



Isotopic composition of skeleton-bound organic nitrogen in reef-building symbiotic corals: A new method and proxy evaluation at Bermuda

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Abstract

The skeleton-bound organic nitrogen in reef-building symbiotic corals may be a high-resolution archive of ocean nitrogen cycle dynamics and a tool for understanding coral biogeochemistry and physiological processes. However, the existing methods for measuring the isotopic composition of coral skeleton-bound organic nitrogen (hereafter, CS- $\delta^{15}\text{N}$) either require too much skeleton material or have low precision, limiting the applications of this relatively new proxy. In addition, the controlling factors on CS- $\delta^{15}\text{N}$ remain poorly understood: the $\delta^{15}\text{N}$ of source nitrogen and the internal nitrogen cycle of the coral/zooxanthellae symbiosis may both be important. Here, we describe a new (“persulfate/denitrifier”-based) method for measuring CS- $\delta^{15}\text{N}$, requiring only 5 mg of skeleton material and yielding a long-term precision better than 0.2‰ (1 σ). Using this new method, we investigate CS- $\delta^{15}\text{N}$ at Bermuda. Ten modern *Diploria labyrinthiformis* coral cores/colonies from 4 sampling sites were measured for CS- $\delta^{15}\text{N}$. Nitrogen concentrations (nitrate + nitrite, ammonium, and dissolved organic nitrogen) and $\delta^{15}\text{N}$ of plankton were also measured at these coral sites. Among the 4 sampling sites, CS- $\delta^{15}\text{N}$ shows an increase with proximity to the island, from $\sim 3.8\text{‰}$ to $\sim 6.8\text{‰}$ vs. atmospheric N_2 , with the northern offshore site having a CS- $\delta^{15}\text{N}$ 1–2‰ higher than the $\delta^{15}\text{N}$ of thermocline nitrate in the surrounding Sargasso Sea. Two annually resolved CS- $\delta^{15}\text{N}$ time series suggest that the offshore-inshore CS- $\delta^{15}\text{N}$ gradient has persisted since at least the 1970s. Plankton $\delta^{15}\text{N}$ among these 4 sites also has an inshore increase, but of only $\sim 1\text{‰}$. Coral physiological change must explain the remaining ($\sim 2\text{‰}$) inshore increase in CS- $\delta^{15}\text{N}$, and previous work points to the coral/zooxanthellae N cycle as a control on host tissue (and thus carbonate skeletal) $\delta^{15}\text{N}$. The CS- $\delta^{15}\text{N}$ gradient is hypothesized to result mainly from varying efficiency in the internal nitrogen recycling of the coral/zooxanthellae symbiosis. It is proposed that, in more productive inshore waters, greater food uptake by the coral causes a greater fraction of its low- $\delta^{15}\text{N}$ regenerated ammonium to be excreted rather than assimilated by zooxanthellae, raising the $\delta^{15}\text{N}$ of the inshore corals. If so, coral tissue- and CS- $\delta^{15}\text{N}$ may prove of use to reconstruct and monitor the state of the coral/zooxanthellae symbiosis over space and time.

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1. INTRODUCTION

Because reef-building corals are widespread in the tropical and subtropical ocean and have a relatively fast growth rate (0.5–2 cm/year), coral skeleton represents one of the most promising recorders of climate variability in the low-latitude ocean. Applications to date include the reconstruction of high-frequency climate variability associated with the El Niño-Southern Oscillation (ENSO) (Dunbar et al., 1994; Gagan et al., 2000; Watanabe et al., 2011; Cobb et al., 2013), the Pacific Decadal Oscillation (Linsley et al., 2000), and the North Atlantic Oscillation (Kuhnert et al., 2005; Goodkin et al., 2008). Previous coral-based paleoceanographic studies have focused on the inorganic components of coral skeleton, with attention to the organic matter within coral skeleton only recently (e.g., LaVigne et al., 2008), primarily due to the analytical challenges of working with this relatively organic-poor material.

The coral's aragonite forms at the interface of coral tissue and the underlying skeleton. Seawater containing calcium ions and dissolved inorganic carbon is transported via paracellular pathways (Cohen and McConnaughey, 2003) to an extracellular space between the calicoblastic epithelium and the existing skeleton, where crystals are nucleated and grown. The organic matrix is synthesized in the calicoblastic epithelium and then transported into the space below (Muscatine et al., 2005; Drake et al., 2013), and it has been hypothesized to serve as the calcification nucleus and control the crystallographic direction of the aragonite precipitation (Cuif et al., 1999; Mass et al., 2013). The skeleton-bound organic matter reflects the carbon and nitrogen sources of the coral-zooxanthellae symbiotic system (Drake et al., 2013) and may thus provide insights into past ocean biogeochemical conditions and/or the physiological and ecological history of corals.

Several studies have explored the isotopic composition of coral skeleton-bound organic nitrogen (hereafter, CS- $\delta^{15}\text{N}$) in reef-building symbiotic corals, using a variety of methods. Muscatine et al. (2005) did a CS- $\delta^{15}\text{N}$ survey in the modern ocean, finding that the CS- $\delta^{15}\text{N}$ in symbiotic coral ($4.09 \pm 1.51\text{‰}$, $n = 24$) is much lower than CS- $\delta^{15}\text{N}$ in non-symbiotic corals ($12.28 \pm 1.81\text{‰}$, $n = 17$). They suggested that CS- $\delta^{15}\text{N}$ is a proxy for the coral host/zooxanthellae symbiosis, using their data to argue that this symbiosis may have started as early as the Triassic. Marion et al. (2005) generated CS- $\delta^{15}\text{N}$ downcore records at Bali, Indonesia. They interpreted CS- $\delta^{15}\text{N}$ as a proxy for the $\delta^{15}\text{N}$ of the fixed nitrogen sources to the reef and found that low- $\delta^{15}\text{N}$ synthetic fertilizer has been affecting coastal coral reefs since the 1970s. Yamazaki et al. (2011) also interpreted CS- $\delta^{15}\text{N}$ as a proxy for the $\delta^{15}\text{N}$ of nitrogen sources. On a subtropical island near Taiwan, where the riverine nitrate is the dominant nitrogen source to the reef, they observed a correlation between an onshore-offshore CS- $\delta^{15}\text{N}$ gradient and the gradient in surface water nitrate $\delta^{15}\text{N}$.

