed that symbiont density, chlorophyll concentration and host soluble proteins were significantly higher in FF colonies than SF colonies (Table 2, Fig. 3). Symbiont density and total chlorophyll concentrations were strongly and positively correlated ($r[16] = 0.97$, $p < 0.001$).

Average TDN and NO_x concentrations in the inlet water were 8.60 ± 0.16 and 0.12 ± 0.02 µmol l^{-1} respectively. The $\delta^{15}N$ of the inlet water TDN was on average 6.50 ± 0.82‰ (n = 4). No statistical differences in NO_x or TDN content were found between the different treatments (all $p \gg 0.05$).

Total N content in coral tissue was significantly higher than in algal tissue for all feeding and ^{15}N-enrichment regimes (pair-wise test, p = 0.0001) (Fig. 4A). For both coral and algal tissues, N content was significantly higher in the FF corals than in the SF colonies (Table 2). These results were consistent among univariate and multivariate ANOVA analyses.

The $\delta^{15}N$ values of coral or algal tissues for FF colonies did not reach the $\delta^{15}N$ of their food source. For the –3, 5 and 10‰ treatments, the final enrichment of the coral tissue of FF colonies was 3.90, 6.30 and 7.20‰ respectively, whereas for the SF colonies it was 5.40, 5.20 and 4.70‰ respectively. The $\delta^{15}N$ values of –3‰ treated coral tissues were found to be significantly different from both the 5‰ (pairwise test, p = 0.007) and 10‰ (pairwise test, p = 0.001) treatments (Table 2), but no differences were found between the 5 and 10‰ (pairwise test, p = 0.07) treatments. Average change in enrichment in the FF coral tissues relative to the starting

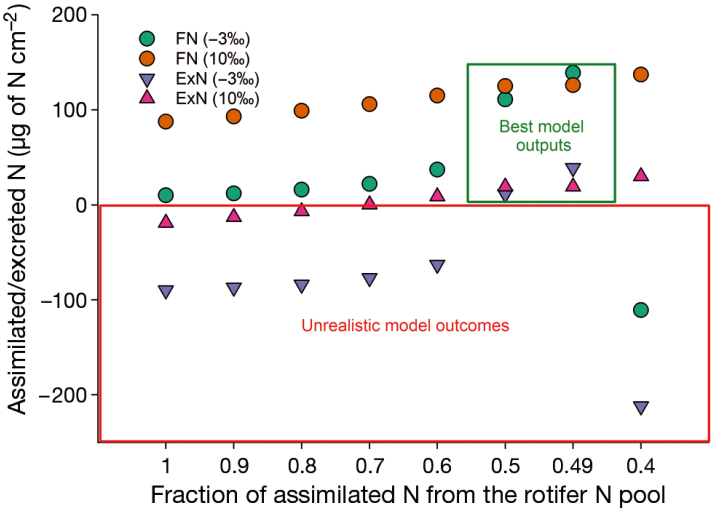

Fig. 2. Adjustments of the nitrogen mass-balance model considering the potential contribution of N from heterotrophic feeding (^{15}N-enriched rotifers) and external total dissolved nitrogen. FN: nitrogen consumed; ExN: nitrogen excreted from the holobiont to the water column

values (referred to as Δcoral-δ^{15}N) grew with increases in the food enrichment (Fig. 4B); in contrast, the coral or algal tissue δ^{15}N in the SF colonies did not change relative to the starting value. Endosymbiont algal tissues of FF colonies displayed a final enrichment of 3.40, 5.20 and 6.10‰, for the –3, 5 and 10‰ treatments respectively; whereas SF colonies showed mean δ^{15}N values of 5.60, 4.80 and 5.20‰ respectively. For the FF treatment, the δ^{15}N values of –3‰ treated algal tissues were significantly different from the 10‰ treated samples (pairwise test, p = 0.002) (Table 2), but no differences were found in the treatment pairs –3 and 5‰ or 5 and 10‰ (pairwise test, all p > 0.052). The average change in enrichment in the algal tissues of FF colonies relative to the starting value (referred to as Δalgae-δ^{15}N) also grew with increases in food enrichment (Fig. 4B).

When comparing coral and algal FF treatments, coral δ^{15}N mean (±SE) values were significantly different (pairwise test, p = 0.04) and slightly higher (5.80 ± 0.90‰) than algae δ^{15}N mean values (4.90 ± 0.90‰) (Table 2). Changes in enrichment of FF coral tissue and endosymbiont algae with respect to SF colonies (i.e. Δcoral-δ^{15}N vs. Δalgae-δ^{15}N) showed a positive but non-significant correlation (r = 0.96, p = 0.13) (Fig. 5A).

