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HOST MICROBE INTERACTIONS

Trophic Ecology of the Tropical Pacific Sponge *Mycale grandis* Inferred from Amino Acid Compound-Specific Isotopic Analyses

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Abstract

Many sponges host abundant and active microbial communities that may play a role in the uptake of dissolved organic matter (DOM) by the sponge holobiont, although the mechanism of DOM uptake and metabolism is uncertain. Bulk and compound-specific isotopic analysis of whole sponge, isolated sponge cells, and isolated symbiotic microbial cells of the shallow water tropical Pacific sponge *Mycale grandis* were used to elucidate the trophic relationships between the host sponge and its associated microbial community. $\delta^{15}N$ and $\delta^{13}C$ values of amino acids in *M. grandis* isolated sponge cells are not different from those of its bacterial symbionts. Consequently, there is no difference in trophic position of the sponge and its symbiotic microbes indicating that *M. grandis* sponge cell isolates do not display amino acid isotopic characteristics typical of metazoan feeding. Furthermore, both the isolated microbial and sponge cell fractions were characterized by a similarly high ΣV value—a measure of bacterial-re-synthesis of organic matter calculated from the sum of variance among individual $\delta^{15}N$ values of trophic amino acids. These high ΣV values observed in the sponge suggest that *M. grandis* is not reliant on translocated photosynthate from photosymbionts or feeding on water column picoplankton, but obtains nutrition through the uptake of amino acids of bacterial origin. Our results suggest that direct assimilation of bacterially synthesized amino acids from its symbionts, either in a manner similar to translocation observed in the coral holobiont or through phagotrophic feeding, is an important if not primary pathway of amino acid acquisition for *M. grandis*.

Keywords Dissolved organic matter · Symbioses · Amino acid translocation · $\delta^{13}C$ · $\delta^{15}N$

Introduction

Sponges are ubiquitous members of benthic coral reef communities and many species are known to host diverse and metabolically active symbiotic microbial communities [1]. Sponges are essential to sustaining high secondary productivity on oligotrophic coral reefs through their rapid uptake of dissolved organic matter (DOM) and subsequent shedding of particulate organic matter in the form of their choanocyte cells. This sponge detritus is then available to a variety of benthic detritivores [2], driving an efficient transfer of DOM to higher trophic levels via the sponge-loop pathway [3, 4].

The mechanisms by which DOM is assimilated and transferred by the sponge is not well known but may be facilitated by a close symbiosis between sponges and their microbial communities [2, 5–12]. This symbiosis is often functionally mutualistic and is among the most ancient of metazoan symbioses [13]. The sponge-microbe symbiosis is fundamental to sponge ecology, as even distantly related sponges from geographically disparate regions share a common set of associated microbes, some of which are unique to sponges and cannot be acquired from the surrounding environment [14–19]. Sponge-associated symbionts also contribute to the carbon (C) and nitrogen (N) requirements of sponges [10, 20], and some sponge symbionts produce secondary metabolites that provide for their hosts chemical defenses [21–24].

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The invasive sponge *Mycale grandis* is found in Kāne ohe Bay and other partially degraded coral reef habitats around the main Hawaiian Islands. It is unknown whether *M. grandis* obtains C and N from its microbial symbionts in addition to, or instead of, heterotrophic filter feeding. Some sponges are able to obtain half of their energy budget and more than half of their C budget from their photosymbionts [25], whereas DOM can comprise 90% of the diet in other species [7, 9, 10, 26]. For instance, shallow water Caribbean sponge species derive much of their required metabolic C from dissolved organic carbon (DOC), while Indo-Pacific species rely more heavily on photoautotrophy [25, 27]. Since many sponges harbor over 10^9 bacterial cells g^{-1} of sponge wet weight [16], we investigated whether bacterially derived DOM may be a significant source of DOM to sponges.

The main producers of DOM on coral reefs are primary producers, particularly macroalgae and the endosymbiotic dinoflagellates (Family Symbiodiniaceae) [28] of scleractinian corals, which directly or indirectly release a substantial fraction of their excess photosynthate into the water column [29]. As a result, early studies sought to investigate specific sources of DOM available to sponges. de Goeij et al. [26] used ^{13}C-enriched diatom DOM and particulate organic matter (POM) to positively demonstrate that algal-derived DOC was assimilated by the sponge and furthermore shed as sponge cells (e.g., choanocytes), and this detritus was consumed by other reef fauna at higher trophic levels. Rix et al. [12] used ^{13}C- and ^{15}N-labeled warm and cold-water coral mucus DOM as food sources for a warm-water Red Sea sponge and a North Atlantic cold-water sponge, respectively, and demonstrated assimilation of coral mucus into sponge tissue and subsequent sponge-derived detritus production. Subsequently, studies using ^{13}C-labeled DOM have shown ^{13}C uptake of coral-derived DOM by sponge cells and algal-derived DOM by the symbiotic microbes [8]. Although the pathways of acquisition of DOM may vary across species, DOM from the two sources were shown to be assimilated by the sponge and subsequently released as sponge-derived detritus; however, algal-derived DOM was released as sponge cell detritus at a higher rate. Sponge cells, however, are probably capable of taking up the larger colloidal fraction in DOM such as viruses and free amino acids, while bacteria can assimilate the smaller, truly dissolved fraction of DOM [26, 30, 31].

The study of diverse and complex consortia of symbionts within sponges is challenging because multiple functions and interactions can take place simultaneously and it is difficult to resolve the specific roles and contribution of the symbionts and host. We use stable C and N isotope analyses of whole sponge, sponge cell, and microbial cell fractions, as well as isotopic analyses of individual amino acids (AAs) in each cell fraction to elucidate the trophic pathways utilized by *M. grandis*. Carbon isotopic composition (δ^{13}C values) is used to determine the sources of an organism's dietary C. Nitrogen isotopic composition (δ^{15}N values) has been used in earlier studies of marine food webs as an indicator of relative trophic position of an organism based on the consistent increase in ^{15}N/^{14}N ratios from food source to consumer [32, 33]. However, understanding key dietary details can be limited due to multiple factors influencing bulk tissue δ^{13}C and δ^{15}N values, including differences in baseline values and uncertainty regarding the magnitude of trophic discrimination factors of organisms.

Compound-specific isotopic measurements of AAs can be used to address some of these challenges. δ^{15}N values of individual AAs allows for determination of trophic positions (TP) based on the differential fractionation of AAs from food source to consumer. In samples of consumer tissues, "source" AAs retain the isotopic composition of N sources at the base of the food web, whereas "trophic" AAs are greatly enriched in ^{15}N relative to source AAs with each trophic transfer [34–37]. The utility of the amino acid compound-specific isotopic analysis (AA-CSIA) approach is that the N isotopic composition of an individual AA reflects the degree of isotopic fractionation associated with various biochemical reactions that involve C-N bond breakage (deamination and transamination) in individual AAs involved in N metabolism. However, the exact biochemical mechanisms are complex [38, 39]. The δ^{15}N value of an organism also inherently reflects the isotopic composition of inorganic N sources (nitrate, nitrite, ammonia, and urea) assimilated by primary producers at the base of the food web. Since source AAs retain δ^{15}N values from the base of the food web, AA-CSIA provides information about N metabolism, constrain TP, and identify the source and transformation of dissolved and detrital organic matter in marine waters and sediments [40–42]. Similar to δ^{15}N values of source AAs, the δ^{13}C values of essential amino acids (EAA) in consumers retain the carbon isotopic composition of the plants and bacteria that produced those AAs [43].

Here, we provide experimental evidence that the means of transferring nutrition from microbial symbiont to the sponge *M. grandis* is through the uptake of AAs synthesized by its symbiotic bacteria, and bacterially produced AAs are a major substrate by which C and N are transferred from microbial symbiont to the sponge. The efficiency of this transfer mechanism is facilitated by the high concentrations of microbial communities hosted within the sponge mesohyl.