At this point, two major issues limit the utility of CS- $\delta^{15}\text{N}$. First, the methods so far employed have had major drawbacks. At least three different methods have been used

to measure CS- $\delta^{15}\text{N}$ (Muscatine et al., 2005; Uchida et al., 2008; Yamazaki et al., 2013), and these methods either require large quantities of skeletal material (e.g., >1 g; Marion et al., 2005; Muscatine et al., 2005) and/or have very low precision (e.g., 1 SD greater than 0.75‰; Uchida et al., 2008; Yamazaki et al., 2013). Because of the difficulty of the measurements, there has also been limited method testing (e.g., of cleaning protocols). Second, beyond the methodological issues, the controlling factors on CS- $\delta^{15}\text{N}$ remain poorly understood. The $\delta^{15}\text{N}$ of the nitrogen sources to the reef is expected to affect CS- $\delta^{15}\text{N}$, and there are now some data in support of this expectation (Yamazaki et al., 2011). However, coral physiology, including the symbiosis with zooxanthellae, may also be important (Muscatine et al., 2005; Swart et al., 2005).

In this study, we seek to make inroads on both of these problems. First, we report the development of a robust, highly sensitive method for measuring CS- $\delta^{15}\text{N}$, based on previous foraminifera-bound $\delta^{15}\text{N}$ studies (Ren et al., 2009, 2012). This method requires ~5 mg of coral skeleton material and yields a long-term precision better than 0.2‰. Second, with this method, we measured CS- $\delta^{15}\text{N}$ from 10 cores/colonies from 4 sites on the pedestal of Bermuda, as well as nutrient concentrations and plankton $\delta^{15}\text{N}$ from the same sites. We find a ~3‰ inshore-offshore gradient in CS- $\delta^{15}\text{N}$, at least ~2‰ of which requires a physiological (rather than environmental N isotopic) explanation. We propose that variation in the efficiency of the internal nitrogen recycling between the coral host and its zooxanthellae is responsible for the observed CS- $\delta^{15}\text{N}$ difference among the sites.

2. METHODS

2.1. Background

Several groups have undertaken in-depth studies of the nitrogen isotopic composition of coral skeleton-bound organic matter. Hoegh-Guldberg et al. (2004) and Muscatine et al. (2005) generated powder from coral, cleaned it with sodium hypochlorite and hot NaOH, and decalcified the product with HCl. The dissolved organic matrix was then separated from other solutes by dialysis, evaporated to dryness, and combusted to N_2 , which was analyzed by isotope ratio mass spectrometry (IRMS). As published, their protocol requires 15,000–50,000 mg of coral skeleton, which compromises its utility.

Marion et al. (2005) used a similar approach. However, cleaning was not included in their protocol. They dissolved their coral powder samples with 1 N HCl and then filtered out the acid-insoluble residue, which they regarded as the coral skeleton-bound organic matter. The sample was then combusted to N_2 and analyzed by IRMS. This protocol yielded a precision of 0.40‰ (1 σ) and required >750 mg of coral powder. The potential for loss of soluble organic N in the filtration step was an additional concern.

Uchida et al. (2008) applied a novel methodology. A step-wise heating method was used to destroy the structure of aragonite and combust the organic nitrogen to N_2 . On-line with this heating system, a static vacuum mass

spectrometer was used to measure the nitrogen isotopes of the product N_2 . Because of the use of the static vacuum mass spectrometer, sensitivity was substantially improved relative to the previous protocols, with a sample size requirement of 35 mg. However, precision was relatively poor, with an overall precision of $>1.0\text{‰}$ (1σ).

Yamazaki et al. (2011, 2013) undertook measurements in an approach similar to the methods that Robinson et al. (2004) developed for diatom-bound organic nitrogen and that Ren et al. (2009) developed for foraminifera-bound organic nitrogen. Coral powder was cleaned with hot NaOH, dissolved with acid, and the exposed material oxidized to nitrate with basic potassium persulfate. The nitrate was converted to NO_2^- by cadmium reduction, followed by reduction of NO_2^- to N_2O with sodium azide (McIlvin and Altabet, 2005), and the N_2O was then analyzed with a purge-trap system coupled to a mass spectrometer, similar to that of Casciotti et al. (2002). This method requires ~ 30 mg of coral powder, but coral sample replicates indicate only modest reproducibility ($1\sigma = 0.75\text{‰}$, Yamazaki et al., 2013).

2.2. Protocol

Adapting the method developed for foraminifera shell-bound organic N isotopes by Ren et al. (2009), we have established a protocol for measuring the $\delta^{15}N$ of coral skeleton-bound organic matter. 5–10 mg of coral powder per sample is generated from coral skeleton and cleaned oxidatively with sodium hypochlorite (reagent grade, 10–15%) for 24 h in a 15 mL polypropylene centrifuge tube. The centrifuge tube is oriented horizontally and shaken vigorously every 6 h during the 24-h cleaning period. The reagent is decanted, and the sample is rinsed three times with deionized water. The sample is transferred into a 12 mL pre-combusted glass vial, and a pre-combusted glass pasteur pipette connected to house vacuum is used to remove the liquid above the sample. The sample is dried at 60 °C. Once dry, the sample is weighed and transferred into combusted 4 mL glass vials. The sample is dissolved by reaction with 4 N HCl. An aliquot of 1 mL freshly combined persulfate oxidizing reagent (1 g recrystallized low-N potassium persulfate and 2 g ACS grade NaOH in 100 mL deionized water) is added, and the sample is autoclaved for 1.5 h to completely oxidize to nitrate the organic N released during decalcification. After oxidation, 1 mL deionized water is added to the sample. After centrifugation for 10 min at 3000 rpm, the clear supernatant is transferred to a pre-combusted 4 mL glass vial. The pH of the supernatant is adjusted to neutral with 4 N HCl and 2 N NaOH. The nitrate concentration of the sample solution is analyzed by chemiluminescence (Braman and Hendrix, 1989), and the $\delta^{15}N$ of the nitrate is measured by conversion to N_2O with the “denitrifier method” (Sigman et al., 2001), followed by extraction, purification, and isotopic analysis of the N_2O product (Casciotti et al., 2002).

The N blank of the persulfate reagent, after recrystallization of the potassium persulfate, yields 0.2–0.4 μM nitrate, which is less than 5% of the total N content in an oxidized sample. In order to correct for this blank, amino

acid standards with known $\delta^{15}N$ (USGS 40, $\delta^{15}N = -4.5\text{‰}$; and USGS 41, $\delta^{15}N = 47.6\text{‰}$) are included in each batch of samples. For each set of analyses, three “blanks” with only persulfate reagent are used to determine the blank N concentration. A linear regression based on equation (1) was used to determine the $\delta^{15}N$ of the persulfate blank, where AA refers to the amino acid standard:

$$\delta^{15}N_{AA\text{-true}} \times \left(1 - \frac{\text{Blank N}}{\text{Total N}}\right) + \delta^{15}N_{\text{blank}} \times \frac{\text{Blank N}}{\text{Total N}} = \delta^{15}N_{AA\text{-measured}} \quad (1)$$

2.3. Method validation

Because our method has much higher sensitivity (only 5 nmol $N_2O\text{-N}$ is introduced on-line to the mass spectrometer) and requires multiple manipulations in the lab, nitrogen contamination must be minimized throughout the process. An in-house coral standard was established to help with quality control. A ~ 5 g piece of *Porites* from Great Barrier Reef was ground and homogenized and is now used as an in-house standard for method testing and consistency control. In the processing of each sample batch, three replicates of the coral standard are included. The long-term precision of our method as calculated from CS- $\delta^{15}N$ of the coral standard for 13 batches of analyses is 0.19‰ (1σ , Fig. 1).