Most parameters calculated with the mass-balance model (FN, δFN, CN, ExN and δExN; Fig. 6A), assuming the assimilation of N from rotifers and TDN, were higher for the 10‰ treatment than the –3‰. Endosymbiont algae N biomass (ZN) was the only exception, being higher in the –3‰ treatment. The calculated isotopic value of excreted N (δExN) was 2.95‰ higher for 10‰ treated corals than for those treated with –3‰ enriched rotifers (Fig. 6B,C).

The coral tissue turnover value, which is calculated using the mixed N source determined from the model (i.e. a partial contribution of N assimilated from rotifers and TDN), was 87 ± 10 d (average ± propagated error) with a difference of ~1‰ between the coral and the food source; whereas for the algae it was 111.30 ± 8 d, with a difference of –0.13‰ between the endosymbiont and the food source.

Table 2. Statistical outcomes of univariate permutational ANOVA analyses, testing the effects of feeding frequency, ^{15}N-enriched food and fraction type on *Porites lutea* coral colonies. **Bold** indicates statistically significant effects (p < 0.05). Interactions including the factor 'fraction type' were not included in the table as they were never significant

Variables	Fraction	Feeding		Enrichment		Fraction		Feeding × enrichment	
		Pseudo-*F*	p-value	Pseudo-*F*	p-value	Pseudo-*F*	p-value	Pseudo-*F*	p-value
Total nitrogen content	Coral tissue	8.5327	**0.0196**	1.5406	0.2629	–	–	0.3589	0.703
	Endosymbiont algae tissue	5.6448	**0.0361**	0.7415	0.496	–	–	0.6687	0.5263
	Coral + algae tissues	5.5254	**0.0245**	1.6841	0.197	64.828	**0.0001**	1.1254	0.3429
δ^{15}N value	Coral tissue	1.7418	0.2163	14.52	**0.0002**	–	–	10.088	**0.0024**
	Algae tissue	0.25338	0.6365	4.9718	**0.0206**	–	–	5.1547	**0.0156**
	Coral + algae tissues	0.8635	0.3737	11.835	**0.0001**	4.345	**0.0439**	10.405	**0.0003**
	Coral + algae + skeleton	0.005	0.9694	1.4348	0.2441	7.017	**0.0032**	2.636	0.0873
	Skeletal material	0.9924	0.3392	0.3816	0.692	–	–	2.3312	0.1466
	Before + during feeding skeletal portions	4.0497	0.0549	0.2019	0.8255	4.417	**0.0441**	4.8061	**0.0182**
Symbiont density	Endosymbiont algae tissue	15.386	**0.003**	0.43514	0.6511	–	–	0.42998	0.6496
Chlorophyll $a + c_1$	Endosymbiont algae tissue	11.431	**0.0068**	3.49×10^{-2}	0.9719	–	–	0.80177	0.4773
Host soluble proteins	Coral tissue	19.037	**0.0009**	0.28561	0.7637	–	–	0.51517	0.608
Growth rates	Tissue + skeleton	2.87×10^{-2}	0.9095	0.49232	0.6148	–	–	4.89×10^{-2}	0.9625