Methods

Sampling Locations

Kāne ohe Bay on the northeast coast of O ahu, Hawai'i, is characterized by an extensive system of scleractinian coral dominated fringing reefs, patch reefs, and a large barrier reef. Sponge samples for bulk tissue isotopic analysis were

collected on May 6, 2015 from two patch reefs just northwest of Coconut Island (21° 26′ 07.5″ N 157° 47′ 43.9″ W) in the southern portion of Kāne ohe Bay ($n = 34$), from a patch reef in mid Kāne ohe Bay (21° 27′ 37.5″ N 157° 49′ 19.7″ W) ($n = 10$), and from a patch reef in the northern portion of Kāne ohe Bay (21° 28′ 37.6″ N 157° 49′ 54.7″ W) ($n = 10$). Sponge samples for bulk and compound-specific isotopic analysis of isolated cell fractions ($n = 8$) were collected from ~ 1 m depth on May 3, 2017 from the fringing reef located beneath the Lilipuna Pier in south Kāne ohe Bay (21° 25′ 46.0″ N 157° 47′ 31.2″ W). Sponge specimens were inclusive of surface pinacoderm, inner mesohyl, and choanocyte chambers. Visible debris and epiphytes were removed from the surface of the samples. Specimens from north, mid, and south Kāne ohe Bay collected for bulk isotopic analysis were immediately frozen at − 80 °C and stored until analysis. Whole sponge samples for epi-fluorescence microscopy were collected from under the Lilipuna Pier, rinsed with filtered seawater, and preserved in 1% paraformaldehyde. Isotope values for planktonic end members were determined using oblique net tows (63 μm mesh) on January 19, 2018, west of Coconut Island and east of Lilipuna Pier (21° 25′ 48.5″ N 157° 47′ 27.0′ v W). Plankton were size-fractioned using nylon mesh between 63 and 250 μm and consisted predominantly of copepods and crab zoea. Plankton were concentrated on a GF/F filter (0.7 μm nominal poe size), rinsed with distilled H_2O, dried (60 °C) and ground to a powder, and stored until AA-CSIA. While these samples only contained zooplankton, $\delta^{15}N$ values of source AAs and $\delta^{13}C$ values of EAA will reflect that of the phytoplanktonic end member.

Separation of Microbial Cells and Sponge Cells from Sponge Tissue

Sponge and microbial cells of *M. grandis* were separated through size fractionation using modification of the methods described in Freeman and Thacker [44, 45]. Samples were homogenized with mortar and pestle in filtered seawater to dissociate the sponge cells and vacuum filtered (Whatman No. 4 filter, 20–25 μm nominal pore size). The resulting filtrate was centrifuged (430×*g*) for 6 min to form a sponge cell pellet. The supernatant containing microbial cells was decanted and stored at − 20 °C. To rinse the sponge cell pellet, it was suspended in filtered seawater and centrifuged (twice, 5 min, 4 °C, 770×*g*). Further rinsing was accomplished by suspension and centrifugation (twice, 2 min, 3900×*g*). The isolated sponge cell pellet was stored frozen (− 20 °C) until analysis.

To isolate microbial cells, the previously frozen supernatant containing the microbial fraction was thawed and centrifuged (17,000×*g*) for 17 min at 4 °C. The supernatant was decanted, the pellet resuspended, and the centrifuge step repeated. The pellet was resuspended and transferred to a 1.5 mL centrifuge tube and the microbial cell fraction centrifuged (12,800×g) for 2 min, and the supernatant was carefully removed and discarded. The resulting microbial pellet was rinsed twice using the same procedure and the remaining pellet was frozen at − 20 °C until analysis.

Cell Abundance and Identification (Flow Cytometry, Microscopy)

A small volume (40 μL) of the separated sponge cell and microbial cell fractions were fixed in paraformaldehyde (1% final concentration), and frozen (− 80 °C) until batch analyses for cell abundances using flow cytometry. The flow cytometer was a Beckman Coulter EPICS Altra flow cytometer with a Harvard Apparatus syringe pump for volumetric sample delivery. Simultaneous (co-linear) excitation of the cells was provided by two water-cooled 5-W argon ion lasers tuned to 488 nm (1 W) and the UV range (200 mW). Samples were thawed and diluted with 800 μL of filtered seawater and stained with the DNA-specific stain Hoechst 33342 (1 μg mL^{-1} final concentration) for 1 h in the dark. Aliquots of 100 μL were analyzed for *Synechococcus* (SYN), photosynthetic eukaryotes, and non-pigmented bacterial abundances. Discrete populations were enumerated on the basis of chlorophyll *a* (red fluorescence, 680 nm), phycoerythrin (orange fluorescence, 575 nm), DNA (blue fluorescence, 450 nm), forward scatter, and 90° scatter signatures. Listmode files were processed using FlowJo software (Treestar Inc., www.flowjo.com). The DNA fluorescence detector was set at 700 V for the microbial samples, whereas the sponge cell samples were analyzed at a much lower voltage (400 V) to eliminate microbial cells from the gathered data files.

Cell abundances were converted to carbon for each population, assuming 30 fg $cell^{-1}$ for bacteria [46], 200 fg $cell^{-1}$ for *Synechococcus* and other picophytoplankton, 800 fg $cell^{-1}$ for nanophytoplankton [47], and 3 pg $cell^{-1}$ for sponge cells [48].

Epi-fluorescence microscopy (Olympus BX-41) was used to examine sponge cell and microbial isolates. Preserved samples were thawed, stained with 4′,6-diamidino-2-phenylindole (1 μg mL^{-1} final concentration) and proflavin 3,6-diaminoacridine, hemisulfate salt for protein-staining (0.33% *w*/*v*, final concentration), then filtered onto black 0.8 μm 25 mm PCTE filters and mounted on glass slides. Samples were observed (× 200 and × 400) under blue and UV excitation wavelengths, to determine pigment (chlorophyll, phycoerythrin) presence and observe DNA contents.

Bulk Sponge and Cell Isotope Analysis

The $\delta^{13}C$ and $\delta^{15}N$ values of lyophilized bulk sponge tissue (1.7–2.1 mg), isolated sponge (0.2–0.3 mg), and microbial cells (0.3–0.4 mg) were determined using a Costech elemental

combustion system (Model 4010) coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer (IRMS) through a Conflo IV interface. Isotopic compositions are reported in typical δ-notation relative to the internationally recognized standards V-PDB and atmospheric AIR, respectively. Accuracy and precision were < 0.2‰, as determined from multiple laboratory reference materials extensively calibrated using National Institute of Science and Technology reference materials and analyzed every 10 samples.

Preparation of Samples for Amino Acid Isotope Analysis

Isolated sponge, microbial cells, and plankton were prepared for compound-specific amino acid $\delta^{13}C$ and $\delta^{15}N$ analysis. Due to insufficient materials, cell fractions isolated from separate sponges were combined prior to analysis. Individual sponge samples are designated sponge *n*, whereas sponge and microbial cell isolates are S*n* and M*n* respectively, where *n* represents the sponge genotype. Microbial fractions for sponges 1 and 3 were combined, as were the sponge cell fractions. For sponges 4, 6, and 7, sufficient materials were available for AA-CSIA of microbial cell fractions for each sponge; however, these three sponge cell isolates were combined for $\delta^{13}C$ and $\delta^{15}N$ analysis.

Samples for AA-CSIA were hydrolyzed and trifluoroacetyl/isopropyl ester derivatives created according to the methods of Popp et al. [36] and Hannides et al. [49]. Samples (4–11 mg) were hydrolyzed (trace-metal grade 6 M HCl, 150 °C, 70 min) and the hydrolysate purified using low protein-binding filters and cation exchange chromatography. Purified samples were esterified using 4:1 isopropanol:acetyl chloride and derivatized using 3:1 methylene chloride:trifluoroacetyl anhydride. Trifluoroacetyl/isopropyl ester derivatives were additionally purified using solvent extraction [50] and stored at − 20 °C for up to 2 weeks before analysis. Samples were prepared with an additional vial containing a mixture of 15 pure AAs purchased commercially (Sigma Scientific).

Nitrogen Isotope Analysis of Amino Acids

The $\delta^{15}N$ values of AA trifluoroacetyl/isopropyl ester derivatives were determined using gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS, [51]). The isotope ratio mass spectrometer (IRMS; Thermo Scientific Delta V) was interfaced to a gas chromatograph (GC; Thermo Scientific Trace) fitted with a 60 m BPX5 *forte* column (0.32 mm internal diameter with 1.0 μm film thickness; SGE, Inc.) through a GC-C III combustion furnace (980 °C), reduction furnace (650 °C), and liquid nitrogen cold trap. Helium (1.2 mL min^{-1}) was used as the carrier gas. Prior to analysis, samples were dried and redissolved in an appropriate volume of ethyl acetate. Each sample was analyzed in at least triplicate, with norleucine and aminoadipic acid internal reference compounds co-injected in each run. The suite of 15 pure amino acids was also analyzed every 3 injections to provide an additional measure of instrument accuracy. The $\delta^{15}N$ values of all pure amino acid reference compounds were previously determined using the bulk isotope technique described above. Nitrogen isotope values are reported in standard δ-notation relative to atmospheric AIR. For replicate injections of samples, amino acid $\delta^{15}N$ standard deviations averaged 0.5‰ and ranged from 0.1 to 1.0‰.

