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Complex interactions between marine sponges and their symbiotic microbial communities

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Abstract

To investigate the importance of symbiont-derived nutrition to host sponges, we coupled manipulative shading experiments with stable isotope analyses of isolated symbiont and host cell fractions. Experiments were conducted with four common reef sponges: *Aplysina cauliformis*, *A. fulva*, *Neopetrosia subtriangularis*, and *Niphates erecta*. The sponge *N. erecta* lacks photosymbionts, had a higher growth rate under shaded conditions, and displayed no difference in chlorophyll *a* (Chl *a*) concentrations across treatments. Isotope values suggested that this sponge obtains nutrition from particulate organic matter in the water column. In contrast, sponges hosting cyanobacterial symbionts (*Aplysina* spp. and *Neopetrosia*) had lower growth rates and lower Chl *a* concentrations under shaded conditions, suggesting that these hosts rely on photosymbiont nutrition. δ^{15}N and δ^{13}C values of sponge and microbial cell fractions demonstrated that, while both carbon and nitrogen are transferred from symbionts to host cells in *A. cauliformis*, only carbon is transferred in *N. subtriangularis*, and only nitrogen is transferred in *A. fulva*. Under shaded conditions, shifts in symbiont δ^{13}C values were coupled to shifts in host δ^{13}C values in some, but not all, host species, suggesting that the stability of these interactions varies across host species. Symbiont-derived nutrients are transferred to the cells of host sponges, and the variability observed among host species indicates that these interactions are more complex than originally hypothesized.

Mutualistic interactions are based on the reciprocal exchange of goods or services that would be too costly or impossible to obtain without the use of a partner (Boucher 1985). Obligate mutualisms have long been exemplified by photosymbionts living within marine invertebrates in oligotrophic coral reef ecosystems. In these ecosystems, symbionts can confer supplemental nutrition that allows their hosts to compete for space where substrate is limited (Muscatine 1967; Wilkinson 1979). The relationship between reef-building corals and the dinoflagellate *Symbiodinium* (Muscatine and Cernichiari 1969; Muscatine and Porter 1977) is a well-defined example of such a mutualism. Recently, additional invertebrate–photosymbiont symbioses have been documented across numerous host taxa, including turbellarians, anemones, ascidians, tridacnid clams, and sponges (Venn et al. 2008).

In what might be the most ancient microbe–metazoan association, marine sponges host abundant and diverse communities of symbiotic microbes (Webster and Blackall 2009). In exchange for a nutrient-rich environment and the products of host metabolism, these microbes might provide the sponge with access to products of novel metabolic pathways and chemical defenses (Flatt et al. 2005; Taylor et al. 2007*b*). The diversity of sponge-specific symbiotic microbes is staggering, with members of cyanobacteria, proteobacteria, actinobacteria, the candidate phylum "poribacteria," and archaea reported within many species (Taylor et al. 2007*b*). Photosymbionts such as cyanobacteria are dominant members of this consortium, which, as in corals, could provide supplemental nutrition crucial for host performance and survival (Wilkinson 1979; Borowitzka et al. 1988; Taylor et al. 2007*a*).

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Symbioses between sponges and cyanobacterial symbionts are reported in disparate oceanic regions throughout the world (Wilkinson 1979; Steindler et al. 2005; Thacker 2005). These symbionts can be vertically transmitted (Sharp et al. 2007; Collin et al. 2010) and likely represent an ancient evolutionary relationship resulting from a combination of multiple host colonization events and cospeciation (Thacker and Starnes 2003; Thacker et al. 2007). Although the common mode of carbon metabolism in sponges is thought to be heterotrophic filter feeding, some sponges can obtain 50% of their energy budget and more than half of their carbon budget from their photosymbionts (Wilkinson 1983; Cheshire and Wilkinson 1991). Nutritional inputs might not be limited to photosynthates, as fixed atmospheric nitrogen or other products of microbial nitrogen metabolism could potentially be transferred to the sponge host (Taylor et al. 2007*b*; Mohamed et al. 2008). Although several studies have inferred a transfer of carbon and nitrogen from microbial symbionts to sponge hosts, direct evidence of such nutrient transfer is lacking.

Recent reports on the interaction between host sponges and microbial symbionts have used either manipulative shading experiments or survey-based approaches to document the benefits of symbiosis (Wilkinson 1983; Weisz et al. 2007; Erwin and Thacker 2008*a*). Shading experiments suggest that the reliance of the sponge host on symbiont nutrition differs across host species. While some host species appear to compensate for reduced symbiont nutrition and are thus unaffected by shading experiments, others seem to be less flexible in their dominant mode of nutrition and thus undergo drastic reductions in growth rates (Thacker 2005; Erwin and Thacker 2008*a*). Compensation mechanisms of sponges under reduced irradiance and the responses of sponge–microbe interactions to reduced irradiance remain unexplored. In a recent survey

of stable isotope values of bulk tissue from sponges in the Florida Keys, Weisz et al. (2007) reported that sponges hosting abundant microbial communities (i.e., high microbial abundance [HMA] sponges) had isotope values suggestive of inputs of symbiont-derived nutrition, while sponges with sparse microbial communities (i.e., low microbial abundance [LMA] sponges) had isotope values suggestive of inputs from heterotrophic filter feeding. While this method supports the use of stable isotopes in the delineation of symbiont- vs. heterotrophically derived nutrients in sponges, the use of bulk sponge tissue greatly limits the conclusions that can be drawn. Since symbiont cell density in some sponges can be up to 40% of the sponge volume and bacterial cells can be present within sponges at high levels (6.4×10^8 cells per gram of sponge tissue [Friedrich et al. 2001] and 10^5 to 10^{10} cells per gram of sponge tissue [Hentschel et al. 2006]), the disparity of stable isotope values between HMA sponges and LMA sponges might simply reflect the presence of significantly higher microbial biomass in HMA sponges. Thus, conclusions regarding the transfer of symbiont nutrition cannot be made from these surveys. The utility of segregating host and symbiont cells prior to stable isotope analyses has long been recognized in the coral–zooxanthellae system (Muscatine et al. 1989) but has not been applied to sponge–microbe symbioses.