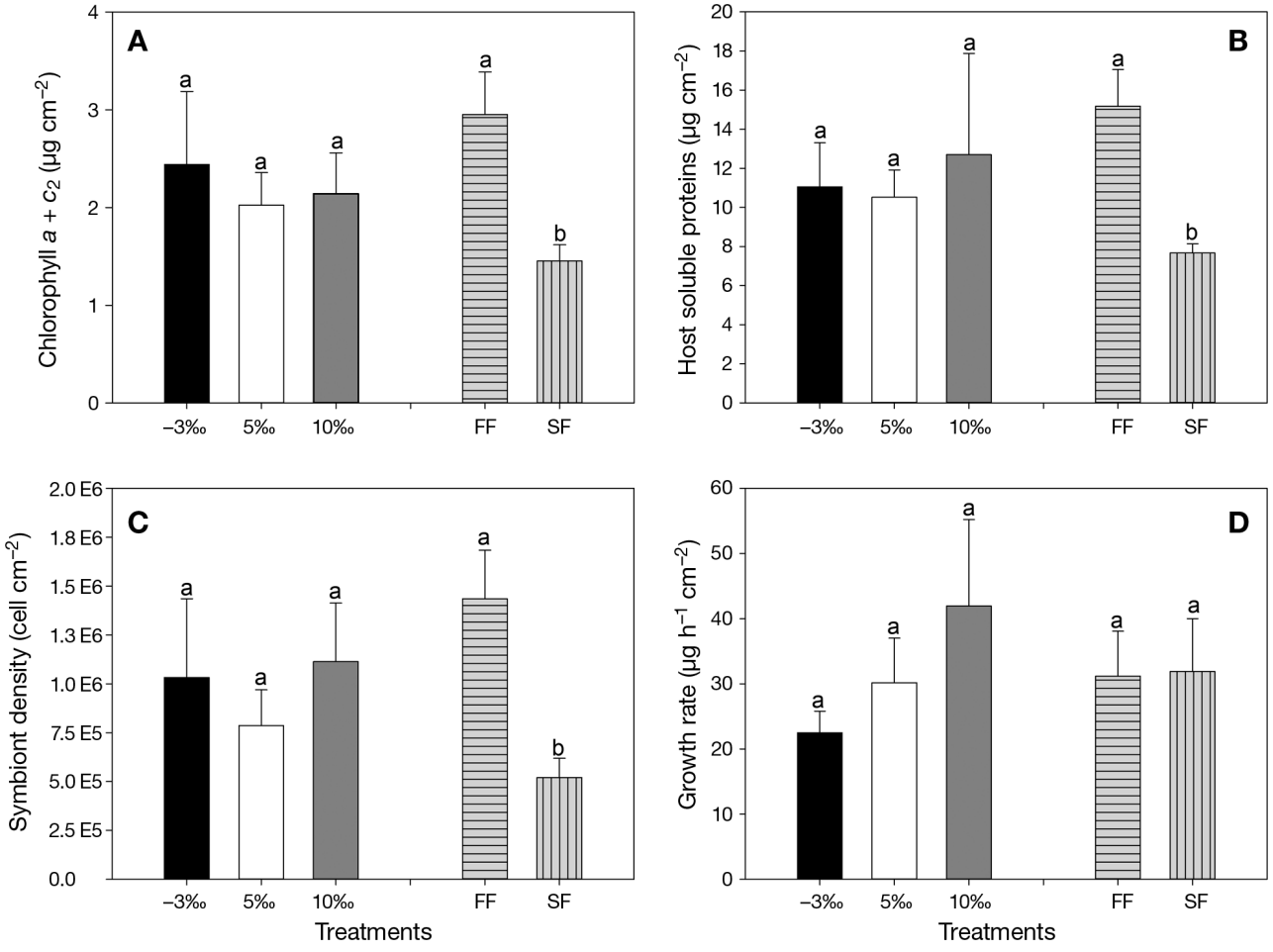

Fig. 3. Coral health parameters in terms of (A) chlorophyll $a + c_2$; (B) host soluble proteins; (C) symbiont density; and (D) growth rates of *Porites lutea* under different ^{15}N-enrichment regimes and feeding frequencies. Bars represent the means (±SE) of the main effects, as there was no significant interaction between feeding frequency and rotifer enrichment. Significant groups (at $p < 0.05$), derived from pairwise post hoc tests (comparing levels of each factor among themselves: −3‰ vs. 5‰ vs. 10‰, whereas frequently fed [FF] vs. sparsely fed [SF]) are indicated with different letters

3.2. Skeletal $\delta^{15}N$

Skeletal $\delta^{15}N$ values of FF colonies did not reach the $\delta^{15}N$ of their food source. For FF corals, we compared the CS-$\delta^{15}N$ after the experimental period to that of the SF treatments; this assumes that, like the tissue, the CS- $\delta^{15}N$ of the SF colonies did not change over the course of the feeding period. The Δskeletal-$\delta^{15}N$ (i.e. CS-$\delta^{15}N$ of the FF colonies minus the CS-$\delta^{15}N$ of the control colonies) for −3, 5 and 10‰ treatments were (average ± propagated error) −0.53 ± 0.50, −0.16 ± 0.50 and 0.57 ± 0.50‰ respectively. These values were all less than the Δcoral-$\delta^{15}N$. No significant differences between enrichment treatments were found in the ^{15}N values of the skeletal fraction (pairwise test, $p = 0.34$) (Table 2). However, skeletal $\delta^{15}N$ values were significantly different and higher than both coral and algal tissues (pairwise test, p-values for comparison with coral and algae fractions: 0.0005 and 0.0018 respectively; Table 2).

Changes in enrichment of FF colonies with respect to the SF colonies for the coral (Δcoral-$\delta^{15}N$) and skeletal (Δskeletal-$\delta^{15}N$) fractions showed a positive but non-significant correlation ($r = 0.82$, $p = 0.23$) (Fig. 5B), as well as for the endosymbiont algae (Δalgae-$\delta^{15}N$) and skeletal fractions ($r = 0.85$, $p = 0.35$) (Fig. 5C). Similarly, a positive but non-significant correlation ($r = 0.98$, $p = 0.20$) was found between Δskeletal-$\delta^{15}N$ and the corresponding ^{15}N-enrichment of their food. The change in skeletal $\delta^{15}N$ relative to the normalised food source $\delta^{15}N$ was 2 or 2.50 times higher (for the −3 and 10‰ treatments respectively) for the contribution in N from rotifers and TDN, than when considering only rotifers as food (Table 3).