Because the surface of the coral skeleton can be easily contaminated by extraneous N, the cleaning process is critical for removing any contaminant N on the surfaces of the skeleton powder (Ramos-Silva et al., 2013). To characterize the effect of the cleaning, we compared the NaOCl cleaning protocol with an analogous persulfate-based cleaning used for foraminifera-bound N isotope analyses (Ren et al., 2012) and with no cleaning. The coral standard cleaned by either method has much lower nitrogen content and quite different CS- $\delta^{15}N$ than uncleaned standard (Fig. 1),

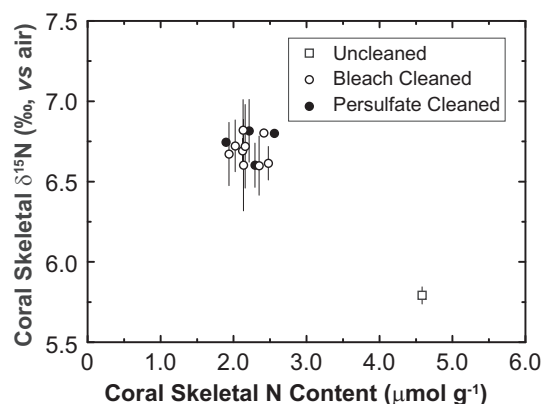


Fig. 1. Comparison of measured N content and $\delta^{15}N$ of an in-house coral standard for the past 13 independent batches of CS- $\delta^{15}N$ analyses and two different cleaning methods. Coral standard was analyzed during each independent run as the quality control. Each point represents an independent run and the error bar (1σ) was calculated from three replicates of the coral standard during each run. The long-term precision is 0.19‰ (1σ).

indicating that cleaning is critical in removing contaminant N. We observe no difference between the two cleaning protocols, indicating that both protocols are appropriate.

One concern with the isotopic analysis of the organic N bound within coral carbonate is that there are no morphological criteria (e.g., the surfaces vs. the interior of a shell wall) by which to distinguish contaminant N from skeleton-protected, coral-derived N. When sample powder is milled out of the coral, much of the N on the powder grain surfaces likely derives from the coral and was protected by the carbonate matrix up to the point of milling. Conversely, it cannot be assumed that the milling exposes all contaminant N, some of which may reside within pores in the powder grains that are effectively isolated on the timescale of the chemical cleaning. To evaluate these issues, a piece of coral skeleton from Oman margin (*Porites*) was ground and sieved into two different sizes, smaller than 63 μm and 63–250 μm . Cleaning, digestion, and isotopic analysis of these two size classes of powder indicated that the

fineness to which the sample was ground prior to cleaning had only minor effects on CS- $\delta^{15}\text{N}$ and nitrogen content (Table 1). If anything, N content was slightly higher for the more finely ground material, in the opposite sense of expectations if contaminant N persisted on external skeletal surfaces. The results suggest that any contaminant N in the corals is evenly disseminated throughout the coral or that any contaminant N exists on skeletal surfaces is fully exposed to cleaning in either grain size. This is most easily explained by the lack of a significant contaminant N pool in oxidatively cleaned coral even if it was only coarsely ground before cleaning. In the standard protocol, we thus do not sieve the coral skeleton powder into different sizes after the powder is drilled out from the coral slabs.

3. MATERIALS AND OCEANOGRAPHIC SETTING

3.1. Corals

Ten coral heads (named BER 008, 009, 010, 011, 012, 013, 014, 015, 016 and 017) were collected from living *Diploria labyrinthiformis* at four sites around Bermuda in July 2005 (Fig. 2). BER 008 and 009 are from John Smith's Bay at a depth of ~ 13 m, off the southern side of the island ~ 400 m from shore and just beyond the boiler reefs; BER 016 and 017 are from Tynes Bay at a depth of ~ 6 m; BER 010, 011 and 012 are from Crescent Reef at a depth of ~ 4 m; and BER 013, 014 and 015 are from Hog Reef at a depth of ~ 10 m. A 1 cm-thick slab was cut from each coral core. The slabs were rinsed with deionized water,

Table 1
Test result of the powder size effect on coral skeletal $\delta^{15}\text{N}$.

Coral ID	Powder size (μm)	N content ($\mu\text{mol N g}^{-1}$)	Skeletal $\delta^{15}\text{N}$ (‰ , vs. air)
A	<63	2.02 ± 0.11	10.33 ± 0.19
A	<63	1.93 ± 0.12	10.29 ± 0.06
B	63–250	1.85 ± 0.14	10.09 ± 0.11
B	63–250	1.75 ± 0.08	10.03 ± 0.14