While recent studies propose that symbiont nutrition is crucial to some sponge hosts (Erwin and Thacker 2008*a*), basic underlying questions about this potential symbiosis remain unanswered: (1) Are symbiont-derived nutrients being transferred to the sponge? (Webster and Blackall 2009), (2) Is nutrient transfer reduced under shaded conditions? and (3) How might host sponges compensate for such a reduction? To address these questions, we conducted manipulative shading experiments with sponge species spanning a range of possible responses to experimental shading. We included a sponge with a dense microbial community that has been reported to undergo a drastic reduction in growth under "shaded" conditions (*Aplysina fulva* [Erwin and Thacker 2008*a*]), a related sponge that also hosts a dense microbial community but has not previously been used in shading experiments (*A. cauliformis*), a sponge hosting photosynthetic symbionts that does not undergo a reduction in growth under shaded conditions (*Neopetrosia subtriangularis* [Erwin and Thacker 2008*a*]), and, as a negative control, a sponge with a sparse microbial community that has not previously been used in shading experiments (*Niphates erecta*). Following these experiments, we isolated sponge cell and microbial cell fractions from bulk sponge tissue samples and conducted stable isotope analyses (δ^{13}C and δ^{15}N) on each fraction separately. We tested the following hypotheses: under shaded conditions, sponges hosting photosymbionts have reduced symbiont densities and reduced growth rates, while growth in *N. erecta* remains unaffected; and, since consumers are typically enriched in δ^{13}C and δ^{15}N relative to prey due to trophic enrichment, sponges hosting photosymbionts have δ^{13}C and δ^{15}N values of sponge cell fractions that are enriched relative to the microbial fractions, while in *N. erecta*, the sponge cell fraction is enriched relative to particulate organic matter (POM), a potential heterotrophic food source for sponges.

Methods

Sponge collection and experimental sites—The sponge *A. cauliformis* was collected from shallow (3–4 m) reefs near the Caribbean Marine Research Center (CMRC; Lee Stocking Island, Bahamas). The sponges *A. fulva*, *N. subtriangularis*, and *N. erecta* were collected at depths of 2–5 m near the Bocas Research Station of the Smithsonian Tropical Research Institute (STRI; Bocas del Toro, Panama). These sponge species are dominant members of the reef community at their respective locations (Olson et al. 2006; Erwin and Thacker 2007) and all have a rope-like growth form that is ideal for attaching to artificial substrates. A shading experiment using *A. cauliformis* was conducted at Big Point, a 3–4-m-deep site north of CMRC in May 2009. A second shading experiment using *A. fulva*, *N. subtriangularis*, and *N. erecta* was conducted at STRI Point, a 1–2-m-deep site adjacent to the Bocas Research Station in June 2009. Although *N. erecta* is commonly found covered with zoanthid symbionts, the pink color morph of *N. erecta* at Bocas del Toro is rarely ever observed with zoanthids, and no zoanthids were observed in any of the *N. erecta* used in this study.

Shading experiments—A total of 10 individuals of *A. cauliformis*, *A. fulva*, and *N. erecta* and 8 individuals of *N. subtriangularis* were collected and divided into five 10-cm-long pieces. A portion of the first piece was cut into ~ 3-mm-thick sections and preserved in 4% paraformaldehyde (PFA) in sterile filtered seawater for histology. The remainder of this piece was rinsed in filtered seawater, wrapped in aluminum foil, and frozen for future quantification of chlorophyll *a* (Chl *a*) and stable isotopes. The remaining four pieces were lightly blotted and weighed to the nearest 0.01 g to obtain an initial blotted wet weight. Two pieces were deployed under a control canopy and two under a shade canopy, yielding a total of 10 control and 10 shade canopies with two pieces of a replicate from each species under each canopy. Each canopy at CMRC had two sponge pieces attached, and each canopy at STRI had six sponge pieces attached.

Canopy design was adapted from Erwin and Thacker (2008*a*), enlarged to accommodate up to six pieces of sponge. Canopies consisted of a 26.5×26.5-cm plastic grid base attached by cable ties to a 30×30-cm acrylic canopy with four 10-cm polyvinyl chloride tubes located in the corners. Control canopies were transparent to visible light, while shade canopies were opaque and blocked up to 93% of ambient irradiance as monitored by data loggers deployed under and above the canopies during the course of the experiments.

Sponges were attached to canopies using cable ties, and canopies were deployed in the field for 4 weeks. Canopies were monitored and cleaned of fouling organisms twice each week. Since POM is a potential source of food for sponges feeding heterotrophically, water was collected from around the canopies weekly, prefiltered through a 105-μm

mesh to remove sediment and zooplankton, and then filtered through a precombusted (450°C for 4 h) glass fiber filter (GFF-Whatman) and frozen for isotope analysis.

At the end of the experiment, a final blotted wet weight was measured for all sponge pieces. A portion of one piece of each species from each canopy was sectioned and preserved in 4% PFA for histology. The remainder of this piece and all other sponge pieces were rinsed in filtered seawater, wrapped in aluminum foil, and frozen. Growth rates were calculated as relative change per day [(final mass – initial mass) (initial mass)$^{-1}$ (29 d)$^{-1}$]. Sponges were not included in these analyses if they displayed predation scars or tissue necrosis. If both pieces from an initial source individual under a canopy were removed from the analysis, then the corresponding pieces under the other treatment canopy were also removed.

Chl a *and cyanobacterial symbiont abundance*—We used two metrics to quantify symbiont abundance: traditional Chl *a* measurements and histology-based cell counts of symbiotic cyanobacteria. Chl *a* analyses were conducted as in Erwin and Thacker (2008*a*). For each sponge sample, ~ 0.25 g of frozen tissue was removed, lyophilized overnight, weighed to the nearest 0.001 g, and added to a 20-mL scintillation vial covered with aluminum foil to prevent photodegradation of chlorophyll. Samples were covered in 5 mL of 90% acetone and extracted for 18 h at 4°C. After extraction, 1.5 mL of extract was added to microcentrifuge tubes and spun at low speed to pellet suspended particles. The supernatant was transferred to a quartz spectrophotometric cuvette, and absorbances were measured at 750, 664, 647, and 630 nm. Concentrations of Chl *a* were determined from these absorbances and formulas from Parsons et al. (1984) and were standardized to sponge dry mass. Chl *a* values are presented as absolute values (for initial, control, and shade).