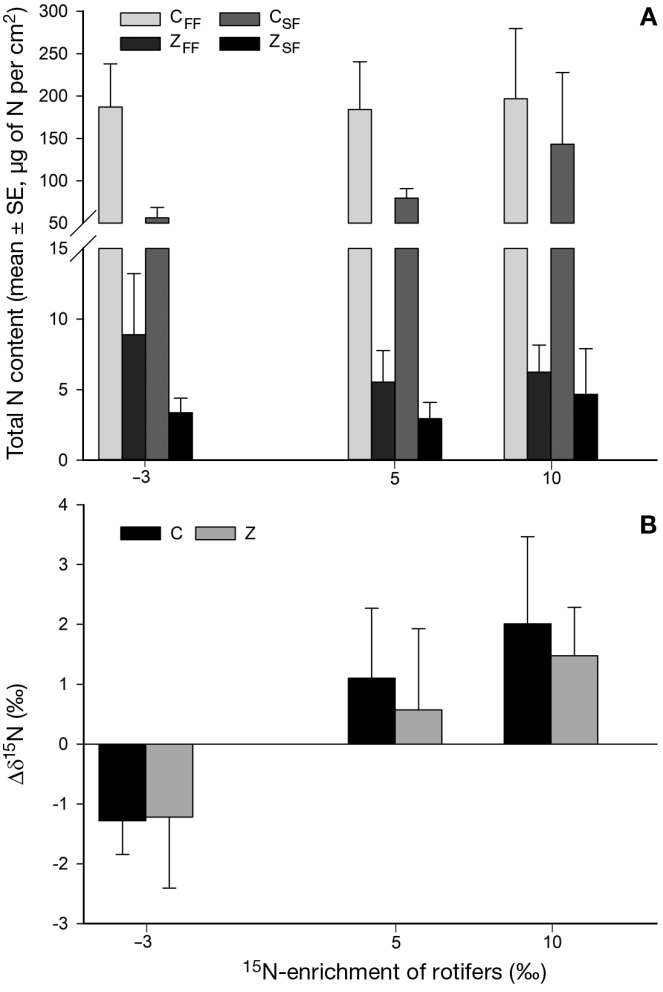

Fig. 4. (A) Total N content and (B) $\Delta\delta^{15}N$ of coral and endosymbiont algae tissues of *Porites lutea* in frequently fed corals under different ^{15}N-enrichment regimes, in relation to control colonies (starting values). Bars represent the means (±SE). C: coral; Z: endosymbiont algae; C_{SF}: sparsely fed coral tissue; C_{FF}: frequently fed coral tissue; Z_{SF}: sparsely fed endosymbiont algae tissue; Z_{FF}: frequently fed endosymbiont algae tissue

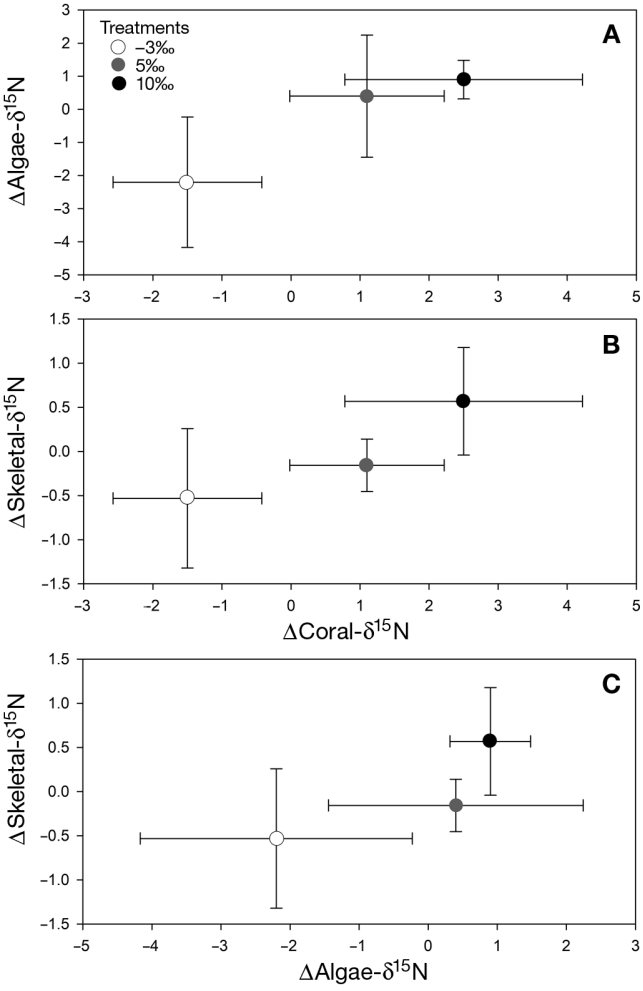

Fig. 5. Mean (±SE) change in enrichment ($\Delta\delta^{15}N$) of frequently fed in relation to sparsely fed corals for (A) endosymbiont algae vs. coral tissues, (B) skeletal vs. coral tissues and (C) skeletal vs. algae tissues of *Porites lutea*