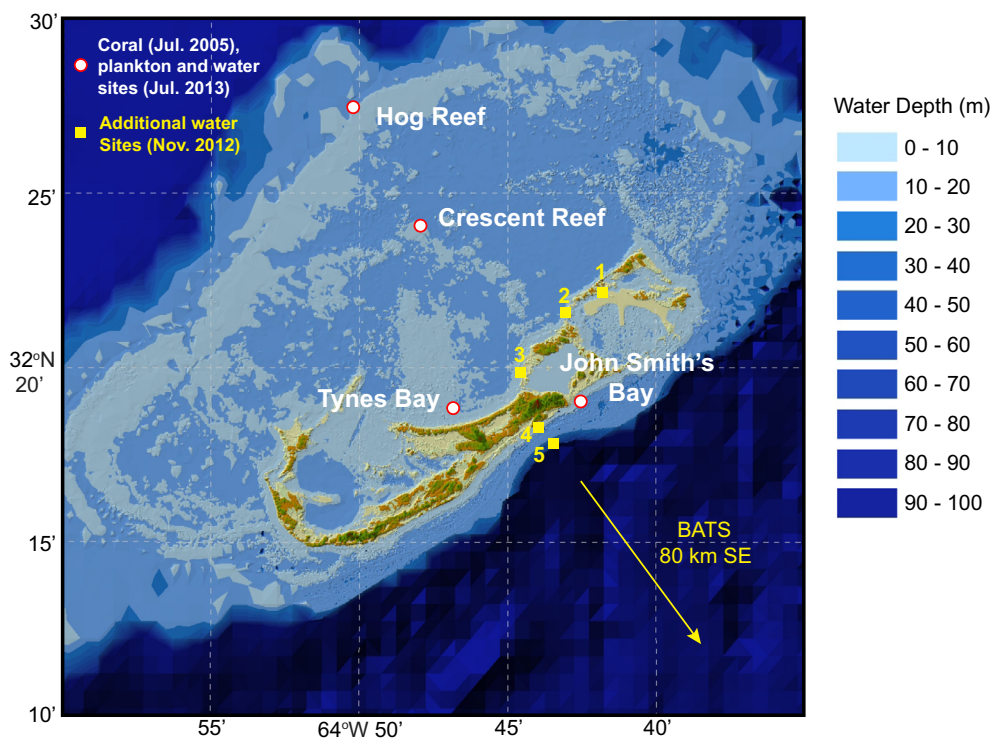


Fig. 2. Bathymetric map of the Bermuda pedestal showing the locations of 10 *Diploria labyrinthiformis* cores collected in Jul. 2005, plankton and water samples collected in Jul., 2013, and additional water samples collected in Nov. 2012. Indicated by the yellow arrow is the direction of Bermuda Atlantic Time-Series Study (BATS). Map credit: M. Shailer, Department of Conservation Services, Government of Bermuda.

dried, and scanned by computed axial tomography (CAT) to determine the maximum growth axis. Age models were determined by counting annual growth bands in CAT scan images. Along the maximum growth axis, 5–6 powder samples were drilled out from the top layer of each slab (0–3 cm) and analyzed for CS- $\delta^{15}\text{N}$ (Fig. 3). Each sample represents approximately one year. From BER 009 (John Smith's Bay) and BER 016 (Tynes Bay), ~ 20 year-long annual CS- $\delta^{15}\text{N}$ records were generated (Fig. 4). In addition, five samples from the bottom part of a longer coral core (BER001 from John Smith's Bay, same location and depth as BER009 but back to ~ 1780 AD, Goodkin et al., 2005) were also analyzed for CS- $\delta^{15}\text{N}$ (Fig. 4).

3.2. Plankton net tows

Plankton samples were collected using a 35 μm net tow at the same 4 sites just below the surface for 15 min in July 2013. Plankton samples were separated into 4 size classes by passing each sample through a series of sieves (35, 250, 500 and 1000 μm) and transferring each size fraction onto a combusted Whatman GF/F filter. The filters were freeze-dried and then subsampled for $\delta^{15}\text{N}$ analysis using an element-analyzer-isotope ratio mass spectrometer (EA-IRMS) system at Princeton University (Thermo DeltaV Advantage

interfaced with a Costech elemental analyzer using a Thermo ConFloIV). USGS40 standard ($\delta^{15}\text{N} = -4.5\text{‰}$) was used as the reference. Each sample was analyzed three times and the standard deviation of each sample was calculated from the triplicates (Fig. 3B).

3.3. Dissolved nitrogen

Although the nitrogen cycle at the Bermuda Atlantic Time-Series sites has been well studied (e.g., Altabet, 1988; Lipschultz, 2001; Knapp et al., 2005; Fawcett et al., 2011), very few nutrient data have been published for reef water around the island of Bermuda, and we know of no published N isotope data. To better constrain the nitrogen cycle on the reefs around Bermuda, water samples (2–3 replicates for each water site) were collected at 5 coastal sites in Nov. 2012 and at the 4 coral sites in Jul. 2013 (Fig. 2). Off-shore water was not sampled in Nov. 2012 due to poor weather. Briefly, all sample bottles (500 mL, Nalgene HDPE) were acid-washed and rinsed three times with deionized water. At the sites, sample bottles were rinsed three times with GF/F filtered sample water, filled to a volume of ~ 400 mL, and stored in a cooler filled with ice (at Water Site 1, 2 and 5, samples were not filtered due to time limitations; a comparison of filtered *vs.* unfiltered seawater

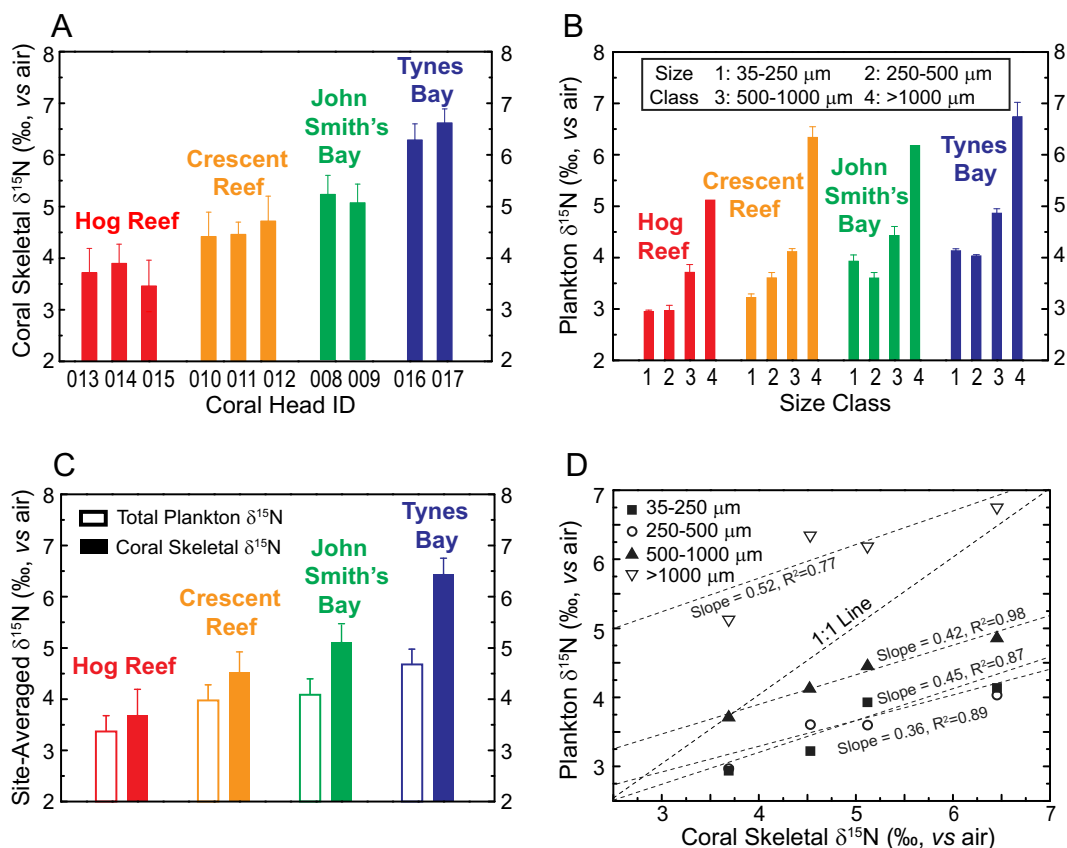


Fig. 3. Bermuda coral skeletal $\delta^{15}\text{N}$ and plankton $\delta^{15}\text{N}$. (A) 5-point temporal average and standard deviation of skeletal $\delta^{15}\text{N}$ on the top layer (0–3 cm) of each coral head collected at each of the four sites; (B) size-fractionated plankton $\delta^{15}\text{N}$ at the same four sites; (C) Comparison of average coral skeletal $\delta^{15}\text{N}$ to total plankton $\delta^{15}\text{N}$ at each site as calculated by mass weighting the measured size fractions; (D) Cross-plot and regression lines of plankton $\delta^{15}\text{N}$ *vs.* coral skeletal $\delta^{15}\text{N}$ for each size class.