Histological methods allowed us to quantify the abundance of cyanobacteria within a sponge section. Initial and final (shade and control) samples of each replicate of *Aplysina* spp. and *N. subtriangularis* were transferred from PFA to histology cassettes and placed in 95% ethanol overnight. *N. erecta* was not analyzed for cyanobacterial abundance because preliminary counts revealed that cyanobacteria were not present within the sponge tissue. Samples were infiltrated with paraffin by incubating cassettes in beakers that contained an increasing ratio of paraffin : Histo-Clear (National Diagnostics) from 0 : 100 to 100 : 0 for 1 h each at 60°C. Infiltrated samples were embedded into paraffin blocks, allowed to dry, and then sectioned at a thickness of 20 μm and mounted on slides. Dried sections were cleared by submersion in 100% Histo-Clear for 1 h, and coverslips were added using Permount (Fisher Scientific). Cyanobacterial epifluorescence was easily distinguished using an epifluorescence microscope at 1000× magnification under oil. Using a digital camera attached to the microscope, we photographed 10 fields of view per sample. We counted the number of cyanobacterial cells present in each image using the "analyze particles" feature of Image-J (http://rsb.info.nih.gov/ij/). The total number of cyanobacterial cells in each field of view was averaged to obtain a mean number of cyanobacterial cells counted per viewing area (2886 μm^2) for each sample. Since we measured Chl *a* concentrations (as μg Chl *a* g^{-1} dried sponge) and counted a mean number of cyanobacterial cells for each sponge sample, we also standardized our Chl *a* measurements by calculating the Chl *a* concentration per mean number of cyanobacterial cells counted per viewing area (hereafter referred to as Chl *a* per cyanobacterial cells counted).

Sponge and microbial cell separations—Methods for cell separation were adapted from Wehrl et al. (2007). Frozen sponge specimens (6 to 10 cm long) were thawed at 4°C, weighed to the nearest 0.01 g, and minced into small pieces using a sterile razor blade. Sponge pieces were added to a 50-mL Falcon tube, covered with ice-cold calcium- and magnesium-free artificial seawater (CMFASW) with ethylenediaminetetraacetic acid (EDTA; CMFASW-E), inverted 3×, and incubated at 4°C for 30 min. CMFASW was prepared by adding 54 g of NaCl, 2 g of Na_2SO_4, 1.6 g of KCl, and 0.36 g of $NaHCO_3$ to 2 liters of filtered water. To prepare CMFASW-E, 900 mL of the above solution was added to 100 mL of 0.5 mol L^{-1} EDTA (8.0 pH). For *Aplysina* spp., the initial CMFASW-E was decanted to remove worms that dissociated from these sponges. Pieces of *Aplysina* spp. were covered a second time with CMFASW-E and incubated as above. Samples were gently homogenized with a mortar and pestle for 75 s to dissociate the sponge cells. This solution was filtered through a Whatman No. 4 filter (20–25-μm pore size) to remove undissociated cells and large spicules and fibers. The resulting filtrate was poured into 50-mL centrifuge tubes and centrifuged at 430 × *g* for 2 min at 4°C. The resulting supernatant (containing microbial cells) was carefully decanted and stored at 4°C, leaving the sponge pellet and approximately 5–10 mL of supernatant. This remaining supernatant was discarded to prevent contamination of the sponge pellet. The pellet was then suspended in 30 mL of CMFASW and centrifuged at 770 × *g* for 2 min at 4°C. This wash was repeated twice, discarding the supernatant, after which the pellet was resuspended in 1.0 mL of CMFASW and transferred to a 1.5-mL microcentrifuge tube. Microcentrifuge tubes were spun at 3800 × *g* for 2 min, after which the supernatant was removed, yielding a fraction consisting of sponge cells. The isolated sponge pellet was rinsed twice with CMFASW, discarding the CMFASW wash each time, and then frozen at −20°C for future stable isotope analyses. The supernatant containing microbial cells was centrifuged at 17,000 × *g* for 15 min at 4°C to pellet the microbial fraction. The supernatant was discarded, and the resulting microbial pellet was rinsed with CMFASW, resuspended, and transferred to 1.5-mL microcentrifuge tubes. These samples were centrifuged at 12,800 × *g* for 2 min, the supernatant was discarded, and the resulting microbial pellet was rinsed twice and frozen. The purity of sponge and microbial fractions was assessed using epifluorescence and light microscopy. The sponge fraction always contained large (8–10-μm diameter) cells and low epifluorescence, while the microbial fraction contained only small (< 1–2-μm diameter) cells and high epifluorescence, attributable to pigments of symbiotic cyanobacteria.

Stable isotope analyses and mixing models—Lyophilized GF filters and pellets of sponge and microbial cells were transferred to glass vials and placed in a 60°C vacuum oven for 24 h. Samples were then acidified to remove any residual carbonate by exposure to 12 mol L^{-1} HCl vapors for 24 h in a sealed desiccator. Residual acid was removed following acidification by placing samples into a 60°C vacuum oven for 24 h. Dried samples were weighed into tared tin capsules (Costech) to the nearest 0.001 mg. Samples were analyzed on a Europa ANCA-SL elemental analyzer coupled to a Europa 20-20 isotope ratio mass spectrometer at the Stable Isotope Laboratory at the Marine Biological Laboratory (MBL), Woods Hole, Massachusetts. We report mean values (± 1 standard error) for the $\delta^{13}C$ and $\delta^{15}N$ of sponge and microbial fractions. Analytical precision at the MBL is ± 0.1‰, and the mean precision of sample replicates for $\delta^{13}C$ and $\delta^{15}N$ was ± 0.1‰ and ± 0.3‰, respectively. In stable isotope analyses, the abundance of the heavier stable isotope for the elements carbon (^{13}C) and nitrogen (^{15}N) in samples is compared to that of the standards Vienna Pee Dee Belemnite and atmospheric N_2 and is expressed using the delta (δ) or the permil notation (‰) in the following equation:

$$\delta X = \left(R_{sample}/[R_{standard} - 1]\right) \times 1000 \quad (1)$$

in which X is the heavier isotope (^{13}C or ^{15}N) and R = $^{13}C:{}^{12}C$ or $^{15}N:{}^{14}N$ (Lajtha and Michener 1994).