Carbon Isotope Analysis of Amino Acids

$\delta^{13}C$ values of individual amino acid trifluoroacetyl/isopropyl ester derivatives were determined using an IRMS (MAT 253) interfaced with a Trace GC Ultra via a combustion furnace (1000 °C) and ConFlo IV interface (Thermo Scientific). Samples were injected using a PTV (pressure/temperature/volume) injector, held at 40 °C for 3 s, heated to 87 °C (400 °C min^{-1}), heated again to 200 °C and transferred at 200 °C using a 1:10 split. Helium (1 mL min^{-1}) was used as the carrier gas. The gas chromatograph was fitted with a BPX5 *forte* capillary column (30 m × 0.32 mm internal diameter with 1.0 μm film thickness; SGE, Inc.). The oven temperature for the GC started at 40 °C and was held for 1 min before heating at 15 °C min^{-1} to 120 °C, then 3 °C min^{-1} to 190 °C, and finally 5 °C min^{-1} to 300 °C where it was held for an additional 10 min. Isotope values are reported in standard δ-notation relative to V-PDB. Each sample was analyzed in at least triplicate with a perdeuterated *n*-C_{20} alkane with a well-characterized $\delta^{13}C$ value co-injected as an internal reference. The 15 AA reference suite was analyzed every three injections, and sample $\delta^{13}C_{AA}$ values corrected relative to this AA suite following Silfer et al. [52]. For statistical analysis, $\delta^{13}C_{AA}$ values were compared to those previously published by Larsen et al. [43, 53, 54]. To account for inter-laboratory differences, corrections based on results of previous extensive calibrations were used [55, 56].

Trophic Proxy and Trophic Position

Trophic position was estimated using the difference in $\delta^{15}N$ values between the trophic amino acid glutamic acid (Glx) and the source amino acid phenylalanine (Phe). This calculation assumed a β value of 3.4‰ for the difference in $\delta^{15}N$ values between Glx and Phe in primary producers and assumed that Glx was enriched in ^{15}N relative to Phe (Δ value) by 7.6‰ with each trophic transfer, e.g.: $TP = ((\delta^{15}N_{Glx} - \delta^{15}N_{Phe} - 3.4) / 7.6) + 1$ [34]. Uncertainty in calculations of trophic position was determined using propagation of errors [57, 58]. The propagated error in this TP calculation assumed the

uncertainty in β value is ± 0.9‰ and the error in Δ is ± 1.1‰ and used the measured analytical uncertainty in $\delta^{15}N_{Glx}$ and $\delta^{15}N_{Phe}$ based on at least triplicate analyses.

A proxy for trophic position was also determined using the difference in averaged $\delta^{15}N$ values between trophic (Alanine [Ala], Leucine [Leu], Glx) and source amino acids (Lysine [Lys], Phe). A second trophic position proxy was also calculated using weighted mean $\delta^{15}N$ values of trophic (Ala, Leu, Glx) and source amino acids (Lys, Phe) using:

$$\delta^{15}N_{\overline{x_w}} = \frac{\sum \frac{\delta^{15}N_x}{\sigma_x^2}}{\sum \frac{1}{\sigma_x^2}}, \quad (1)$$

where $\delta^{15}N_x$ is the nitrogen isotopic composition of a specified AA and σ_x is the standard deviation of the $\delta^{15}N$ value of that AA determined by triplicate isotopic measurements [51, 57]. Errors for proxy trophic position calculations were propagated using the measured reproducibility of individual AA in at least triplicate.

Summed Variance in $\delta^{15}N$ Values of Trophic Amino Acids (ΣV)

McCarthy et al. [40] introduced an index for microbial resynthesis of amino acids: ΣV, which is the sum of variance among individual $\delta^{15}N$ values of trophic amino acids. ΣV values for isolated sponge cell and microbial cell fractions were calculated using $\delta^{15}N$ values of Ala, Leu, Proline (Pro), Aspartic acid (Asx), and Glx.

Data Analysis

Statistical tests were conducted using SigmaPlot (v.13, Systat Software) and R [59]. For all statistical analyses, we considered p values < 0.05 statistically significant. Statistical differences between geographic regions in Kāne ohe Bay were tested using one-way analysis of variance tests (ANOVAs). Data distributions were assessed using histograms, with log-transformations used to improve normality when necessary. When ANOVA results were significant, a post-hoc Tukey's honest significant difference (HSD) test was conducted to determine which groups differed from others. Hierarchical cluster analysis of the $\delta^{13}C$ values of essential amino acids and $\delta^{15}N$ values of source amino acids of microbial cells, sponge cells, and plankton were performed in R using scaled data in a Euclidian dissimilarity matrix with the Ward clustering criterion. Clusters were validated using silhouette plots in the package *factoextra* [60].

Results

Flow Cytometry

In the sponge cell samples, two sponge cell populations were observed with flow cytometry: cells with very high DNA and chlorophyll fluorescence (Sponge-1), which were most likely sponge cells containing phytoplankton prey, and sponge cells without prey or with non-photosynthetic bacterial prey in them (Sponge-2, low DNA, and chlorophyll fluorescence per sponge cell). Both populations had high light scatter indicative of larger cells (Online Resource Figure 1). There were ca. six times more sponge cells present than residual phytoplankton or bacterial cells in these samples (Online Resource Table 1). The carbon contribution of sponge cells averaged 94 ± 4% (range 85% to 98%), while the residual microbial cell C ranged from 1.8 to 15% and averaged 6.3 ± 4% (Table 1). Sponge cell fractions contained a mean of 6×10^3 cells g^{-1} wet weight (range 1.4 to 12.1 × 10^3 cells g^{-1}, $n = 8$, Online Resource Table 1). Sponge-1 cell counts averaged 2×10^3 cells g^{-1}, while Sponge-2 cell counts averaged 4×10^3 cells g^{-1}. Residual microbial cells in these samples had a mean of 1×10^3 cells g^{-1} wet weight, with approximately equal cell contributions (both ~ 0.5×10^3 cells g^{-1}) of Microbe-1 (smaller phytoplankton and bacterial cells) and Microbe-2 (larger phytoplankton).

Table 1 Composition (percent of total) of microbial (M) and sponge (S) cell fractions by carbon contribution, after determination of cell abundances by flow cytometry. Populations shown are non-pigmented, heterotrophic bacteria (HBACT), phytoplankton without *Synechococcus* (PHYTO), *Synechococcus* (SYN), and sponge cells (SPONGE). Populations determined as described in the text

Sample	HBACT	PHYTO	SYN	Sponge
M1	79	21	0	0
M2	77	23	0	0
M3	33	66	1	0
M4	84	16	0	0
M5	41	58	1	0
M6	82	18	0	0
M7	83	17	0	0
M8	73	26	1	0
S1	8	0	0	92
S2	7	0	0	93
S3	16	0	0	84
S4	2	0	0	98
S5	7	0	0	93
S6	4	0	0	96
S7	3	0	0	97
S8	4	0	0	96

The sponge cell fraction samples S6 through S8 contained significantly more Sponge-2 cells ($p = 0.040$) and Sponge-1 cells ($p < 0.001$) than samples S1 through S5. The standard deviations do not differ significantly, and there were no significant differences for the other flow cytometry parameters in other sponge or microbial samples.

The microbial fraction samples contained ~10,000 times higher counts of bacteria (HBACT) and ~200 times higher counts of phytoplankton relative to the sponge cell samples (Online Resource Table 2). Microbial cell samples contained between 3.9 and 29.0 × 10^6 HBACT g^{-1} wet weight. Two subpopulations of HBACT were found: high DNA-containing HBACT (Bact-1) and low DNA-containing HBACT (Bact-2) (Online Resource Figure 2). Bact-1 ranged from 0.8 to 22.5 × 10^6 cells g^{-1}, and Bact-2 from 3.0 to 12.0 × 10^6 cells g^{-1}. *Synechococcus* (SYN) abundance ranged from 0.6 to 2.4 × 10^4 cells g^{-1}. Phytoplankton were divided into smaller (< 10 μm, Phyto-1) and larger (10–20 μm, Phyto-2) size fractions (Online Resource Figure 2). Phyto-1 abundance ranged from 4.9 to 30.6 × 10^4 cells g^{-1} wet weight. Phyto-2 abundance ranged from 0.3 to 1.6 × 10^4 cells g^{-1} wet weight. Microbial C was mostly HBACT (33% to 84%, average 69 ± 20%), with phytoplankton C ranging from 16 to 66% (average 30 ± 20%), while SYN C contributed only 0 to 1% (average 1.0 ± 0.4%) (Table 1).