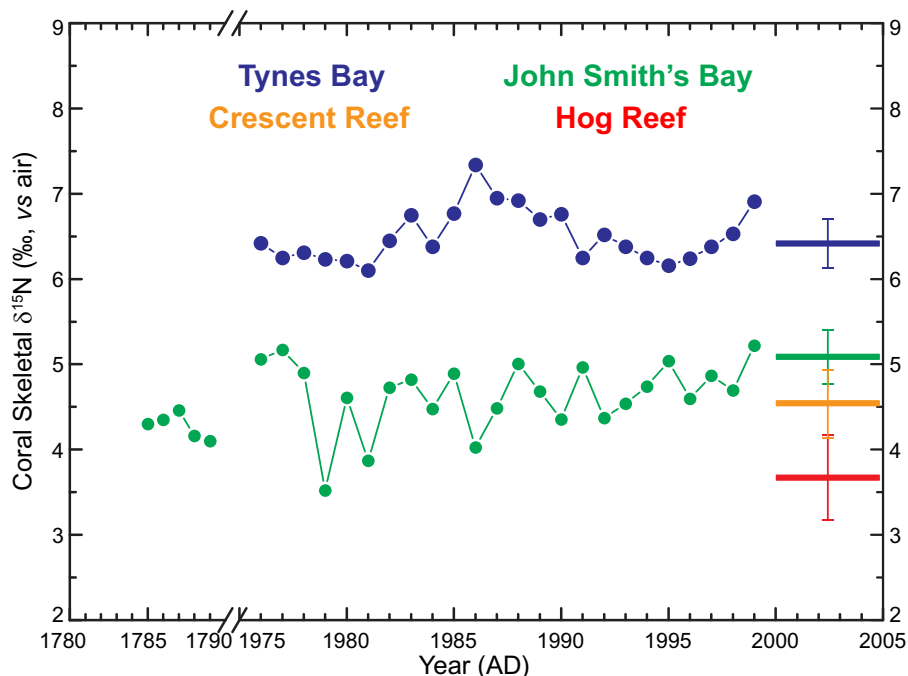


Fig. 4. Bermuda coral skeletal $\delta^{15}\text{N}$ time series. The horizontal bars are site-averaged coral skeletal $\delta^{15}\text{N}$ from Fig. 3, with the error bars indicating the standard deviations of all measured samples at a given site. The Tynes Bay $\delta^{15}\text{N}$ record is generated from BER016. The John Smith's Bay $\delta^{15}\text{N}$ record is generated from BER 009 and BER001.

at Water Site 2 and 3 showed no significant differences in any of the measured properties). Once back on the island, all samples were frozen, shipped to Princeton University, and kept frozen until analysis. Ammonium concentrations were measured using the OPA method (Holmes et al., 1999). Nitrate + nitrite concentrations were measured using the chemiluminescence method (Braman and Hendrix, 1989). Total nitrogen (dominated by dissolved organic nitrogen) concentration and $\delta^{15}\text{N}$ were measured in the Nov. 2012 samples following the persulfate/denitrifier protocol (Knapp et al., 2005).

4. RESULTS

At Bermuda, modern CS- $\delta^{15}\text{N}$ shows good reproducibility ($< 0.5\text{‰}$) among different colonies at the same sites (Fig. 3A). The average CS- $\delta^{15}\text{N}$ are 3.8‰ , 4.5‰ , 6.8‰ and 5.1‰ for Hog Reef, Crescent Reef, Tynes Bay and John Smith's Bay, respectively. CS- $\delta^{15}\text{N}$ shows a $\sim 3\text{‰}$ increase from the outer reef toward the island (from Hog Reef to Tynes Bay). The Tynes Bay CS- $\delta^{15}\text{N}$ record from 1976 to 1999 varies from 6.0‰ to 7.5‰ , while that of John Smith's Bay varies from 3.5‰ to 5.2‰ (Fig. 4). The five samples from 1785 to 1789 AD show a CS- $\delta^{15}\text{N}$ of 4.0‰ to 4.5‰ .

The plankton $\delta^{15}\text{N}$ also shows an inshore increase for each size class (Fig. 3B and C), but the magnitude is only $\sim 1\text{‰}$. The plankton $\delta^{15}\text{N}$ shows a $2\text{--}3\text{‰}$ increase with increasing size at each site, in agreement with previously published plankton $\delta^{15}\text{N}$ data from the North Atlantic open ocean (Montoya et al., 2002). Cross plots of plankton $\delta^{15}\text{N}$ vs. CS- $\delta^{15}\text{N}$ show correlation at all size classes

($R^2 > 0.75$, Fig. 3D). However, the slopes from the cross plots are much lower than 1.

Ammonium and nitrate + nitrite measurements around the island of Bermuda are generally consistent with the oligotrophic nature of the waters overlying coral reefs (Table 2), except at the heavily populated coastal sites (in particular, Water Sites 1 and 2). Although there is no clear inshore increasing trend in the nutrient concentrations, the higher ammonium concentrations at Water Sites 1 and 2 suggest that the populated areas of the island are nitrogen sources to the reefs (Table 2). Water Sites 1 and 2 have higher total nitrogen (TN) concentration and $\delta^{15}\text{N}$ because of the substantial contribution of dissolved inorganic nitrogen (DIN) (Table 2). Total nitrogen at Water Sites 3–5 is primarily composed of dissolved organic nitrogen (DON), and all of these sites have indistinguishable TN $\delta^{15}\text{N}$ ($4.5\text{--}4.6\text{‰}$), similar to but slightly ($\sim 0.6\text{‰}$) higher than the average TN $\delta^{15}\text{N}$ measured at BATS (Table 2, Knapp et al., 2005, 2011).

5. DISCUSSION

5.1. Bermuda CS- $\delta^{15}\text{N}$ and plankton $\delta^{15}\text{N}$

Multiple modern coral cores/colonies from each site at Bermuda were used to examine the natural heterogeneity of CS- $\delta^{15}\text{N}$ for colonies growing in a single environmental regime. Good reproducibility ($< 0.5\text{‰}$) among different colonies at the same site (Fig. 3A) suggests the CS- $\delta^{15}\text{N}$ reliably reflects the local conditions. However, we also observe a $\sim 3\text{‰}$ shoreward increase in CS- $\delta^{15}\text{N}$, from Hog Reef to Tynes Bay. The two CS- $\delta^{15}\text{N}$ records from 1976

Table 2

Concentrations of ammonium, nitrite + nitrate, total nitrogen; and total nitrogen $\delta^{15}\text{N}$ on Bermuda coral reefs.