The process of trophic enrichment allows us to infer whether symbiont-derived C and N are translocated to sponge cells by comparing the $\delta^{13}C$ and $\delta^{15}N$ values measured in the microbial and sponge cell fractions. In trophic investigations, carbon isotopes permit the identification of the primary producers that act as a carbon source for higher trophic levels because consumers typically have $\delta^{13}C$ values that either directly reflect their ultimate carbon source or are enriched by 0.5 to 1.0‰ relative to their diet (Michener and Schell 1994). In food webs, $\delta^{15}N$ can often be used to establish trophic structure because the $\delta^{15}N$ of a consumer reflects the $\delta^{15}N$ of its diet, with an enrichment of between 2‰ and 5‰ (average of 3.4‰; Michener and Schell 1994; Freeman 2001). In some host–symbiont systems, however, the enrichment of $\delta^{15}N$ might vary due to nutrient recycling (Tanaka et al. 2006). Little is known about how sponges fractionate C and N obtained from POM or from the translocation of symbiont-derived nutrition; thus, the fractionation values listed above are based on published values from studies on other organisms (Freeman 2001). To determine the percentage of symbiont-derived C assimilated by a host sponge, we used a two–end-member mixing model (Fry 2006) with a fractionation correction of 1‰ for the $\delta^{13}C$ of the sponge fraction to account for trophic enrichment:

$$\%\ \text{symbiont C} = \left[1 - \left(\delta^{13}C_{sponge} - \delta^{13}C_{symbiont}\right) / \left(\delta^{13}C_{POM} - \delta^{13}C_{symbiont}\right)\right] \times 100 \quad (2)$$

This model estimates the percentage of C assimilated from each of the two end members (POM and the microbial cells isolated from bulk sponge tissue). Since both *Aplysina* spp. and *N. subtriangularis* host abundant microbial symbiont communities, we suggest that values for $\delta^{13}C_{symbiont}$ are predominantly from symbiotic photosymbionts and other microbial symbionts resident within these hosts but might include transient microbial cells present in the sponge's canals at the time of collection. However, since *N. erecta* hosts only a sparse community of nonphotosynthetic microbial symbionts, $\delta^{13}C_{symbiont}$ values for this sponge are likely to be dominated by transient microbial cells. Stable isotope values of *N. erecta* should thus reflect heterotrophic feeding on nonsymbiotic microbes regardless of experimental treatment.

Data analysis—Since each canopy contained two portions of an individual of each species, we compared differences in the mean values of relative growth rates, Chl *a* concentrations, cyanobacterial cells counted, Chl *a* concentration per cyanobacterial cells counted, and the percentage of symbiont-derived C between control and shade treatments by using a general linear model (GLM; Systat, version 11, Systat Inc.), with the individual serving as a blocking variable. In addition, we compared differences in the mean initial values of the percentage of symbiont-derived C among sponge species using a one-way analysis of variance (ANOVA), followed by multiple pairwise comparisons with a Bonferroni correction. Data not meeting the assumptions of normality and equal variances were rank-transformed prior to these analyses. A significance level of $\alpha = 0.05$ was used for all analyses.

Results

Photosymbiont abundance, as estimated by Chl *a* concentration, was significantly lower under shaded conditions in three species of sponges hosting abundant photosymbiont communities (GLM on rank-transformed data: $F_{1,24} = 17.85$, $p < 0.001$; $F_{1,26} = 76.90$, $p < 0.001$; $F_{1,23} = 15.05$, $p = 0.001$ for *Aplysina cauliformis*, *Aplysina fulva*, and *Neopetrosia subtriangularis*, respectively; Fig. 1). The sponge *Niphates erecta* does not host a large community of microbial symbionts, and there was no significant difference in Chl *a* concentrations between the shade and control treatments for this sponge (GLM on rank-transformed data: $F_{1,29} = 1.99$, $p = 0.168$; Fig. 1). For the three species hosting photosymbionts, cyanobacterial cell counts declined (GLM: $F_{1,7} = 6.70$, $p = 0.036$ for *A. cauliformis*; Fig. 2A) or remained unchanged (GLM: $F_{1,6} = 0.36$, $p = 0.571$ for *N. subtriangularis*; $F_{1,7} = 1.12$, $p = 0.328$ for *A. fulva*; Fig. 2A); and the concentration of Chl *a* per cells counted was lower under shaded conditions for all three species (GLM: $F_{1,8} = 6.96$, $p = 0.030$; $F_{1,8} = 9.76$, $p = 0.014$; $F_{1,6} = 8.23$, $p = 0.028$ for *A. cauliformis*, *A. fulva*, and *N. subtriangularis*, respectively; Fig. 2B). Growth rates of the sponges *A. fulva* and *N. subtriangularis* were reduced under shaded conditions (GLM: $F_{1,17} = 11.50$, $p = 0.003$ and $F_{1,18} = 19.49$, $p < 0.001$, respectively), but there was no significant difference between treatments for *A. cauliformis* (GLM: $F_{1,12} = 2.64$, $p = 0.130$; Fig. 3). Growth of *N. erecta* was higher under shaded conditions (GLM: $F_{1,24} = 7.21$, $p = 0.013$; Fig. 3).

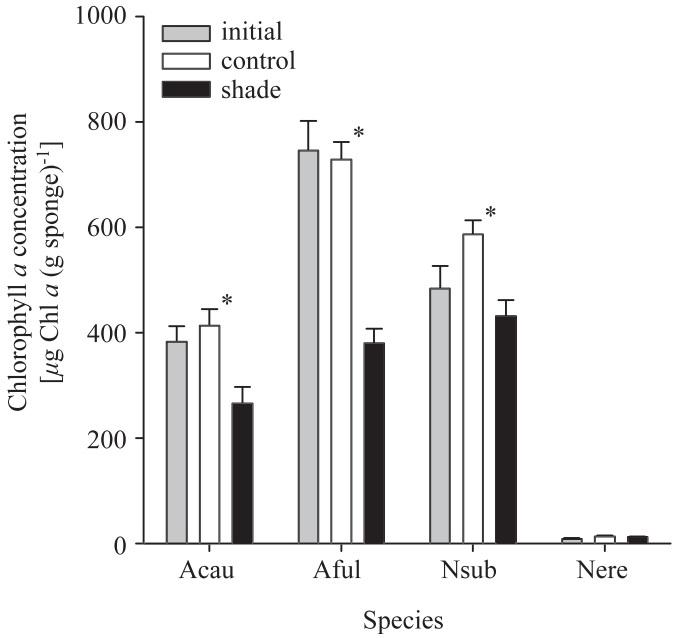

Fig. 1. Chl *a* concentrations of initial and experimental samples from the sponges *Aplysina cauliformis* (Acau), *A. fulva* (Aful), *Neopetrosia subtriangularis* (Nsub), and *Niphates erecta* (Nere). Initial values are included for reference. $n = 10$ for Acau, Aful, and Nere and $n = 8$ for Nsub. Bars represent means ± 1 SE; asterisks indicate statistically significant differences between control and shade treatments ($\alpha = 0.05$).