C:N Molar Ratios of Whole Sponge and Isolated Sponge and Microbial Cell Fractions

C:N molar ratios for whole sponge samples averaged 4.5 and ranged from 4.0 to 5.4 but varied systematically with geographic location (Online Resource Table 3). Mean C:N ratio of the isolated sponge cell fraction (6.0 ± 0.2) were significantly different ($p = 0.016$) from the mean C:N ratio of the isolated microbial cell fraction (7.3 ± 1.3, Table 2). Whole sponge samples in the north bay had significantly higher C:N ratios than samples from mid bay ($p = 0.005$) and the south bay ($p < 0.001$). C:N ratios of whole sponge samples collected from the mid bay did not differ from those from south bay ($p = 0.744$). The C:N ratios of both the sponge cell fractions and the microbial cell fractions were significantly higher than the C:N ratios of all whole sponge samples from all locations ($p < 0.001$). The C:N molar ratio of the plankton sample was 4.4.

Bulk $\delta^{15}N$ and $\delta^{13}C$

Bulk tissue $\delta^{15}N$ and $\delta^{13}C$ values of whole sponge samples averaged 5.1‰ and − 18.0‰, respectively, but varied systematically with geographic location within Kāne ohe Bay (Online Resource Table 3). The mean bulk $\delta^{15}N$ values of whole sponge samples from the north bay were significantly higher than mid bay ($p = 0.014$) and south bay ($p = 0.003$). However, $\delta^{15}N$ values of sponges from the mid bay and south bay were not significantly different ($p = 0.798$). The mean bulk $\delta^{13}C$ values of sponges from the north bay did not differ from mid bay sponges ($p = 0.146$), but north bay sponge mean bulk $\delta^{13}C$ values were significantly higher than south bay sponges ($p < 0.001$). The mean $\delta^{13}C$ values of sponges from the mid bay were also significantly higher than the mean $\delta^{13}C$ values of sponges from the south bay ($p = 0.026$). The $\delta^{15}N$ value of the whole plankton sample is 7.1‰, whereas the $\delta^{13}C$ value is − 21.8‰.

Table 2 $\delta^{15}N$ (‰, vs. AIR) and $\delta^{13}C$ (‰, vs. V-PDB) values, as well as carbon:nitrogen (C:N) molar ratios, of isolated bulk microbial and sponge cell fractions. M = microbial fraction; S = sponge cell fraction

Sample	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	C:N
M1	8.1	− 31.2	5.7
M2	8.3	− 23.0	8.9
M3	8.0	− 23.0	8.4
M4	7.8	− 27.2	6.9
M5	8.1	− 23.0	8.8
M6	7.3	− 28.3	6.3
M7	7.3	− 29.1	6.2
M8	8.0	− 22.8	7.5
S1	7.6	− 22.4	6.0
S3	8.1	− 22.2	6.0
S4	8.4	− 21.9	5.9
S5	8.3	− 22.4	6.1
S6	7.9	− 23.3	6.1
S7	7.9	− 22.4	5.7
S8	8.6	− 22.3	6.2

Average bulk $\delta^{15}N$ value of the isolated sponge cell fraction was 8.1 ± 0.3‰ and average $\delta^{13}C$ value was − 22.4 ± 0.4‰ (Table 2). Average bulk $\delta^{15}N$ value of the isolated microbial cell fraction was 7.9 ± 0.4‰ and average $\delta^{13}C$ was − 26.0 ± 3.4‰ (Table 2). The mean $\delta^{15}N$ values of the sponge cell and microbial cell fractions were not significantly different ($p = 0.200$). However, the mean $\delta^{13}C$ value of the sponge cell fraction was significantly higher than the $\delta^{13}C$ values of the microbial cell fraction ($p = 0.018$). At all locations, the $\delta^{15}N$ value of both the sponge cell and the microbial cell fractions were significantly higher than the $\delta^{15}N$ value of whole sponges ($p < 0.001$), and the $\delta^{13}C$ value of both the sponge cell and microbial cell fractions were significantly lower than the $\delta^{13}C$ values of whole sponges ($p < 0.001$).

The $\delta^{15}N$ values of whole sponge samples and sponge cell fraction are significantly and positively correlated with C:N molar ratios (Online Resource Figure 3). In addition, the $\delta^{13}C$ values of the microbial cell fractions were significantly and positively correlated with C:N molar ratios (Online Resource Figure 4).

Compound-Specific Amino Acid Analysis

Carbon isotopic composition of individual amino acids was determined on six sponge samples with some fractions

combined in order to have sufficient material for analyses (Table 3). Microbial cell fractions M1 and M3 as well as sponge cell fractions S1 and S3 were combined into a single microbial and a single sponge sample, respectively for AA-CSIA. Three microbial cell fractions M4, M6, and M7 were analyzed individually; however, their equivalent sponge fractions had to be combined into a single sample (S4 + S6 + S7). Although 14 amino acids were detected, the $\delta^{13}C$ values of only 12 were reliably measured in all samples (Table 3). The $\delta^{13}C$ values of amino acids ranged from − 27.0 to 4.5‰. The essential amino acid (Thr, Val, Leu, Phe, and Lys) average $\delta^{13}C$ value of microbial cell fraction M1 + M3 (− 21.1 ± 0.6‰) are similar and shows overlapping error with that of sponge cell fraction S1 + S3 (− 21.6 ± 0.6‰) (Fig. 1). The essential amino acid $\delta^{13}C$ values of individual microbial cell fractions M4, M6, and M7 were not significantly different from those of the combined sponge cell fraction S4 + S6 + S7 ($p = 0.922$) (Fig. 1).

The carbon isotopic composition of 13 amino acids from the plankton sample ranged from − 27.8 to − 11.0‰. The average $\delta^{13}C$ value of essential amino acids in the single plankton sample collected is − 23.1 ± 1.1‰.

Recovery of sufficient material limited nitrogen isotope analyses even more than carbon isotope analysis and only five samples had sufficient material for analysis. In addition, only eight amino acids were reliably measured in all samples (Table 4). $\delta^{15}N$ values ranged from 2.3 to 16.4‰ and average trophic amino acid (Ala, Leu, Pro, Asx, Glx) $\delta^{15}N$ values were higher (10.6 ± 3.3‰) than those of source amino acids (Lys, Phe, 4.1 ± 1.3‰). Direct comparison of nitrogen isotopic compositions between microbial and sponge cell fractions could only be made for M4, M6, and M7 and S4 + S6 + S7 (Fig. 2). The source amino acid $\delta^{15}N$ values of microbial cell fractions M4, M6, and M7 were not significantly different from those of sponge cell fraction S4 + S6 + S7 ($p = 0.288$). In addition, the trophic amino acid $\delta^{15}N$ values of microbial cell fractions M4, M6, and M7 were not significantly different from those of sponge cell fraction S4 + S6 + S7 ($p = 0.267$).

The $\delta^{15}N$ values of 13 amino acids from the plankton sample ranged from − 2.0 to 12.5‰. The average $\delta^{15}N$ values of source acids in the single plankton sample collected is 2.2 ± 0.7‰. A dendrogram produced from data clustering analysis showed two distinct data clusters. Plankton comprised the most distinct cluster and different from those of sponge and microbial cell fractions, which did not separate from each other in the cluster analysis (see Online Resource Figure 5).

Trophic position calculated from the difference in $\delta^{15}N$ values of Glx and Phe ($TP_{Glx\text{-}Phe}$) for the combined sponge sample (S4 + S6 + S7) is 2.1 (propagated SD = ±0.2) and the average trophic position for samples M4, M6, and M7 is 1.9 (propagated SD = ±0.3) (Table 5). $TP_{Glx\text{-}Phe}$ of these sponge cell and microbial cell fractions were not significantly different ($p = 0.622$).