4. DISCUSSION

4.1. Changes in $\delta^{15}N$ of coral tissue and endosymbiont algae

The $\delta^{15}N$ of organic material in the coral skeleton is being increasingly used to reconstruct spatial and/or temporal patterns in water column $\delta^{15}N$ (Yamazaki et al. 2013, 2015, Erler et al. 2016, Wang et al. 2016). However, there is little understanding about the time required for the $\delta^{15}N$ of the water column N source to be reflected in the coral tissue or skeletal $\delta^{15}N$. This study revealed that, at least for *Porites lutea*, it takes about 3 mo for the $\delta^{15}N$ of the food source to be fully recorded in the coral tissue when the food source is unlimited. Results from SF colonies indicate that if the $\delta^{15}N$ of the food source changes, but availability is limited, then the time taken for the tissue $\delta^{15}N$ to emulate the food source $\delta^{15}N$ is much greater than 3 mo (data not shown). This has important implications for the reconstruction of past patterns in water column $\delta^{15}N$. For instance, short-term N inputs, such as sewage contamination or flood discharge events, will only be recorded in the coral tissue of *P. lutea* if the water column N pool is enriched for a relatively long period of time. In highly dynamic or well-flushed environments, substantive changes in water column $\delta^{15}N$ may simply not be recorded in coral tissues.

The coral tissue turnover calculated here for the FF colonies (considering only the contribution of rotifer N) is lower than the reported rates in *Acropora pul-*

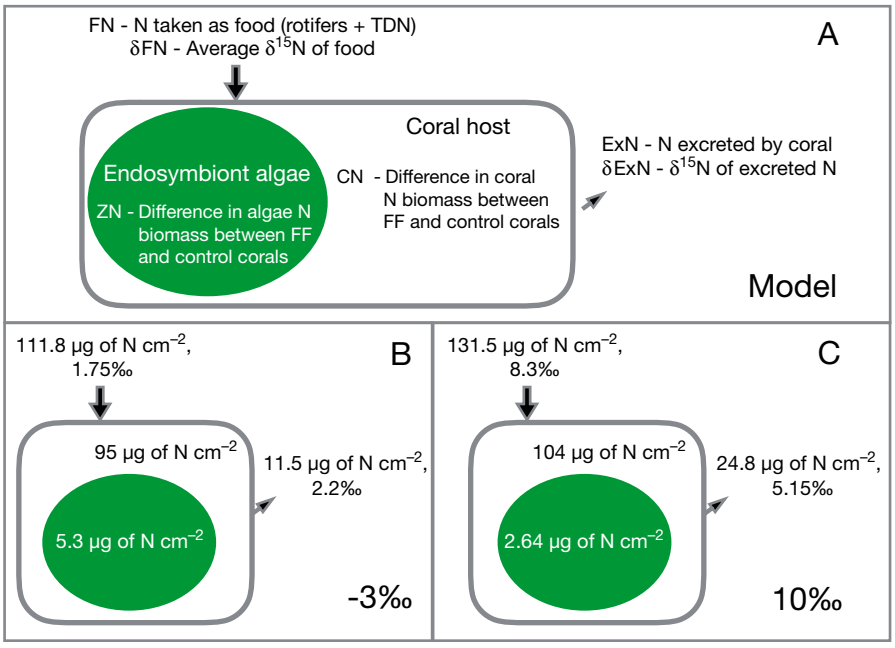

Fig. 6. (A) Conceptual N mass-balance model for N pools of the coral holobiont, describing the correspondent parameters shown in (B) and (C): model outputs for the –3‰ (B) and 10‰ (C) treatments using ^{15}N-enriched rotifers as the N source. FF: frequently fed corals; TDN: total dissolved nitrogen

chra (210 d) supplied with dissolved inorganic nitrogen (DIN), and suggest that coral hosts tend to store N with a long turnover time (Tanaka et al. 2015, 2018). It is known that turnover rates of isotopes are linearly correlated with metabolic rates (MacAvoy et al. 2005), and as a consequence, we can assume that coral tissues of *P. lutea* have great longevity and low growth rates; which is consistent with the fact that these massive corals grow only a few cm every year (between 1.3 and 1.6 cm yr^{-1}; Cantin & Lough 2014).

Other studies have shown that isotopic values in coral may not directly reflect food sources due to low tissue turnover rates (Teece et al. 2011). Furthermore, isotopic values of consumers (such as the coral host) depend on the relative proportion in the diet of the isotopic element of interest, but also on how important the synthesis of compounds containing such isotopic elements is in relation to their absorption from the diet and their turnover rates (Treignier et al. 2009).