Stable isotope values (δ^{13}C and δ^{15}N) for POM differed across the two experimental sites and were more variable in the samples collected from the Bahamas (compare POM values of Fig. 4A to Fig. 4B–D). Both δ^{13}C and δ^{15}N in the sponge fraction of *A. cauliformis* were enriched relative to those of the microbial fraction isolated from this sponge, suggesting that this sponge obtains both C and N from its microbial symbionts (Fig. 4A). In *A. fulva*, the sponge cell fraction was enriched in δ^{15}N relative to both the microbial fraction and POM, suggesting that this sponge may obtain N from its symbionts and also from POM (Fig. 4B). The sponge fraction of *N. subtriangularis* was enriched in δ^{13}C compared to the microbial cell fraction, suggesting that this species obtains C from its symbionts (Fig. 4C). Finally, δ^{13}C and δ^{15}N values of the sponge cell and microbial cell fractions from *N. erecta* were almost identical (Fig. 4D). Because the sponge fraction was enriched in δ^{13}C and δ^{15}N relative to POM, *N. erecta* likely obtains the majority of its C and N from POM.

When sponges were shaded, the δ^{13}C values of the microbial cell fraction shifted towards more enriched values in *A. fulva*, *A. cauliformis*, and, to a lesser extent, in *N. subtriangularis* (Fig. 4A–C). In *A. cauliformis* and *N. subtriangularis*, this change was accompanied by a shift in the δ^{13}C values of the sponge cell fraction, while in *A. fulva* the sponge cell fraction did not undergo a shift toward more enriched values (Fig. 4A–C). In *N. erecta*, the trend of almost identical values for δ^{13}C and δ^{15}N between the sponge and microbial fractions continued in both shade and control samples, but the δ^{13}C values of both fractions

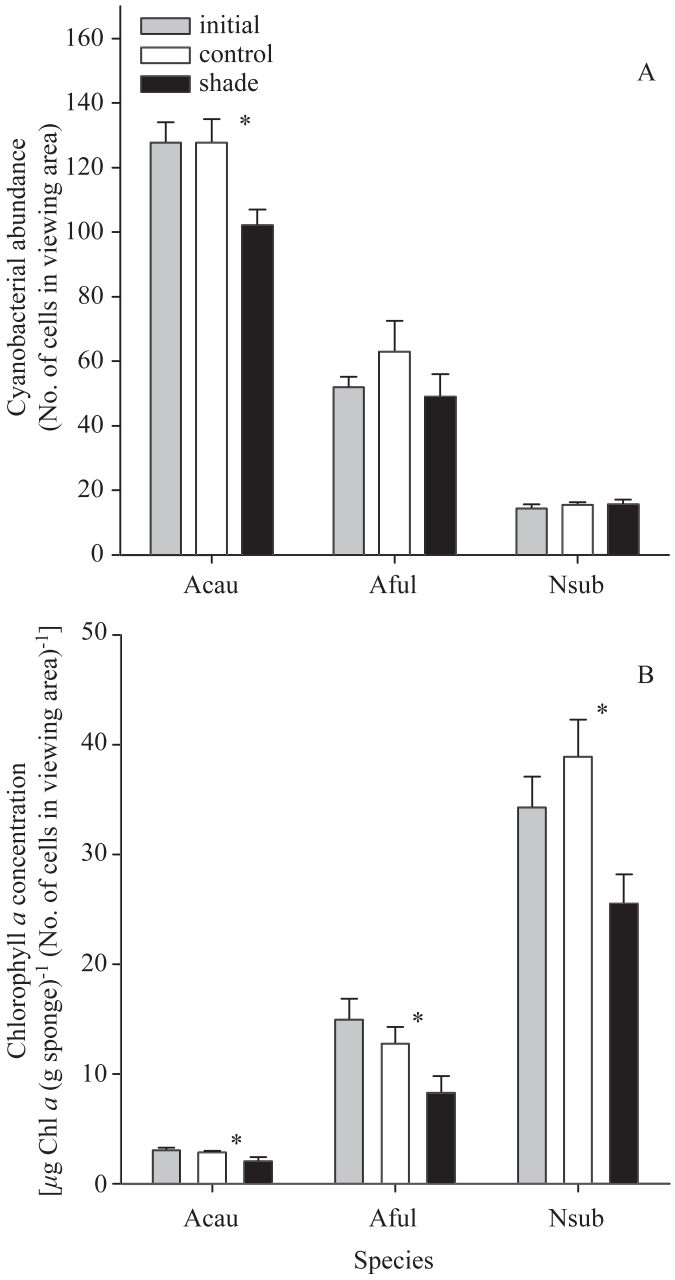

Fig. 2. (A) Abundance of cyanobacterial cells in the species Acau, Aful, and Nsub. $n = 10$ for Acau, $n = 9$ for Aful, and $n = 8$ for Nsub. (B) Chl *a* concentrations per cyanobacterial cell counted in initial and experimental samples of the sponges Acau, Aful, and Nsub. Initial values are included for reference. $n = 10$ for Acau and Aful and $n = 8$ for Nsub. Bars represent means ± 1 SE; asterisks indicate statistically significant differences between control and shade treatments ($\alpha = 0.05$).

from these experimental samples were more enriched than those found in initial samples (Fig. 4D).