Sample Site	Sample date	Sample depth (m)	[Ammonium] (μM)	[Nitrite + Nitrate] (μM)	[TN] (μM)	TN $\delta^{15}\text{N}$ (‰)
Hog Reef	Jul. 2013	6	0.046 ± 0.005^a	0.62 ± 0.04	nd ^b	nd
Crescent Reef	Jul. 2013	3	0.103 ± 0.001	0.25 ± 0.01	nd	nd
Tynes Bay	Jul. 2013	2	0.057 ± 0.001	0.29 ± 0.02	nd	nd
John Smith's Bay	Jul. 2013	5	0.075 ± 0.001	0.43 ± 0.04	nd	nd
Water Site 1	Nov. 2012	0	0.378 ± 0.006	0.81 ± 0.08	7.08 ± 1.21	4.88 ± 0.24
Water Site 2	Nov. 2012	0	0.311 ± 0.013	0.63 ± 0.02	6.03 ± 0.71	4.77 ± 0.32
Water Site 3	Nov. 2012	0	0.165 ± 0.006	0.52 ± 0.03	5.63 ± 0.61	4.55 ± 0.19
Water Site 3	Jul. 2013	1	0.131 ± 0.002	0.39 ± 0.03	nd	nd
Water Site 4	Nov. 2012	0	0.028 ± 0.006	0.15 ± 0.02	4.85 ± 0.46	4.57 ± 0.26
Water Site 5	Nov. 2012	0	0.011 ± 0.002	0.01 ± 0.00	4.93 ± 0.26	4.54 ± 0.21
Water Site 5	Jul. 2013	0	0.017 ± 0.001	0	nd	nd
BATS ^c	Jul. 2000	0	0	0	4.2	4.2
BATS ^c	Nov. 2000	0	0	0	4.0	4.0

^a All the standard deviations (1σ) are calculated from at least two replicates.

^b nd: not determined.

^c Data from Knapp et al., 2005.

to 1999 show only modest variation ($<1.5\text{‰}$), suggesting that the local nitrogen cycle dynamics at each site have been roughly stable since the 1970s. Accordingly, the CS- $\delta^{15}\text{N}$ gradient between Tynes Bay and John Smith's Bay has been maintained since the 1970s, implying that our reconstructed CS- $\delta^{15}\text{N}$ pattern is not a transient feature. As one minor wrinkle, the John Smith's Bay CS- $\delta^{15}\text{N}$ record shows a weak increasing trend since the late 1970s, with the lowest value close to the modern Hog Reef CS- $\delta^{15}\text{N}$. The CS- $\delta^{15}\text{N}$ in the 1780s at John Smith's Bay is also close to that of the modern Hog Reef CS- $\delta^{15}\text{N}$. A plausible explanation for higher CS- $\delta^{15}\text{N}$ in modern John Smith's Bay than Hog Reef is that anthropogenic N sources to John Smith's Bay have been increasing modestly over the last 40 years (Jones et al., 2011).

A similar shoreward increase in CS- $\delta^{15}\text{N}$ was observed in the previous coral $\delta^{15}\text{N}$ study of Yamazaki et al. (2011) near Taiwan, the cause of which was attributed to high- $\delta^{15}\text{N}$ riverine nitrate input. However, at Bermuda, the plankton $\delta^{15}\text{N}$ in all size classes showed only $\sim 1\text{‰}$ shoreward increase (Fig. 3B and 3C), indicating that the corals' food source cannot explain the full coral $\delta^{15}\text{N}$ gradient. Moreover, the observed plankton $\delta^{15}\text{N}$ gradient should respond to changes in the $\delta^{15}\text{N}$ of DIN due to its assimilation by phytoplankton, implying that the $\delta^{15}\text{N}$ gradient in DIN is similar to that of the plankton. Thus, it appears that the $\sim 3\text{‰}$ difference in CS- $\delta^{15}\text{N}$ apparently cannot be explained solely by variation in the $\delta^{15}\text{N}$ of N sources to the corals, and we must look to the coral system itself to explain the residual $\sim 2\text{‰}$ shoreward increase in CS- $\delta^{15}\text{N}$.

For the $\sim 1\text{‰}$ offshore decrease in plankton $\delta^{15}\text{N}$, there are a number of possible explanations. First, the release of ^{15}N -enriched human waste N from the island into the coastal waters through marine outfalls (Jones et al., 2011) or groundwater input (Simmons and Lyons, 1994) could be communicated throughout the food web by phytoplankton uptake and subsequent heterotrophy. Second, the isotopic signal of benthic N loss should be greater inshore, which would similarly increase the $\delta^{15}\text{N}$ of the plankton inshore (Granger et al., 2011). Whatever the origin of this plankton

$\delta^{15}\text{N}$ variation, the Bermuda pedestal might serve as a model system for understanding the N cycle of coral reefs adjacent to the oligotrophic open ocean, with the benefit that it does not have a freshwater runoff with properties specific to its continental region.

The nitrogen isotopes have been used extensively to study the internal nitrogen cycle of the coral/symbiont system over the past several decades (Muscatine and Kaplan, 1994; Heikoop et al., 1998; Hoegh-Guldberg et al., 2004; Swart et al., 2005; Lesser et al., 2007; Reynaud et al., 2009; Ferrier-Pages et al., 2011). It has been broadly observed that the $\delta^{15}\text{N}$ of the zooxanthellae is lower than that of the coral host (Muscatine and Kaplan, 1994; Swart et al., 2005; Reynaud et al., 2009), with the implication that the algal symbionts prevent the efflux of low- $\delta^{15}\text{N}$ ammonium from the coral, eventually returning this N to the host as new photosynthate (Reynaud et al., 2009). In addition, it has been observed by Muscatine et al. (2005) that non-symbiotic corals have higher tissue $\delta^{15}\text{N}$ (8.08 ± 1.23 , $n = 5$) than symbiotic corals ($\delta^{15}\text{N} = 4.56 \pm 1.40$, $n = 17$). This difference was originally explained as symbiotic corals assimilating ^{15}N -depleted ammonium while non-symbiotic corals can only acquire its N sources from heterotrophic feeding. However, we argue that this difference is more easily explained by a much reduced ammonium excretion out of the symbiotic corals than non-symbiotic corals (Szmant-Froelich and Pilson, 1984): low- $\delta^{15}\text{N}$ ammonium is retained within the symbiotic corals, causing a low tissue $\delta^{15}\text{N}$ relative to non-symbiotic corals. Similar explanations have been applied to the $\delta^{15}\text{N}$ difference between symbiotic and non-symbiotic foraminifera (Ren et al., 2012).