In general, assimilation of nutrients can differ between species and depends on the type of food available, making it difficult to establish a unique relationship between what is consumed and how much is assimilated (Pearson et al. 2003). As such, the response of a holobiont's tissue $\delta^{15}N$ is not simply a reflection of the $\delta^{15}N$ of the available food source, but rather a complex interplay between the types of N available and the transfer of N between host and symbiont. For instance, when DIN is available, scleractinian coral species such as *Porites cylindrica*, *Montipora digitata* and *Turbinaria reniformis* displayed higher average endosymbiont algae

Table 3. Changes in holobiont tissues and skeletal-$\delta^{15}N$ (Δtissue/skeletal-$\delta^{15}N$) of fed corals relative to the tissue/skeletal values prior to the start of the feeding period (i.e. $\delta^{15}N_{tissue\ control}$). The Δtissue/skeletal-$\delta^{15}N$ is then presented as a fraction of the difference between the rotifer enrichment and the $\delta^{15}N_{tissue\ control}$, i.e. Δtissue-$\delta^{15}N/\delta^{15}N_{food(rotifers)}$–$\delta^{15}N_{tissue\ control}$, and the rotifer plus total dissolved nitrogen (TDN) enrichment and the $\delta^{15}N_{tissue\ control}$, i.e. Δtissue-$\delta^{15}N/\delta^{15}N_{food(rotifers+TDN)}$–$\delta^{15}N_{tissue\ control}$. Values are means ± propagated error

Tissue	Treatment (‰)	Δtissue/ skeletal-$\delta^{15}N$ (‰)	Δtissue-$\delta^{15}N$ (rotifers vs. controls) (‰)	Δtissue-$\delta^{15}N$ (rotifers+TDN vs. control)(‰)
Coral	–3	–0.18 ± 0.1	0.16 ± 0.08	0.4 ± 0.3
	10	0.28 ± 0.2	0.42 ± 0.46	0.6 ± 0.9
Endosymbiont algae	–3	–0.17 ± 0.2	0.16 ± 0.16	0.4 ± 0.5
	10	0.21 ± 0.1	0.28 ± 0.25	0.4 ± 0.5
Skeleton	–3	–0.07 ± 0.07	0.05 ± 0.05	0.1 ± 0.4
	10	0.08 ± 0.07	0.2 ± 0.32	0.5 ± 1.6

$\delta^{15}N$ values than those of the coral host (Béraud et al. 2013, Tanaka et al. 2015). This study, using rotifers as a heterotrophic food source, showed the contrary: higher $\delta^{15}N$ values of coral tissues compared with algal tissues, and unexpected lower values in both coral and algal tissues compared with their food source, which is similar to the results reported by Reynaud et al. (2009) for *Stylophora pistillata* fed with zooplankton. Predators are expected to be enriched by 3.5‰ compared to their prey, due to the excretion of light N. However, within the coral–endosymbiont algal symbiosis these light waste products seem to be efficiently recycled, therefore muting the expected isotopic enrichment of both algae and coral (Reynaud et al. 2009).

Considering the contribution of TDN and rotifer N in the coral diet, the response of the endosymbiont algae to the change in the $\delta^{15}N$ of the dietary ^{15}N-enriched rotifers (111 d) was slower than for the coral tissue (87 d). It is well-known that corals exert strong control on the number of algae per host cell, as part of the homeostatic process of maintaining a cell-specific symbiont density (Davy et al. 2012). As food availability can lead to higher endosymbiont algal densities in corals (Borell et al. 2008), even mechanisms such as the digestion of symbiont cells can play a key role in controlling the excess of algal cells (Tang 2016). Inside cnidarian host cells, reported duplication times of symbionts are between 10 and 70 d (Davy et al. 2012), and scleractinian corals usually show the lowest cell turnover times (Wilkerson et al. 1988, Jones & Yellowlees 1997). *Porites* species commonly contain clade C15 symbionts (Rodriguez-Lanetty et al. 2004, Fitt et al. 2009), which seem to have lower growth rates than other endosymbiont algae clades (Tremblay et al. 2015), and *P. lutea* has been previously characterised with a low mitotic index (Mwaura et al. 2009).