Results from the stable isotope mixing model suggest that the percentage of host C assimilated from microbial cells in the initial samples differed among the four host species (ANOVA: $F_{3,27} = 14.55$, $p < 0.001$; Fig. 5). In initial samples, *A. cauliformis* and *N. subtriangularis* assimilated from 70% to 77% of their C from microbial

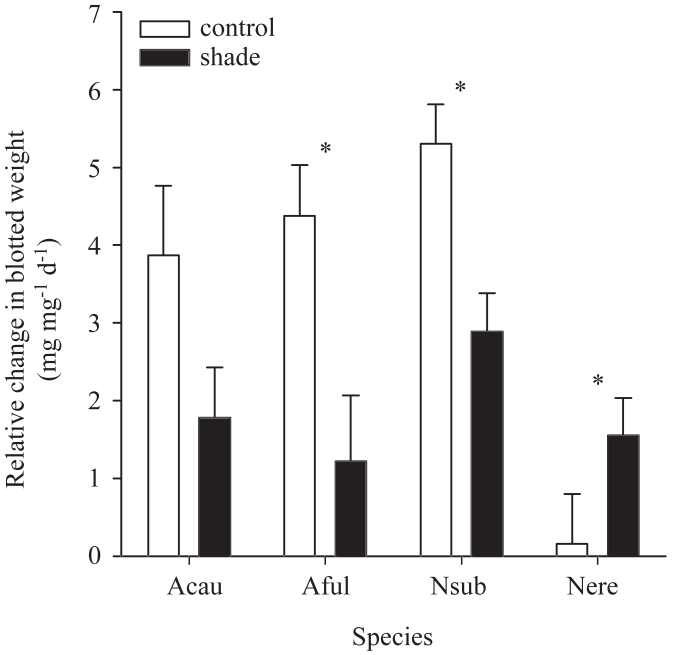

Fig. 3. Relative change in growth rate per day under control and shade treatments for the species Acau, Aful, Nsub, and Nere. $n = 8$ for Aful and Nsub, $n = 6$ for Acau, and $n = 9$ for Nere. Bars represent means ± 1 SE; asterisks indicate statistically significant differences between control and shade treatments ($\alpha = 0.05$).

cells, while *A. fulva* assimilated approximately 47% of its C from microbial cells and *N. erecta* assimilated only 27% of its C from microbial cells (Fig. 5). There was no significant difference in the percentage of host C assimilated from microbial cells between control and shade treatments for any of the four species (GLM: $F_{1,6} = 0.93$, $p = 0.373$; $F_{1,7} = 0.14$, $p = 0.718$; $F_{1,7} = 3.96$, $p = 0.087$; $F_{1,6} = 0.08$, $p = 0.789$ for *A. cauliformis*, *A. fulva*, *N. subtriangularis*, and *N. erecta*, respectively; Fig. 5).

Discussion

Our results demonstrate that the transfer of nutrition from microbial symbionts to a host sponge is highly variable across host species, with some, but not all, sponges obtaining significant amounts of nutrition from their symbiont community. We initially hypothesized that sponges hosting photosymbionts would have decreased symbiont abundance under shaded conditions, yielding reduced growth rates. As predicted, the three species hosting photosymbionts (*Aplysina* spp. and *N. subtriangularis*) displayed lower Chl *a* concentrations under shaded conditions, while the very low Chl *a* concentrations found in the sponge not hosting photosymbionts (*N. erecta*) did not differ across treatments. Although the mean number of counted cyanobacterial cells was significantly lower only in *A. cauliformis*, all three of the sponges hosting photosymbionts displayed lower concentrations of Chl *a* per cyanobacterial cell count under shaded conditions. These findings counter the hypothesis that these cyanobacteria might photoacclimate to low light conditions by increasing Chl *a* concentrations within each cell (Six et al. 2004). Instead, it appears as though either cell number is decreasing due to expulsion of these symbionts or due to consumption by the host sponge (Maldonado and Young 1998); alternatively, the cyanobacterial cells remaining in these sponges might have reduced photosynthetic capacity.

If photosymbiont-derived nutrition is crucial to maintain host performance, then shaded conditions are expected to reduce host growth rates (Thacker 2005; Roberts et al. 2006; Erwin and Thacker 2008*a*). Growth rates of *A. fulva* and *N. subtriangularis* were significantly lower under shaded conditions, but were still positive, while growth rates of *A. cauliformis* were not significantly reduced under shaded conditions. This maintenance of positive growth rates under low-irradiance conditions implies a strong compensatory ability of a host or its symbionts. In all three hosts, such compensation might arise from increased heterotrophic feeding (Grottoli et al. 2006; Erwin and Thacker 2008*a*) or an ability to acquire symbiont-derived nutrition under low-irradiance conditions. Photosymbionts might maintain some photosynthetic ability under shaded conditions or might switch to heterotrophic metabolism. Higher growth rates of *N. erecta* under shaded conditions implies that this sponge obtains its nutrition strictly from filter feeding on POM and that growth of this sponge could be inhibited by high irradiances. The outcomes of our shading experiments suggest that sponge–photosymbiont interactions differ across sponge species but do not answer the fundamental questions of if and how much symbiont nutrition is transferred to the sponge and provide no insight into how some sponges might compensate for reduced symbiont-derived nutrition.

Previous investigations of sponge–symbiont interactions using stable isotopes were based on large-scale surveys of sponges in the Florida Keys. While stable isotope analyses appear to be useful for resolving symbiont- vs. heterotrophically derived nutrition (Weisz et al. 2007), their utility for assessing inputs of symbiont nutrition at a fine scale remained unknown because previous studies analyzed bulk sponge tissue that included both sponge and microbial biomass. In order to determine whether or not symbiont nutrition is transferred to the host, bulk tissue must be segregated into host and symbiont cell fractions, which must then be analyzed separately, as has been demonstrated in corals (Muscatine et al. 1989). We used a combination of tissue dissociation and centrifugation to separate bulk sponge tissue; using published values for trophic enrichment, we inferred whether symbiont-derived C and N are translocated to sponge cells by comparing the $\delta^{13}C$ and $\delta^{15}N$ values from the microbial and sponge cell fractions. While we hypothesized enrichment in $\delta^{13}C$ and $\delta^{15}N$ of the sponge cell fractions relative to the symbiont fractions across all three species hosting photosymbionts, we found that each of these sponges forms a unique relationship with its symbiotic microbial community. Additionally, the disparity in these interactions across species was further supported by construction of a two–end-member stable isotope mixing model to quantify the percentage of symbiont-derived vs. POM-derived carbon assimilated by the sponge host. For instance, isotope values suggest that, while *A. cauliformis* obtains both C and N from its