Table 3 $\delta^{13}C$ values (‰, vs. V-PDB) of individual amino acids from isolated microbial and sponge cell fractions and well as plankton. M = microbial fraction; S = sponge cell fraction. Plus (+) symbols indicate fractions combined to have sufficient material for amino acid isotope analyses. Non-essential amino acids are Ala, Gly, Ser, Pro, Asx, Glx, and Tyr. Essential amino acids are Leu, Phe, Lys, Val, and Thr

Sample	Ala	Gly	Ser	Pro	Asx	Glx	Tyr	Leu	Phe	Lys	Val	Thr
M1 + M3	− 18.3 ± 0.2	− 11.3 ± 0.2	+ 4.0 ± 1.3	− 19.8 ± 0.2	− 15.9 ± 0.1	− 14.6 ± 0.1	− 19.9 ± 0.7	− 26.6 ± 0.3	− 24.4 ± 0.1	− 16.2 ± 0.1	− 25.1 ± 0.4	− 13.5 ± 0.8
M4	− 17.5 ± 0.2	− 8.5 ± 0.1	− 0.5 ± 0.8	− 20.0 ± 0.2	− 16.8 ± 0.3	− 14.9 ± 0.1	− 24.6 ± 0.4	− 26.5 ± 0.1	− 23.5 ± 0.2	− 17.6 ± 0.6	− 24.5 ± 0.1	− 15.3 ± 0.4
M6	− 17.2 ± 0.2	− 7.0 ± 0.7	− 2.9 ± 0.8	− 20.2 ± 0.2	− 17.2 ± 0.3	− 15.4 ± 0.2	− 25.2 ± 0.8	− 25.7 ± 0.1	− 23.4 ± 0.3	− 17.4 ± 0.4	− 24.8 ± 0.4	− 15.6 ± 0.4
M7	− 21.0 ± 0.2	− 9.4 ± 0.9	− 0.5 ± 0.9	− 19.9 ± 0.2	− 16.6 ± 0.1	− 15.9 ± 0.8	− 22.6 ± 0.3	− 26.5 ± 0.5	− 24.4 ± 0.3	− 18.5 ± 0.3	− 24.1 ± 0.3	− 15.2 ± 0.1
S1 + S3	− 19.0 ± 0.4	− 12.4 ± 0.5	− 17.3 ± 0.1	− 19.5 ± 0.3	− 19.1 ± 0.1	− 15.8 ± 0.1	− 26.3 ± 0.4	− 27.0 ± 0.1	− 24.2 ± 0.1	− 18.2 ± 0.3	− 23.9 ± 0.2	− 14.9 ± 0.2
S4 + S6 + S7	− 17.3 ± 0.3	− 13.4 ± 0.3	+ 4.5 ± 1.2	− 19.6 ± 0.4	− 17.2 ± 0.2	− 14.6 ± 0.3	− 23.3 ± 0.2	− 26.3 ± 0.1	− 23.8 ± 0.1	− 17.9 ± 1.0	− 25.3 ± 0.9	− 13.8 ± 0.5
Plankton	− 20.8 ± 0.3	− 19.5 ± 0.3	− 11.0 ± 0.6	− 17.3 ± 0.4	− 15.4 ± 0.2	− 16.1 ± 0.4	− 25.2 ± 0.7	− 27.8 ± 0.2	− 24.9 ± 0.4	− 19.3 ± 1.0	− 27.2 ± 0.1	− 17.9 ± 0.4

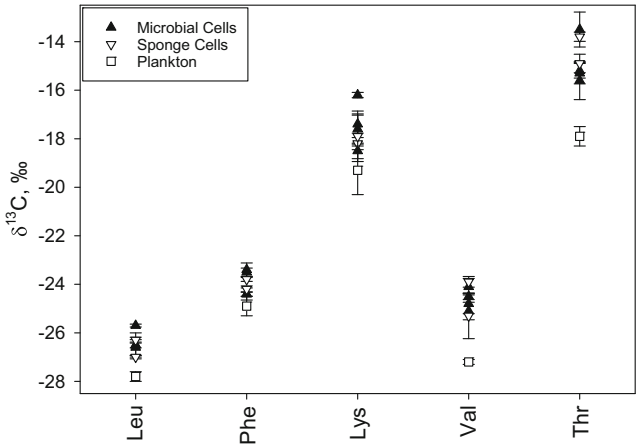

Fig. 1 Essential amino acid $\delta^{13}C$ values (‰, vs. V-PDB) in microbial and sponge cell fractions isolated from *Mycale grandis* and from a plankton tow collected in south Kāne ohe Bay

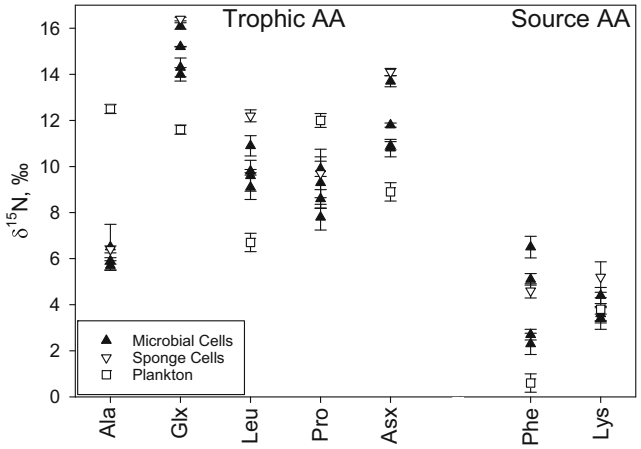

Fig. 2 Trophic and source amino acid $\delta^{15}N$ values (‰ vs. AIR) in microbial and sponge cell fractions isolated from *Mycale grandis* and from a plankton tow collected in south Kāne ohe Bay

The difference in the average $\delta^{15}N$ values of trophic and source amino acids, a proxy measure of trophic position, of the sponge cell fraction (sample S4 + S6 + S7) was 6.9 ± 1.3‰ and that of the microbial cell fractions (samples M4, M6, and M7) was 5.9 ± 1.2‰ (Table 5). These proxy measurements of trophic position of the microbial and sponge cell fractions were not significantly different ($p = 0.540$). In addition, the difference in the weighted mean $\delta^{15}N$ values of trophic and source amino acids of the sponge cell fraction (sample S4 + S6 + S7) was 9.4 (propagated SD = 0.4) and 8.9 (propagated SD = 0.3) for the microbial cell fractions (samples M4, M6, and M7). This trophic proxy of the microbial and sponge cell fractions was also not significantly different ($p = 0.286$).

Summed Variance in $\delta^{15}N$ Values of Trophic Amino Acids (ΣV)

V values of cell fractions ranged from 2.0 to 3.0 (Table 6). V values of microbial cell fractions M4, M6, and M7 were not significantly different from those of sponge cell fraction S4 + S6 + S7 ($p = 0.230$).

Discussion

Sponge-associated microbial symbionts play an important role in the trophic ecology of sponges. While the sponge holobiont has been shown to be capable of DOM uptake and ultimately the incorporation of both carbon and nitrogen, the exact mechanisms and the role(s) microbes may play in this process has not been fully described. Separate analysis of sponge and symbiotic microbial cells here reveal details about the relationship and mechanism of nutrient transfer from symbiont microbes to the sponge host. The separated sponge cell and microbial cell fractions contained distinct cell compositions, with the sponge fraction dominated by large (> 5 μm diameter) sponge cells and the microbial cell fraction predominantly composed of heterotrophic bacteria. Despite large and systematic variation of $\delta^{15}N$ and $\delta^{13}C$ values in whole sponge samples throughout Kāne ohe Bay, the $\delta^{13}C$ values of the essential amino acids and $\delta^{15}N$ values of the source amino acids were not statistically different between the two cell fractions. The indistinguishable amino acid isotopic compositions and high ΣV values suggest that *M. grandis*, despite having chlorophyll-containing cells within some sponge cells, primarily acquires C and N through direct transfer of bacterially

Table 4 $\delta^{15}N$ values (‰ vs. AIR) of individual amino acids from isolated microbial and sponge cell fractions as well as plankton. M = microbial fraction; S = sponge cell fraction. Plus (+) symbols indicate fractions combined to have sufficient material for amino acid isotope analyses. Trophic amino acids are Ala, Glx, Leu, Pro, and Asx. Source amino acids are Phe, Tyr, and Lys. ND indicates no data