With sufficient nutrient supply, doubling times of the endosymbiont algae would be stimulated (Chang et al. 1983), thereby reducing the algal tissue turnover. However, our results in relation to the lack of differences in growth between FF and SF colonies suggest that the corals were strongly controlling their endosymbiont populations, possibly increasing the ingestion of endosymbionts and resulting in an algal tissue turnover larger than previously reported (Tanaka et al. 2006). Other possible explanations for this lack of responsiveness in growth between SF and FF colonies are (1) increases in the excretion of dissolved organic matter into the seawater (Wild et al. 2004); or (2) increases in respiration rates due to a larger production of photosynthates caused by higher chl *a* concentrations and symbiont densities in FF colonies. Imbalances in the rates of C-fixation due to photosynthesis and calcification, as well as respiration, may affect long-term coral growth (Tanaka et al. 2007). In fact, under nutrient-enriched scenarios leading to increases in chl *a*, Tanaka et al. (2007) found that calcification was left behind with respect to C-fixation rates producing tissue growth, possibly due to competition for the internal dissolved inorganic carbon (DIC) pool between photosynthesis and calcification (Marubini & Davies 1996).

Most heterotrophic organisms show an average ^{15}N-enrichment of 3.5‰ relative to their food, mainly due to the excretion of isotopically light N (Minagawa & Wada 1984, Chikaraishi et al. 2007). Results from the present study identified a fractionation between the coral and its food source of 1‰ (from the tissue turnover model) and a measured coral tissue enrichment of only 2‰ relative to the starting value (for the 10‰ treatment). This subdued enrichment is perplexing given that the corals in the FF treatments clearly increased N content, even when considering a low tissue turnover. It is possible that the coral was not excreting assimilated N and therefore the food source enrichment was not fully expressed in the tissues. An efficient recycling and conservation of N within the coral–algae system has been suggested as one of the possible reasons why corals are unlikely to display the typical trophic level effect under heterotrophic conditions (Heikoop et al. 1998). Additionally, corals display a plastic and opportunistic trophic behaviour in response to the availability of resources in the water column, and as a consequence, they cannot be considered completely autotrophic or heterotrophic at any time (Risk et al. 1994, Sammarco et al. 1999).

Consequently, it is likely that the corals were actively assimilating TDN (which had a $\delta^{15}N$ value of 6.5‰ and a concentration of 8.6 µmol l^{-1}) in addition to rotifer N (Tanaka et al. 2015). Corals were fed with rotifers every second day for 2 h in the FF treatments, and therefore could have been assimilating TDN during the non-feeding periods. While we observed active feeding of rotifers during the experiment (average feeding rate of 0.03 ± 0.01 rotifers $polyp^{-1}$ h^{-1}), it is likely that the holobiont tissue was in some way influenced by the $\delta^{15}N$ of TDN in the inlet water (Tanaka et al. 2015). Measurements of DIN concentration in the inlet water to the experimental units were consistently below 1 µM. As such, it is more likely that there was TDN uptake rather than DIN uptake into the holobiont. The isotope mass-balance proved to be an effective way of accounting for the

possible assimilation of external TDN by the corals during the feeding experiments (see Section 4.2).

4.2. Estimating the assimilation and excretion of N using an isotope mass balance

The cycling of N within the coral holobiont when heterotrophic food sources are available remains poorly understood (Gustafsson et al. 2013). By analysing the change in total N content and isotope ratio of the coral and endosymbiont algal tissues of the FF corals in relation to initial conditions (controls), we were able to construct a mass-balance model of N movement during heterotrophic feeding. We were interested in assessing the amount of assimilated N versus the amount of N excreted by the holobiont, and estimating the possible consumption of water column TDN by the holobiont.

As the endosymbiont lives within the host coral tissue, it has been challenging to understand the internal recycling of N under different feeding conditions since the specific mechanisms of coupling between the uptake of inorganic nutrients by the endosymbiont algae and heterotrophic feeding by the coral host still remain unclear (Ezzat et al. 2016). However, the mass-balance model presented here allows calculation of rates of N assimilation and excretion under the combination of heterotrophic feeding and TDN consumption (Fig. 6C,D). The estimated amount of ingested N from the food (including rotifers and TDN available in the water column) was substantially higher (between 3 and 5 orders of magnitude) than previously reported in other scleractinian corals relying on available N from suspended sediments (Mills et al. 2004), but very similar to what has been reported for the temperate coral *Oculina arbuscula* fed with brine shrimps (Piniak & Lipschultz 2004). The average amount of excreted N represented around 15% of the average amount of assimilated N for the –3 and 10‰ treatments.

Different interpretations of the flux of N within corals have been provided. The nitrogen-recycling hypothesis predicts large bidirectional flows of N between the coral and its symbiont algae (Piniak & Lipschultz 2004). In contrast, studies supporting the nitrogen-conservation hypothesis suggest that corals should preferentially respire photosynthetic C from the endosymbiont algae, reduce amino acid catabolism and rates of ammonium production, and as a consequence, increase the N stores in the host and decrease the amount of N recycling (Wang & Douglas 1998); however, even authors supporting this theory consider that N recycling may allow corals to survive with nutrient restriction for short periods (Piniak & Lipschultz 2004).