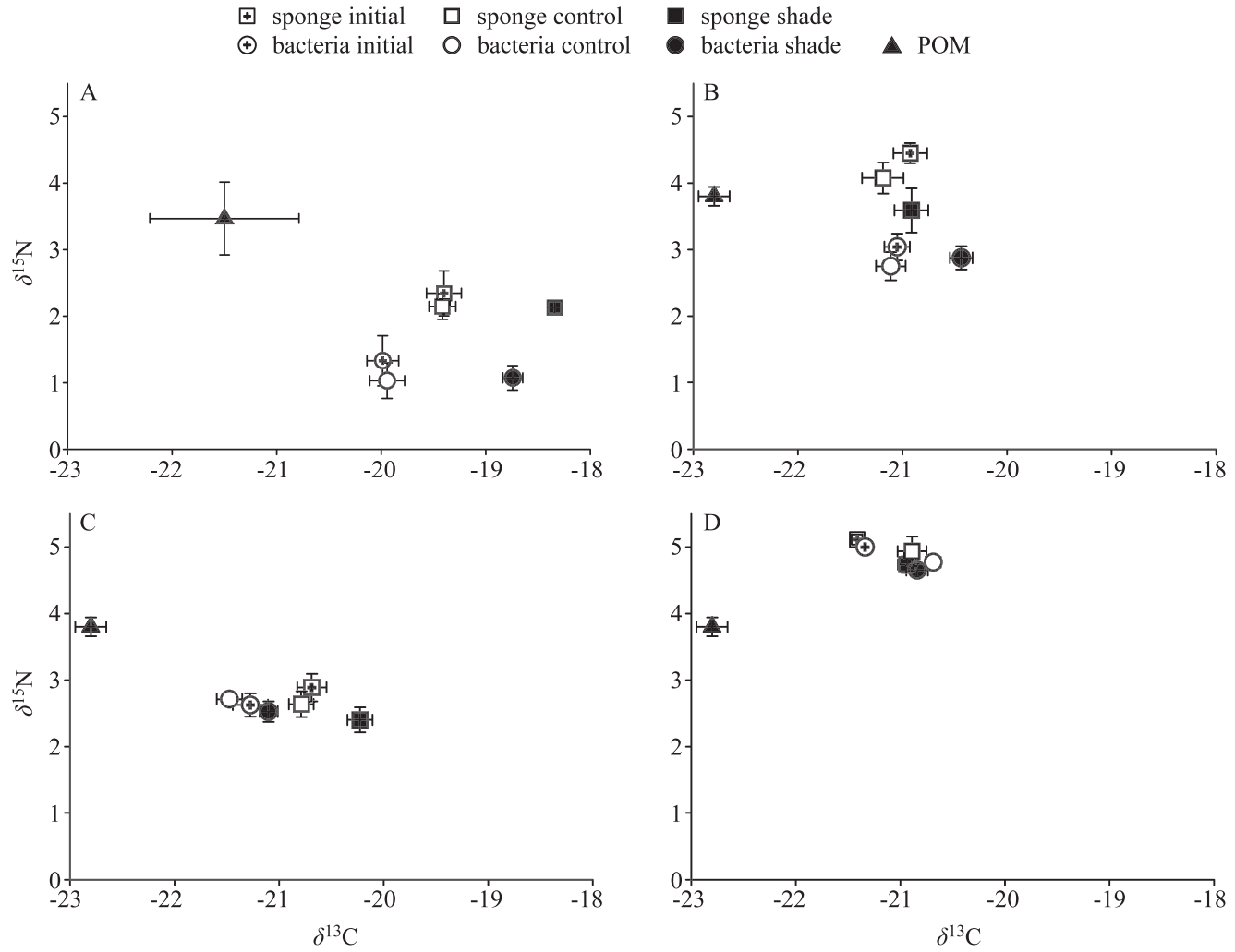

Fig. 4. δ^{13}C and δ^{15}N values for the sponge cell and microbial cell fractions and POM. (A) δ^{13}C and δ^{15}N values for Acau. (B) δ^{13}C and δ^{15}N values for Aful. (C) δ^{13}C and δ^{15}N values for Nsub. (D) δ^{13}C and δ^{15}N values for Nere. $n = 8$ for Aful, Nsub, and Nere and $n = 7$ for Acau. Symbols and error bars represent means ± 1 SE.

microbial symbionts, *N. subtriangularis* obtains only C from its symbionts. High levels of symbiont-derived carbon transfer to the host in these species was supported by the stable isotope mixing model, which suggests that both of these sponges assimilate approximately 75% of their C from symbionts in full light. These values imply that *A. cauliformis* and *N. subtriangularis* obtain more C from their symbionts than from heterotrophy (compare Acau and Nsub to Nere in Fig. 5). In contrast, although the sponge *A. fulva* hosts photosynthetic symbionts, it seems to obtain minimal C nutrition from them, suggesting that even closely related sponge species (e.g., within the genus *Aplysina*) have extremely different interactions with their symbionts. Stable isotope values, however, do suggest that *A. fulva* obtains a portion of its N from its symbionts.

Stable isotope analyses supported our hypothesis that the sponge *N. erecta* is strictly heterotrophic, obtaining its carbon and nitrogen by filtering POM from the water column. The mixing model implies that this sponge obtains about 25% of its C from microbial cells isolated from bulk tissue. Since this sponge does not host large communities of symbiotic microbes and any bacteria within this sponge are most closely related to those from the water column (Weisz et al. 2007), we hypothesize that the isolated microbes are transients that were present in the sponge canals at the time of sampling. *Niphates erecta* has extremely low Chl *a* values, shows no evidence of sponge-specific cyanobacterial symbionts (Erwin and Thacker 2007), grew faster under the shade treatment in this study, and is considered an LMA sponge (Weisz et al. 2007), all indications that a dominant portion of its nutrition should come from POM. Enriched δ^{15}N values for both the sponge and microbial fractions suggest that the microbial fraction most likely consists of heterotrophic bacteria that assimilate nutrient inputs similar to those of the host. In addition, the δ^{13}C values in *N. erecta* underwent a slight shift between the initial samples and those from the experimental treatments. Since this shift is identical in both shaded and control samples, we believe that this shift is evidence for continued heterotrophic feeding after transplantation from the collection site to the experimental site. This pattern might be explained by more enriched δ^{13}C values in the water-