Sample	Ala	Glx	Leu	Pro	Asx	Phe	Tyr	Lys
M1 + M3	5.9 ± 0.1	16.1 ± 0.1	10.9 ± 0.4	9.9 ± 0.3	13.7 ± 0.2	2.7 ± 0.2	2.5 ± 0.5	4.4 ± 0.4
M4	5.7 ± 0.2	15.2 ± 0.0	9.8 ± 0.1	8.6 ± 0.4	11.8 ± 0.1	5.1 ± 0.2	4.2 ± 1.3	3.4 ± 0.2
M6	6.5 ± 1.0	14.3 ± 0.4	9.6 ± 0.7	9.3 ± 1.1	10.9 ± 0.2	6.5 ± 0.5	ND	3.6 ± 0.3
M7	ND	14.0 ± 0.3	9.1 ± 0.5	7.8 ± 0.6	10.8 ± 0.4	2.3 ± 0.5	1.1 ± 0.3	3.4 ± 0.5
S4 + S6 + S7	6.4 ± 0.2	16.4 ± 0.1	12.2 ± 0.3	9.7 ± 1.1	14.1 ± 0.2	4.6 ± 0.3	2.4 ± 0.3	5.2 ± 0.7
Plankton	12.5 ± 0.2	11.6 ± 0.2	6.7 ± 0.4	12.0 ± 0.3	8.9 ± 0.4	0.6 ± 0.4	4.6 ± 0.7	3.8 ± 0.5

Table 5 *Mycale grandis* isolated sponge (S) and microbial (M) cell fractions trophic position ($TP_{Glx\text{-}Phe}$) and trophic position proxies (TPP). TPP calculated using average of $\delta^{15}N$ values of trophic and source amino acids (average), and TPP calculated using weighted mean $\delta^{15}N$ values of trophic and source amino acids (weighted), along with propagated standard deviations

Sample	$TP_{Glx\text{-}Phe}$	TPP (average)	TPP (weighted)
S4 + S6 + S7	2.1 ± 0.2	6.9 ± 1.3	9.4 ± 0.3
M4	1.9 ± 0.2	6.0 ± 1.0	11.1 ± 0.2
M6	1.6 ± 0.2	5.1 ± 1.4	7.9 ± 0.4
M7	2.1 ± 0.2	6.6 ± 1.1	7.7 ± 0.4
M4 + M6 + M7 (average)	1.9 ± 0.3	5.9 ± 1.2	8.9 ± 0.3

synthesized amino acids, and not from feeding on planktonic microbes or detrital components from the water column. Therefore, microbial symbionts appear to play a major role in the acquisition of C and N from DOM uptake and its subsequent transfer and utilization by *M. grandis*.

Separation of Sponge and Microbial Cell Fractions

Determining the distinct isotopic composition of the sponge and its associated microbes required separation of these two fractions. Flow cytometry revealed fractions distinguished by size (light scatter), pigment (chlorophyll and phycoerythin fluorescence), and DNA content. Epi-fluorescence microscopy showed large (~ 5 μm nuclei) cells within the sponge tissue samples that were largely devoid of fluorescence and hence composed of only sponge cells. Consequently, these results indicate that the sponge fractions contained predominantly sponge cells and that the microbial fractions contained predominantly smaller prokaryotic cells.

Bulk $\delta^{15}N$ and $\delta^{13}C$ values in the whole sponge, sponge, and microbial cell fractions also suggest good separation of the cell fractions (Fig. 3). Although there is overlap in $\delta^{13}C$ and $\delta^{15}N$ values between some sponge and microbial cell fractions, the average difference between these two fractions

Table 6 Summed variance in the $\delta^{15}N$ values of select trophic amino acids (ΣV) of isolated sponge (S) cell and microbial (M) cell fractions of *Mycale grandis*

Sample	Summed variance (ΣV)
S4 + S6 + S7	3.0
M1 + M3	2.9
M4	2.6
M6	2.0
M7	2.4
Average microbial (all)	2.5
Average microbial (M4, M6, M7)	2.3

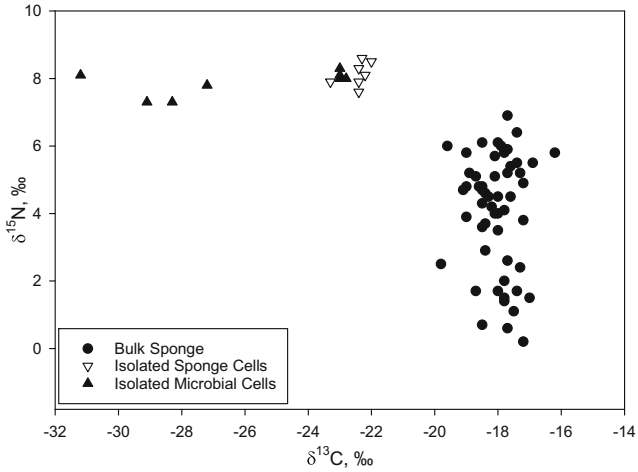

Fig. 3 Bulk $\delta^{13}C$ (‰, vs. V-PDB) and $\delta^{15}N$ values (‰ vs. AIR) of whole sponges and the sponge and microbial cell isolates. The whole sponge carbon and nitrogen isotopic composition of the sponges that the cells were isolated from was not measured. Note that the sum of isolated sponge cell + microbial cell isolates ≠ whole sponge, since whole sponge samples include structural material not present in either fraction

is quite large (3.5‰) and the mean C:N ratio of sponge cell and microbial cell fractions are statistically different.

A weak correlation exists between the $\delta^{13}C$ values of bulk microbial cells and the C contribution of bacteria to the microbial cell fraction (Online Resource Figure 6). A higher C contribution from bacterial cells in this fraction results in lower $\delta^{13}C$ values. In addition, a plot of $\delta^{13}C$ values versus the inverse of the bacterial biomass (1/HBACT) yields a y-intercept close to − 29‰, which closely resembles samples M1, M4, M6, and M7. Some microbial cell fractions (M2, M3, M5, and M8) were also found to have unexpectedly high C:N ratios and the $\delta^{13}C$ values of the microbial cell fractions are significantly and positively correlated with C:N ratios (Online Resource Figure 4), suggesting some microbial cell fractions may have contained carbonate that would increase both C:N ratios and $\delta^{13}C$ values.

Based on these relationships, we suggest that microbial cell fractions with higher C:N values are not representative of the pure microbial fraction, while the lower C:N values found in samples M1, M4, M6, and M7 are more representative and distinctly different from the composition of sponge cell fractions.

Isolated sponge and microbial cell fractions differ significantly from whole sponge tissue samples in C:N, $\delta^{15}N$, and $\delta^{13}C$ values. In the Demospongiae, the internal sponge matrix is dominated by gelatinous collagen-rich mesohyl reinforced by a dense network of fibrous spongin that provides structure and flexibility. Spongin is a modified collagen protein secreted by collagen-producing sponge cells within the mesohyl, and together spongin and mesohyl constitute the majority of bulk sponge tissue. Diet-to-collagen isotope fractionation in bone collagen is known to result in ^{13}C enrichment in bone collagen of about 5‰ relative to other biomolecules from plants and

animals (e.g., see Fernandes et al. [61] and references therein). We suggest that similar carbon isotope fractionation can explain the ~5‰ difference between the spongin-rich whole sponge and sponge cell fraction $\delta^{13}C$ values.

The C:N ratio of the sponge cell fraction (average of S4 + S6 + S7) was significantly lower relative the C:N of the microbial fraction (average of M4 + M6 + M7), indicating fundamental differences in the composition of the two fractions. In addition, the microbial cell fractions also exhibited lower average bulk $\delta^{13}C$ values compared to the bulk $\delta^{13}C$ values of sponge cells. Higher lipid-to-biomass content drives $\delta^{13}C$ values lower and increases C:N ratio [62, 63]. Thus, higher lipid content in microbial cells may contribute to observed $\delta^{13}C$ values and C:N ratios. While the C:N ratio and the relative carbohydrate-lipid-protein composition of sponge-associated bacteria are not known, the sponge cell fraction may have a higher protein and carbohydrate composition relative to lipid than the microbial samples, which may be reflected in the observed C:N ratios. Aside from lipid content effects on C:N and $\delta^{13}C$ differences, the protein fraction contains dietary information about the transfer of amino acids, and the fractionation between trophic and source amino acids in N and non-essential and essential amino acids in C.