The quantity of N available to corals depends on the contribution of feeding activity and the uptake of dissolved nutrients (Piniak & Lipschultz 2004). In fact, the observed changes in $\delta^{15}N$ for the coral and algal fractions relative to the normalised food $\delta^{15}N$ (considering both rotifers and TDN) support this (Table 3), with higher uptake of ^{15}N occurring when the coral uses both sources of nitrogen. As inorganic nutrients can be scarce (D'Elia & Webb 1977, 1990), and plankton is usually found in low abundance or is patchily distributed in coral reefs (Johannes et al. 1970), it has been proposed that corals may develop nutrient conservation mechanisms (Piniak & Lipschultz 2004). According to our results and the model (Fig. 6), the coral tissue is the main reservoir of N in the symbiosis, equivalent to what Tanaka et al. (2015) showed from a DIN perspective. It is reasonable to think that a large host N biomass could provide a more stable internal pool of N for the endosymbiont algae, and as consequence, could promote stability of the symbiotic relationship (Tanaka et al. 2015) by providing enough N availability to support high symbiont densities (Rädecker et al. 2015). Additionally, it reinforces the idea that frequent heterotrophic feeding enhances the general health condition of corals, reflected in higher density of symbionts, chlorophyll and protein content (Ferrier-Pagès et al. 2003, Tagliafico et al. 2017, 2018b).

4.3. Skeletal $\delta^{15}N$

The change in the coral and endosymbiont algae $\delta^{15}N$ values of the FF colonies relative to the control colonies was higher than skeletal $\delta^{15}N$ values, which raises concerns over the use of CS-$\delta^{15}N$ as a proxy for tissue and water column $\delta^{15}N$. When these changes in enrichment were re-calculated considering the simultaneous contribution of N from rotifers and TDN, the skeletal fractions showed a better differentiation between the –3 and 10‰ treatments, compared with the coral and algae fractions; however, the magnitude of the propagated errors of these calculations prevent us from making a more firm assertion (Table 3).

Notwithstanding this, and considering the newly calculated $\delta^{15}N$ of the food source N (i.e. including both rotifer and TDN), the fraction of incorporation of food source N into the skeletons changed on average from 0.125 to 0.3 ‰ (Table 3). This implies that ^{15}N

uptake increases when corals were feeding on rotifers and TDN

We suspect that the small change in skeletal enrichment of fed colonies relative to starting values is most likely an analytical rather than a biological issue. A possible explanation for this inconsistency between coral and skeletal values could be the drilling technique. When the drill hole is large (>1 mm), there is a dilution of the powder from the period of interest (in this case the period corresponding to the feeding treatment) with skeletal portions from surrounding periods. Consequently, fine-scale drilling is needed when skeletal samples are used for isotopic analyses, otherwise short-term changes in $\delta^{15}N$ are likely to be missed. The lack of significant change in the CS-$\delta^{15}N$, therefore, is a function of the relatively short enrichment period (2 mo), which possibly was not long enough for the tissue to fully respond to the food source $\delta^{15}N$, making it difficult to isolate the coral powders from the exact period of the experiment.

It is also interesting to note how unusual it was to observe significant increases in tissue parameters (chlorophyll content, proteins and symbiont density) of FF corals but not increases in growth rates compared with SF colonies. A possible explanation for this could be that the buoyant weight method is not sensitive enough to detect the small increases in weight occurring after the 2 mo feeding period. Another possible reason for this lack of difference in growth between treatments could be the low average light level (164.2 ± 215.3 µmol photons m^{-2} s^{-1}) during the experimental period, as light has been shown to have a crucial influence on growth rates (Highsmith 1979, Risk & Sammarco 1991, Lough & Cooper 2011). Additionally, Alizarin treatments can produce negative effects on growth and polyp expansion (Dodge et al. 1984, Holcomb et al. 2013), which can also affect feeding behaviour and consequently could also have affected our results. However, as all colonies were stained with Alizarin, there is no way to isolate and measure its potential effect on our results.

We found evidence of a long N-tissue turnover for the coral and endosymbiont algal fractions when using TDN and a heterotrophic food source; consequently, the $\delta^{15}N$ of the food source was not fully reflected in the skeletal fractions. An important implication of this result is that short-duration changes (i.e. less than 3 mo) in the $\delta^{15}N$ of the water column N may not be fully recorded in coral skeletal organic N pools due to their relatively long tissue turnover rates. Finally, the outputs of our proposed N mass-balance model support the notion of a bidirectional exchange of N between the coral and endosymbiont algal fractions, as well as providing some estimations of the assimilation and excretion of N by the coral–algae symbiosis during heterotrophic feeding.

More experiments are needed to test the effects of different combinations of inorganic and organic sources of N on the tissue and skeletal $\delta^{15}N$ signatures in order to better understand the balance and contribution of autotrophy and heterotrophy in corals.

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