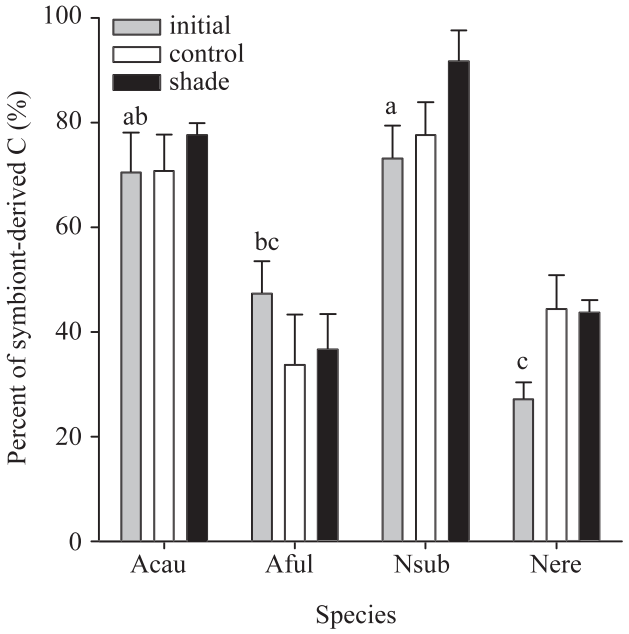

Fig. 5. The percentage of C assimilated by the host from microbial symbionts in comparison to that assimilated from POM for the species Acau, Aful, Nsub, and Nere. Bars represent means ± 1 SE. Initial values are included for reference; different letters above these bars indicate statistically significant pairwise differences between species ($\alpha = 0.05$). $n = 8$ for Aful, Nsub, and Nere and $n = 7$ for Acau. Within all four species, no statistically significant differences were found between control and shade treatments.

column microbial community (including heterotrophic bacteria) at the experimental site compared to the collection site. In contrast, for both *A. cauliformis* and *N. subtriangularis,* δ^{13}C values were similar in initial and control samples but became more enriched in shade samples. If these two sponges were obtaining significant quantities of C via heterotrophic feeding, then one would expect the δ^{13}C values of control and shade samples to be more similar, as observed for *N. erecta*.

Although sponges are known to host a wide variety of microbes capable of N fixation and N transformations (Corredor et al. 1988), evidence of nitrogen inputs to the host is mostly lacking, and the fractionation of N by a sponge or by its microbial community remains largely unknown (Southwell et al. 2008). We attempted to construct a mixing model for δ^{15}N with a variety of fractionation values but were unable to design a model that consistently fit the data across all four species. This lack of fit was due to δ^{15}N values of sponge cell fractions that were not consistently between values for POM and the microbial symbiont fraction, even when accounting for trophic enrichment. For instance, in *A. fulva*, the differences between the sponge δ^{15}N values and those of the sources are negligible and can thus not be resolved; in *N. subtriangularis*, sponge values are lower or equal to source values, so accounting for enrichment would extend these values even further outside the assumptions of this model. Finally, in *N. erecta*, sponge δ^{15}N values were higher than either source, but determining whether this implies 100% microbial N contribution or 100% POM contribution with trophic enrichment is not possible at this time. Our mixing model might have lacked other inputs of N that are currently unknown; furthermore, complex models might be needed to account for diverse nitrogen transformations within the symbiotic microbial community. Even without a mixing model, differences in the δ^{15}N values of sponge and microbial cell fractions in *A. cauliformis* and *A. fulva* suggest that the observed enrichment of the sponge cell fractions is driven by the assimilation of symbiont-derived N (Fig. 4). Studies combining enriched δ^{15}N tracer experiments with cell separations are under way to enhance our understanding of N transfer from microbial symbionts to host sponges.

Finally, the stability of photosymbiont–sponge interactions under reduced light is an important metric for determining host dependence on the products of photosymbiont metabolism. By observing shifts in δ^{13}C values of symbiont and sponge cell fractions under shaded conditions, we were able to investigate this stability at a finer scale than in previous studies and to assess the response of host sponges to reduced photosymbiont abundance. When sponges were shaded, the δ^{13}C values of the microbial cell fraction shifted toward more enriched values in *A. fulva* and *A. cauliformis*, and, to a lesser extent, in *N. subtriangularis*. In *A. cauliformis* and *N. subtriangularis*, this was accompanied by a shift in the δ^{13}C values of the sponge cell fraction; whereas in *A. fulva*, the response of the sponge to shaded conditions was decoupled from symbiont carbon metabolism. Maintenance of high levels of symbiont-derived C under the shade treatment in *A. cauliformis* and *N. subtriangularis* counters the hypothesis that hosts in a facultative mutualism might compensate for shading by increased filter feeding (Erwin and Thacker 2008*a*). The stability of this interaction instead supports an obligate mutualism, which, in sponges, might consist of genetically distinct photosymbiont clades that differ in their capacity to transfer nutrition to host sponges under different environmental conditions (Erwin and Thacker 2008*a*,*b*). While a similar, and perhaps even more intricate, obligate interaction has been reported for many scleractinian corals, in which the genetic diversity of *Symbiodinium* extends below the clade level (Knowlton and Rohwer 2003; LaJeunesse 2005), such an interaction remains to be fully investigated in sponge–photosymbiont interactions. In contrast, although *A. fulva* hosts an abundant cyanobacterial community with multiple genetically distinct clades (Erwin and Thacker 2008*b*), it maintains its ability to feed heterotrophically. While the possibility of heterotrophic plasticity in *A. fulva*, as described in the coral *Montipora capitata* by Grottoli et al. (2006), is an intriguing explanation of these results, further studies are required before the plasticity of this interaction can be confirmed. Our present data suggest that *A. fulva* forms a commensal interaction with its photosymbionts, in which microbes inhabit the sponge without providing C to the host.

Our finding that symbiont-derived nutrient inputs vary across host species raises interesting questions about the complexity of the symbiosis between marine sponges and their microbial communities. For example, variability in

nutrient inputs across sponge species might be due to genetic diversity of sponge-specific cyanobacterium *Synechococcus spongiarum* across sponge hosts. Since each of the three species of sponges used in this investigation hosts at least one unique clade of these symbionts (Erwin and Thacker 2008*b*), this is an intriguing possibility. In addition, although the sponge *N. subtriangularis* did not undergo a drastic reduction in growth rate in Erwin and Thacker (2008*a*), its growth was significantly reduced under shade in the current study. It is not clear whether a change in the photosymbiont community between these two experiments has influenced the change in this interaction, but it once again raises the question of whether clades of sponge-specific cyanobacterial symbionts differ in their ability to supply the host with nutrition under differing environmental conditions. Additional investigations to assess potential physiological variation among clades should include whole-genome sequencing of photosymbionts to elucidate the genetic basis of variability in these interactions. Future experimental manipulations using compounds enriched in $\delta^{13}C$ and $\delta^{15}N$, followed by cell separation and compound-specific isotope analyses, will shed considerable light on both symbiont and host metabolic pathways and on the nature of sponge–symbiont interactions.

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