The $\delta^{15}N$ values of the sponge cell fraction were significantly different from those of whole sponge (Online Resource Figure 3); however, this difference was likely derived from the varying N isotopic composition of nutrients in different regions of Kāne ohe Bay. Nitrogen inputs vary with seasonal patterns and weather (e.g., heavy rainfall and wind). Stream runoff and anthropogenic inputs from urban development, such as coastal residential housing, He'eia Harbor, and commercial properties are also sources of N isotope variability across the bay [64, 65]. The higher $\delta^{15}N$ values measured in samples from the north bay may indicate input from more numerous residential cesspools in the watershed adjacent to the northern portion of the bay [66], as NO_3^- associated with sewage input is typically enriched in ^{15}N relative to that in seawater [67, 68]. Inputs of submarine groundwater discharge [69] would also result in higher $\delta^{15}N$ values [70, 71].

Amino Acid Isotopic Compositions of Sponge Cell Fractions and Plankton

Although the flow cytometry, microscopy, C:N ratios, and carbon isotopic compositions of microbial and sponge cell fractions were distinctly different, the carbon and nitrogen isotopic compositions of amino acids in both fractions were remarkably similar (Tables 3 and 4, Figs. 1 and 2). This similarity in bacterial and sponge cell amino acid $\delta^{13}C$ and $\delta^{15}N$ values strongly suggests that *M. grandis* is acquiring amino acids directly from its heterotrophic bacteria and not through feeding on *Synechococcus* spp. or other planktonic photoautotrophs. This is because it is unlikely that *Synechococcus* or other autotrophs would synthesize amino acids with C and N isotopic compositions identical to the pattern found in the heterotrophic microbial cells that dominate the microbial cell fractions.

Indo-Pacific sponges have long been considered to be phototrophic [25, 72], and *M. grandis* hosts cyanobacterial phylotypes [73]. Among cyanobacteria, *Synechococcus* and *Prochlorococcus* are the dominant groups in most oceans and have been found in at least 26 Demospongiae families [16]. A monophyletic "*Synechococcus spongiarum*" clade that is phylogenetically distinct from the nearest free-living *Synechococcus* relative has been reported to be present in 18 sponge species from various geographic locations and is the most prevalent photosynthetic symbiont present in marine sponges [74, 75]. Although cyanobacterial symbionts can comprise 25 to 50% of a sponge's cellular volume [76], relatively little is known about the metabolic exchange and ecological interactions between sponges and their microbial symbionts.

Synechococcus is the dominant cyanobacteria taxon in Kāne ohe Bay and contributes ≥ 35% of phytoplankton biomass, and is the main autotroph in the microbial to metazoan consumer food web, particularly during dry conditions (i.e., low stream flow) [77]. Consequently, the $\delta^{13}C$ values of essential amino acids and the $\delta^{15}N$ values of source amino acids in the plankton net sample (63–250 μm) should reflect those of *Synechococcus*. Indeed, these average values, -23.4 ± 1.1‰ $\delta^{13}C$ and 2.2 ± 0.7‰ $\delta^{15}N$, were lower than the essential amino acids and source amino acids found in the sponge cell fraction (-21.4 ± 1.5‰ $\delta^{13}C$ and of 4.9 ± 0.7‰ $\delta^{15}N$, Figs. 1 and 2).

Sponge cells with high DNA, chlorophyll, and phycoerythrin signals represented ~ 1/3 of the sponge cell biomass and were likely sponge cells with phytoplankton prey within them. Many *Synechococcus* species contain phycoerythrin, and some sponges have been shown to prey on *Synechococcus* in feeding clearance studies [78–80]. It is unknown whether *M. grandis* consumes *Synechococcus* prey directly and we did not perform *Synechococcus* feeding experiments. However, in our *M. grandis* samples, *Synechococcus* represented < 1% of the carbon contribution in the microbial cell fraction, even though microscopy showed that *Synechococcus* was the most abundant sponge-associated microbe in *M. grandis*. Although *Synechococcus* are ~ 2-3Xs larger than most bacterial cells and some may have been lost during cell fraction separation, their low abundance and differing isotopic signals suggest their negligible importance in the acquisition of amino acids by *M. grandis* sponge cells.

Lastly, using the $\delta^{13}C$ values of essential amino acids and the $\delta^{15}N$ values of source amino acids, the net plankton sample forms a distinct end-member that clusters separately from the microbial and sponge cell fractions (Online Resource Figure 5). This distinct clustering of amino acid $\delta^{13}C$ and

$\delta^{15}N$ values provides support for microbial symbionts as being the source for amino acids in the sponge *M. grandis*, as opposed to plankton-derived nutrition or DOM uptake. Our results however cannot distinguish between the possibility that the essential and source amino acids in *M. grandis* sponge cells fraction were acquired from planktonic heterotrophic bacteria. However, if planktonic heterotrophic bacteria were the source of these amino acids in sponge cells, it would require that they took up DOM and synthesized essential amino acid and source amino acids with $\delta^{13}C$ and $\delta^{15}N$ values significantly different from that we measured in our plankton sample but identical to that in the microbial and sponge cells isolated from *M. grandis*. Given the similarity in isotopic compositions of essential and source amino acids in the microbial and sponge cell fractions isolated from *M. grandis*, the most parsimonious explanation is that microbial symbionts hosted by the sponge were responsible for uptake of DOM and the synthesis of amino acids that were translocated to sponge cells.

Summed Variance in $\delta^{15}N$ Values of Trophic Amino Acids (ΣV)

ΣV is a proxy for heterotrophic bacterial re-synthesis of organic matter, as partial re-synthesis introduces isotopic variability among trophic amino acids in bacterial cells [40, 41, 81]. Heterotrophic reworking of proteinaceous material occurs through a range of processes, including extracellular hydrolysis [42], de novo synthesis and selected re-synthesis, salvage pathways incorporating amino acids into new biomass, and strict catabolism [40]. The ΣV index is a measure of the relative variability in the $\delta^{15}N$ values of select trophic amino acids due to microbial alteration [82]. Patterns in ΣV can differentiate microbial processing from eukaryotic synthesis of amino acids. Values above ΣV ~ 2 are generally considered a threshold for organic matter resulting predominantly from bacterial re-synthesis of detrital materials, although consumption of particles by zooplankton can also result in a slight increase in ΣV values [40].

The V value of the net plankton sample was 2.0, whereas V values in sponge and microbial fractions tended to be higher (2.0–3.0) (Table 6) and were consistent with heterotrophic microbial alteration of amino acids. The V values in microbial and sponge cell fractions were similar to those found in cultures of heterotrophic bacteria and microbially reworked particles in the ocean [40–42] and were higher than values found in phytoplankton and zooplankton [40]. McCarthy et al. [40] observed typical values of ΣV range from ~ 0 to 1 for phytoplankton, 1 to 1.5 for zooplankton, and up to ~ 3 for detrital POM in surface sediment traps along the equatorial Pacific. Yamaguchi and McCarthy [82] also measured high values of ΣV (~ 2 to 4) in high molecular weight dissolved organic matter in the Pacific Ocean north of Hawaii. These researchers also showed that ΣV values increased from surface waters (21 m, ΣV = ~ 2) to mesopelagic depths (ΣV = ~ 2.8 at 915 m and ~ 4 at 670 m). In contrast, they found 500 kDa to ~ 1 μm particles from 25 to 750 m had ΣV values less than ~ 2. Consequently, Yamaguchi and McCarthy [82] suggested that high ΣV values are found in DOM that has been bacterially altered and in bacterial cells which have partly re-synthesized amino acids.

The ΣV of the microbial and sponge cell fractions can reveal the origin of amino acids. The high values observed in the sponge cell fractions (S4 + S6 + S7) suggest that amino acids were not acquired directly from the consumption of fresh phytoplankton. Although higher ΣV values were observed in zooplankton relative to phytoplankton in the equatorial Pacific Ocean [40], those changes were small (increase from ~ 0.7 to 1.5). Sponges have the capacity to incorporate DOM [7, 8, 12, 26]. Thus, the high ΣV values observed in microbial and sponge cell fractions may be evidence of uptake of DOM with high ΣV values. If bacteria in *M. grandis* play a significant role in DOM uptake, these microbes could acquire at least some of their high ΣV values from DOM and transfer those partially re-synthesized amino acids to the host sponge cells. However, the lack of statistical difference between the ΣV values of sponge cell and microbial cell fractions supports the conclusion that the high ΣV values observed in the sponge cell fraction were inherited from their microbial symbionts, rather than the sponge cells directly taking up DOM from the water column.