This interpretation helps to explain our finding that the CS- $\delta^{15}\text{N}$ from the most oceanic reef (Hog Reef) is 3.8‰ and not higher. The Hog Reef CS- $\delta^{15}\text{N}$ is less than 2‰ higher than the $\delta^{15}\text{N}$ of the nitrate supply to the surrounding euphotic zone of the Sargasso (Fawcett et al., 2011, 2014; Knapp et al., 2005), overlapping the range in $\delta^{15}\text{N}$ of the zooplankton in the Sargasso Sea (Montoya et al., 2002; Graham et al., 2010). Accordingly, the CS- $\delta^{15}\text{N}$ of

Hog Reef is also similar to the $\delta^{15}\text{N}$ of the plankton collected over Hog Reef (Fig. 3C and 5). Corals are observed to feed significantly on zooplankton (Houlbreque and Ferrier-Pages, 2009). If the corals behaved as a typical heterotroph, their $\delta^{15}\text{N}$ would be 3–4‰ higher than that of their food source (Deniro and Epstein, 1981; Minagawa and Wada, 1984). On the contrary, the relatively low CS- $\delta^{15}\text{N}$ of the Hog Reef corals is consistent with their low- $\delta^{15}\text{N}$ metabolic ammonium being recycled by the symbionts rather than being effluxed to the environment, causing most coral N loss to be channeled through non-fractionating mechanisms (Reynaud et al., 2009; Ren et al., 2012).

In contrast, CS- $\delta^{15}\text{N}$ at Tynes Bay is $\sim 3\text{‰}$ higher than at Hog Reef and $\sim 2\text{‰}$ higher than the $\delta^{15}\text{N}$ of N sources to the corals as sampled by the plankton tows (Fig. 3C and 5), an observation that is hard to explain if corals are not losing low- $\delta^{15}\text{N}$ N to the environment. Below, we propose a mechanism for changing symbiotic coral $\delta^{15}\text{N}$ that does not involve changing the isotopic composition of its N sources.

5.2. N recycling efficiency/ammonium leakage hypothesis for Bermuda corals

Corals have been reported to capture zooplankton directly with their tentacles (as frequently exploited by purposefully feeding corals kept in experimental systems), collect detritus and microbes using mucus-netting, digest organic material outside their body with mesenterial filaments, and take up dissolved organic matter from sea water (Johannes et al., 1970; Szmant-Froelich, 1981; Lawn and McFarlane, 1991; Grover et al., 2008; Houlbreque and Ferrier-Pages, 2009). Another pathway of N uptake by the coral symbiotic system is DIN assimilation from ambient seawater. Many studies have demonstrated the ability of symbiotic systems to assimilate ammonium and nitrate from seawater (Bythell, 1990; Marubini and Davies, 1996; Hoegh-Guldberg and Williamson, 1999; Grover et al., 2002, 2003; Badgley et al., 2006; Tanaka et al., 2006;

Pernice et al., 2012; Kopp et al., 2013). However, these studies were conducted under the condition of no feeding and/or high DIN concentration, with one exception (Grover et al., 2002). The starvation effects alone will probably up-regulate DIN assimilation. Indeed, Grover et al. (2002) showed that when their corals were fed during a $0.2 \mu\text{M } ^{15}\text{NH}_4^+$ incubation experiment, the ammonium uptake became negligible and manifold lower than in the starved colonies. Given the studies to date, the relative importance of feeding versus DIN uptake by corals in the field is uncertain and may vary substantially across environments. A coral's metabolic nitrogenous waste (mainly in the form of ammonium/ammonia for marine invertebrates, Wright, 1995) as well as any DIN taken up from ambient water is assimilated by the symbionts, amino acids and complex glycoconjugates are synthesized by the symbionts, and a fraction is transferred back to the coral host ("N recycling", Falkowski et al., 1993; Tanaka et al., 2006; Kopp et al., 2013). In this way, the coral symbiotic system is able to sequester and efficiently recycle the nitrogen with little loss to the oligotrophic environment (O'Neil and Capone, 2008). If the coral symbiotic system is very efficient, as is expected under the oligotrophic conditions typical of coral reefs, ammonium leakage will be minimal. Lacking this release of low- $\delta^{15}\text{N}$ ammonium, coral $\delta^{15}\text{N}$ would be similar to the $\delta^{15}\text{N}$ of its N sources. All assimilated N, organic or inorganic, will either accumulate in the colony's biomass or will be "lost" through reproduction or mucus excretion, which do not preferentially remove low- $\delta^{15}\text{N}$ N from the colony. The Hog Reef coral would be an example of efficient N recycling within the coral symbiotic system, giving it a $\delta^{15}\text{N}$ similar to its food source (Fig. 5).

However, several studies have observed that, when corals are well fed, the ammonium leakage out of the symbiotic system becomes significant because the symbionts cannot assimilate all the ammonium excreted by coral host (Szmant-Froelich and Pilson, 1984; Piniak et al., 2003; Piniak and Lipschultz, 2004). Moreover, Szmant-Froelich and Pilson (1984) found that the ammonium leakage rate out of the symbiotic system increases with feeding frequency. If this is the case for Bermuda corals, the $\delta^{15}\text{N}$ difference between coral and its N sources might be explained by more food availability in near-shore waters (Tynes Bay) and thus greater leakage of low- $\delta^{15}\text{N}$ ammonium out of the symbiotic system. Indeed, the suspended particulate N concentrations in the waters over Bermuda are correspondingly higher in the inshore waters (Water Quality Monitoring Program, Bermuda Institute of Ocean Sciences; Welsh et al., 1979). Further, it has been observed that the inshore corals at Bermuda (*Diploria labyrinthiformis* and *Diploria strigosa*) have higher extension rates than the offshore corals (Logan et al., 1994), which could be explained as a response to greater food availability in the inshore waters. Hence, we propose that, as productivity and food supply increase toward the isolated inland waters of the Bermuda reef (e.g., Tynes Bay), corals' greater feeding leads to metabolic ammonium production that significantly exceeds ammonium assimilation by their symbionts, resulting in leakage of ^{15}N -depleted ammonium from the symbiotic