Trophic Position and Trophic Proxy

No significant differences were observed in trophic position determined from AA-CSIA between the sponge cell and microbial cell fractions (Table 5). Although calculation of the trophic position from AA-CSIA has become common in metazoan food webs, questions remain about the accuracy of β and Δ values [34, 57]. In addition, little is known about these values in microbial food webs [83]. Assuming that β and Δ values used in metazoan food webs apply, the average trophic position calculated for the microbial cell fraction (1.9 ± 0.3) is not different from that determined for the sponge fraction analyzed (2.0 ± 0.2). Assuming the β and Δ values remain reasonably constant, calculation of trophic position from AA-CSIA is highly dependent upon the difference between the $\delta^{15}N$ values of trophic and source amino acids.

Trophic position proxy (TPP) removes the reliance on assumed knowledge of β and Δ values, and is therefore based only on the difference in $\delta^{15}N$ values of trophic and source amino acids [57, 84]. Metazoan consumption results in ^{15}N enrichment of trophic amino acids relative to source amino acids [34–36]. TPP values for sponge cells (S4 + S6 + S7) were not greater than the TPP values of the complementary microbial cell samples (M4, M6, and M7) when determined

from average $\delta^{15}N$ values of amino acids (Table 5). TPP using weighted $\delta^{15}N$ averages based on the uncertainty of the measurements also revealed no significant difference in TPP between the sponge and its associated microbes (Table 5).

Phagotrophic protists do not necessarily exhibit the systematic ^{15}N trophic enrichment that is well established for metazoan consumers. Salvage pathway incorporation of amino acids was thought to be restricted to bacteria, but the $\delta^{15}N$ values of trophic and source amino acids in some protistan consumers are not significantly different from those in their prey [85], with the exception of Ala [85, 86]. While a similar effect cannot currently be eliminated as a possibility for the lack of difference observed between isotopic patterns of amino acids in sponge and microbial cell fractions, all other evidence indicates that direct transfer and incorporation of amino acids from microbes to sponge is more likely.

First, the $\delta^{15}N$ values of source amino acids and the $\delta^{13}C$ values of essential amino acids are not statistically different in microbial and sponge cell fractions, suggesting that amino acids in the sponge fraction are directly transferred from the sponge-associated microbes to sponge cells without isotope fractionation. Unfortunately, no C isotope results are available from the experiments of Gutiérrez-Rodríguez et al. [85] or Décima et al. [86] to further corroborate these results. Second, sponge and microbial cell fractions ΣV values are not statistically different and the values are indicative of heterotrophic microbial alteration of amino acids (Table 6). ΣV values of alga *Dunaliella* isolated from the first stage of the chemostat experiments of Gutiérrez-Rodríguez et al. [85] averaged 3.1, whereas ΣV values of protists in stages 2 (light) and 3 (dark) were 1.4. It is unknown why the *Dunaliella* ΣV values in these experiments are so high. Unfortunately, these data are too few however to draw conclusion about whether sponge cells feeding phagotrophically on their host microbial cells are responsible for the similarity in trophic position between these cell types as has been observed elsewhere in protists [10, 87].

Should sponges behave as typical metazoans and consume, digest, and assimilate bacterial cells, they would exhibit a difference in trophic position through obligate deamination and transamination in amino acids derived from their bacterial prey. Instead, it appears that amino acids are transferred from heterotrophic bacteria to the host by translocation in a manner similar to those found in scleractinian corals and its endosymbionts (Family Symbiodiniaceae) in the transfer of nutrients that support growth and metabolism [88, 89]. It is also possible that the transfer of nutrients is from the phagocytosed microbes they host. Corals harbor a variety of symbiotic archaea and bacteria [90], and the translocation of C-rich photosynthates to the host may help the symbionts maintain a favorable C:N ratio [91]. While corals are photoautotrophic, they also rely on prey capture and particle feeding to meet their N requirements. Sponges may likewise be able to undergo shifts between translocation-like nutrient acquisition and heterotrophic feeding as observed in coral [92] under different environmental conditions. The amino acid isotopic compositions of sponge and microbial cells suggest that *M. grandis* acquires C and N predominantly through translocation of amino acids or through some kind of direct assimilation of their host microbes. It is not currently known if translocation of amino acids can occur in sponges; however, our results are consistent with the occurrence of the translocation of products between sponge cells we analyzed and symbiotic microbes that would not produce isotopic fractionation through the severing of amine bonds.

Implications for Amino Acid Metabolism in Sponges

The lack of ^{15}N enrichment in trophic amino acids relative to source amino acids in sponges is surprising and may have implications for amino acid metabolism in sponges. The absence of change in the $\delta^{15}N$ value of Glx between bacterial and sponge cells is particularly surprising since glutamate is a key intermediate in the Krebs cycle and plays a fundamental role in amino acid biosynthesis in metazoans that link protein and energy metabolism [93]. However, as mentioned above, phagotrophic protists do not necessarily exhibit the systematic ^{15}N trophic enrichment that is seen in metazoan consumers and choanocytes phagocytized a significant proportion of the small particles they capture [94]. Early studies [95] showed that phagosomes containing particles were quickly transferred from choanocytes to archaeocytes in the mesophyll where digestion and assimilation continues. Sponges can recognize and discriminate microbes using immune signaling [18] and Leys et al. [10] showed microscopic evidence for large numbers of symbiont microbes being phagocytosed in archaeocyte cells. Although choanocyte and archaeocyte phagocytose, digest, and assimilate prey, excretion of metabolic products occurs primarily in spherulous and granular cells in the sponge mesophyll [96]. Since primary nitrogen isotope effects can only occur when nitrogen bonds are broken or formed, ^{15}N enrichment in trophic amino acids relative to source amino acids might be restricted to spherulous and granular cells in the sponge mesophyll where amino acid transamination and deamination occurs. With regard to our results, spherulous and granular sponge cells may not have been in high concentration in our sponge cell fraction and therefore we did not observe ^{15}N enrichment in trophic amino acids relative to source amino acids typical of metazoans.

In summary, the lack of ^{15}N enrichment in trophic amino acids relative to source amino acids in sponge cells we analyzed may be expected. The separation scheme we used concentrated choanocyte cells. The similarity in bacterial and sponge cell $\delta^{13}C$ values of essential amino acids and $\delta^{15}N$ values of source amino acids strongly suggests that *M. grandis* is acquiring amino acids directly from its heterotrophic bacteria. The isotopic composition of these amino acids in choanocyte cells may have been acquired through translocation of

bacterial amino acids or through bacterial cell phagocytosis. It is possible that amino acid and energy metabolism may occur in spherulous and granular cells not measured in this study. This hypothesis could be tested by analyzing the isotopic composition of spherulous and granular cells or perhaps spongin collagen fibers secreted by spongocyte cells.

Conclusions

Evidence of symbiont-derived nutrition has been studied in phototrophic sponges [44, 97] and the incorporation of algal and coral-derived DOM into sponge tissue [5, 8, 12, 26]. In both cases, evidence suggests that the microbial symbionts provide nutrition to the host sponge. While primary producers have been identified as the original source of C and N to reefs and the sponge holobiont, previous experiments did not directly demonstrate that C and N were transferred as amino acids from their symbiotic microbes directly to sponge cells. Here, we propose that bacterial symbionts in *M. grandis* provide an intermediate step in the transfer of C and N from DOM as suggested by others [9–12]. It is unlikely that identical isotopic compositions of amino acids in sponge and microbial cells would occur from direct transfer of amino acids from planktonic heterotrophic bacteria to sponge cell, as microbial symbionts of *M. grandis* and planktonic heterotrophic bacteria would be expected to have different amino acid isotopic compositions. Rather, we suggest that the heterotrophic bacteria hosted by *M. grandis* are consuming DOM, re-synthesizing the organic material, and passing on nutrition to the sponge as amino acids through translocation. This scenario is consistent with no change in trophic position and absence of amino acid isotope fractionation between the microbe and the host sponge cells analyzed, as it implies that no amine bonds were broken in this transfer and that is inclusive of essential and "trophic" and "source" amino acids. Thus, as previously recognized, nitrogen cycling within marine sponges is likely more complex than previously assumed.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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