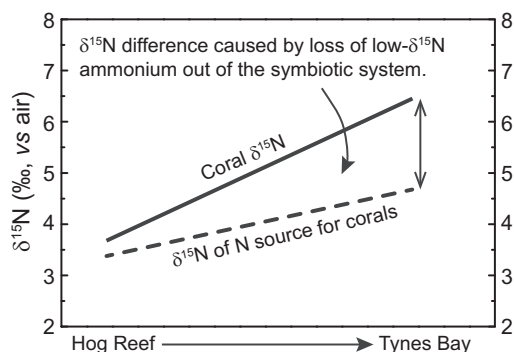


Fig. 5. Schematic plot showing the hypothesis proposed to explain the differences between coral $\delta^{15}\text{N}$ and the $\delta^{15}\text{N}$ of N sources for corals at Bermuda. It is proposed that, in more productive inshore waters (Tynes Bay), greater food uptake by the coral causes a greater fraction of its low- $\delta^{15}\text{N}$ regenerated ammonium to be excreted rather than assimilated by zooxanthellae, raising the $\delta^{15}\text{N}$ of the inshore corals.

system. That is, the higher productivity of Tynes Bay causes the N recycling efficiency of the coral symbiotic system to decrease, with more of the N acquired from feeding leading to ^{15}N -depleted ammonium excretion out of the symbiotic system, yielding a higher CS- $\delta^{15}\text{N}$. As indicated in Fig. 5, we propose that $\sim 2/3$ of the 3‰ shoreward increase (from Hog Reef to Tynes Bay) in CS- $\delta^{15}\text{N}$ is caused by an increase in the leakage of ^{15}N -depleted ammonium out of the symbiotic system. John Smith's Bay, like Tynes Bay, is close to shore. However, the lower CS- $\delta^{15}\text{N}$ of John Smith's Bay corals suggests that the tightness of its symbiotic N cycle is closer to that of Crescent Reef than Tynes Bay. This suggests that inshore nutrient accumulation is less efficient on south shore, presumably because of the steep bathymetry on the south side of the islands.

Despite the evidence in favor of the ammonium leakage-based interpretation of CS- $\delta^{15}\text{N}$ and its variations across Bermuda, alternative possibilities exist. In particular, while our plankton measurements indicate that $\delta^{15}\text{N}$ gradients in N sources cannot explain the full amplitude of CS- $\delta^{15}\text{N}$ variation so far observed on the Bermuda pedestal, this finding cannot be taken as conclusive. First, additional sampling may reveal a stronger inshore $\delta^{15}\text{N}$ increase in plankton than we have documented so far. Second, while plankton $\delta^{15}\text{N}$ should capture significant variations in DIN $\delta^{15}\text{N}$, more direct measures of the latter are needed. The concentrations of ammonium, nitrate, and nitrite are below the range for precise isotopic analysis by routine protocols, but dedicated measurement work should yield useful constraints. N isotopic analysis of co-occurring benthic algae species may provide an approach for characterizing DIN $\delta^{15}\text{N}$ variations. Third, there could be spatial patterns in the forms of N on which corals rely. If these N forms are isotopically distinct, then this could yield variation in the $\delta^{15}\text{N}$ of N acquired by corals even without spatial variation in the $\delta^{15}\text{N}$ of the individual N forms. We suspect that Bermuda corals uniformly rely on heterotrophic feeding as their dominant source of N, as coupled feeding and respiration leave heterotrophic organisms with a natural excess of N and P that they are forced to excrete if they do not develop a symbiosis. Nevertheless, laboratory studies clearly indicate the ability of corals to take up DIN (e.g., Badgley et al., 2006 and references therein). To date, such lab studies have been unable to provide a conclusive view of how corals acquire their N under natural conditions such as are encountered across the Bermuda pedestal, largely because there is no straightforward way to compare the availabilities of the different N forms in the experimental systems to availability in natural environments. In this regard, more extensive CS- $\delta^{15}\text{N}$ measurements in the environment, covering both space and time, represent a promising complement to established approaches for investigating the nutrient cycling of coral colonies and reefs.

6. CONCLUDING REMARKS

One major outcome of the reported study is a method for CS- $\delta^{15}\text{N}$ that has both the needed sensitivity and the precision for high-resolution (interannual or even seasonal) reconstruction of past changes. Our sample size require-

ment of 5 mg is easily recoverable from almost all coral archives, and thus individual samples can derive from within a single annual band. The method's precision of 0.2‰ is equally relevant, as environmental and physiological changes on annual to decadal time scales are unlikely to cause CS- $\delta^{15}\text{N}$ changes greater than $\sim 3\%$. One of the greatest remaining needs is to gain a better understanding of the composition and biochemical origin of the CS-organic N.

In our study of *Diploria labyrinthiformis* from 4 coral sites of Bermuda reefs, we found that CS- $\delta^{15}\text{N}$ at the most exposed site is less than 2‰ higher than the $\delta^{15}\text{N}$ of nitrate supply to the Sargasso Sea euphotic zone, surprisingly low for a heterotroph that feeds to a significant degree on zooplankton. This finding, when combined with previous findings regarding the $\delta^{15}\text{N}$ of coral zooxanthellae as well as comparison of CS- $\delta^{15}\text{N}$ with plankton $\delta^{15}\text{N}$ at each of the Bermuda coral sampling sites, suggests that these corals may lack a portion of the 3–4‰ “trophic elevation” in $\delta^{15}\text{N}$ observed in typical heterotrophs because their low- $\delta^{15}\text{N}$ metabolic ammonium is recycled by the symbionts rather than being effluxed to the environment, such that most coral N loss is channeled through non-fractionating mechanisms. Going inshore, CS- $\delta^{15}\text{N}$ increases by $\sim 3\%$ with increasing proximity to the island. We hypothesize that, inshore corals' greater food uptake causes their metabolic yield of ammonium to exceed the ammonium assimilation rate of symbionts. As a result, there is a leak of this ^{15}N -depleted metabolic N out of the symbiotic system and a complementing elevation in CS- $\delta^{15}\text{N}$. This explanation for CS- $\delta^{15}\text{N}$ variation is analogous to that for the difference in shell-bound $\delta^{15}\text{N}$ between symbiotic and asymbiotic planktonic foraminifera: the symbiont-bearing foraminifera have a consistently lower $\delta^{15}\text{N}$, as expected if the symbiosis reduces the efflux of low- $\delta^{15}\text{N}$ metabolic ammonium (Ren et al., 2012).

The ability of CS- $\delta^{15}\text{N}$ to track the N cycle of the corals' external environment would render this measurement a powerful tool in efforts to reconstruct past oceanographic and biogeochemical change. In this context, our interpretation of a physiological signal might be taken as an unwelcome complication. Future work will address how this physiological signal overlaps with environmental signals and will consider strategies for distinguishing between them, for example, purposefully sampling corals from near the reef margin. Nevertheless, there are questions for which the physiological sensitivity may be a useful indicator. For example, if we are correct in our hypothesis that elevation in CS- $\delta^{15}\text{N}$ results from reduced host/symbiont N cycling within the coral, then abrupt CS- $\delta^{15}\text{N}$ peaks may mark times of past coral bleaching events, during which host/symbiont N cycling is lost (Rodrigues and Grottoli, 2006).

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