

Tracing ^{13}C -enriched dissolved and particulate organic carbon in the bacteria-containing coral reef sponge *Halisarca caerulea*: Evidence for DOM feeding

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Abstract

Here we report on the trophodynamics of the bacteria-containing coral reef sponge *Halisarca caerulea*. The assimilation and respiration of the ^{13}C -enriched substrates glucose, algal-derived dissolved and particulate organic matter (diatom-DOM and -POM), and bacteria were followed in 1- and 6-h incubations. Except for glucose, all substrates were readily processed by the sponge, with assimilation being the major fate. ^{13}C -Enrichment patterns in fatty acid biomarkers revealed that sponge dissolved organic ^{13}C assimilation was both direct and bacteria mediated as tracer carbon was recovered both in bacteria-specific and nonbacterial fatty acid. This is the first direct evidence of DOM incorporation by sponges. The present study demonstrates that the encrusting sponge *H. caerulea* feeds on both DOM and POM and given their dominant coverage of the largest coral reef habitat (coral cavities) it is proposed that organic matter assimilation by cryptic reef sponges may represent an important, largely overlooked ecological function. Quantitatively significant DOM processing may not be the exclusive function of the microbial world on coral reefs; sponges transform DOM to biomass, and thus retain and store organic matter in the reef system.

Sponges are common inhabitants on coral reefs and are widely acknowledged as efficient suspension feeders, preferably filtering small particles ($<10\ \mu\text{m}$) like bacteria and phytoplankton from the passing water (Pile et al. 1996; Ribes et al. 1999). On the open reef sponges co-occur with benthic organisms like corals and algae, whereas in the

cryptic reef framework, which forms the largest habitat on well-developed coral reefs (Ginsburg 1983), sponges dominate the cover (Vasseur 1974; Wunsch et al. 2000; Van Duyl et al. 2006). Coral cavities are sinks of bacterio- and phytoplankton (Richter et al. 2001; Scheffers et al. 2004). Recently it has been demonstrated that the cryptic reef framework is a major sink of dissolved organic carbon (DOC), with DOC removal rates exceeding bacterio- and phytoplankton removal rates by two orders of magnitude (De Goeij and Van Duyl 2007). Heterotrophic bacterio- plankton are the dominant consumers of reactive DOC in the ocean (Harvey 2006). However, although the magnitude of eukaryote uptake of DOC is likely to be small relative to prokaryotic uptake, there is growing evidence that some eukaryotes may be directly fueled by DOC, such as flagellates (Tranvik et al. 1993), and invertebrates like bivalves (Roditi et al. 2000). Moreover, evidence is accumulating that tropical sponges are important removers of bulk DOC from the passing water (Yahel et al. 2003; De Goeij et al. 2008). Substantial bulk DOC removal was found by three common encrusting coral cavity sponges, including the sponge *Halisarca caerulea*, with DOC removal accounting for more than 90% of the total organic carbon removal (De Goeij et al. 2008). The uptake and

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processing, or carbon flow pathways, of DOC compared with particulate sources of nutrition in sponges need to be unraveled.

Sponge-associated bacteria have been assumed to be capable of utilizing dissolved organic matter (DOM) from ambient water (Frost 1987; Ribes et al. 1999). Proline uptake by sponge-associated bacteria in *Chondrosia reniformis* corroborates these assumptions, but direct uptake of amino acids by sponge cells may also occur, considering the presence of required uptake systems (Wilkinson and Garrone 1980). On the actual utilization, partitioning, and possible translocation of natural diets, like DOM, or particulate food sources between sponge cells and sponge-associated bacteria, virtually no data are available.

Fatty acid biomarkers have been repeatedly used as source-specific indicators of DOM and POM both in environmental and food web studies (e.g., Canuel et al. 1995; Hall et al. 2006). In this study, fatty acid source designation was achieved using the different data now available on distinctive fatty acids for bacteria, algae, and sponges (Carballeira et al. 1987; Volkman et al. 1998; Boschker and Middelburg 2002).

We examined the assimilation and respiration of stable carbon isotope-enriched substrates by the sponge *H. caerulea* (Porifera: Demospongiae). *H. caerulea* is an encrusting coral cavity sponge with sponge-associated bacteria and is common on the fringing reefs along Curaçao, Netherlands Antilles (Vacelet and Donadey 1987; De Goeij et al. 2008). In incubation experiments *in situ* with natural seawater only ~40% of the removed DOC by *H. caerulea* was found to be respired, where ~60% of the carbon is assimilated (De Goeij et al. 2008). In this study, tracer assimilation and respiration by the sponge is tracked as excess ^{13}C in tissue (bulk and compound specific) and dissolved inorganic carbon (ΣCO_2) (Middelburg et al. 2000; Moodley et al. 2000). In addition to commercially available ^{13}C -enriched glucose, we utilized organic matter extracted from diatoms enriched in ^{13}C , representing biogenic DOM and POM, and ^{13}C -enriched bacteria.

We used fatty acid analysis for *H. caerulea* to establish assimilation and allocation of DOM and POM in sponge cells and sponge-associated bacteria. We followed ^{13}C -enrichment in fatty acid biomarkers to elucidate sponge-associated bacteria mediated versus direct sponge uptake of DOM, glucose, POM, and bacterioplankton for *H. caerulea*. We investigated the possible translocation of matter from the sponge-associated bacteria and the sponge cells by taking samples at different time intervals (1 h and 6 h respectively).

Methods

Location and sponge collection—This study was conducted on the Caribbean island of Curaçao, Netherlands Antilles (12°12'N, 68°56'W). Incubation experiments were performed at the aquarium building of the Caribbean Marine Biology & Ecology (CARMABI) Foundation. Sponges were collected by scuba diving at Sta. buoy 1 (De Goeij and Van Duyl 2007). We used the common, thin

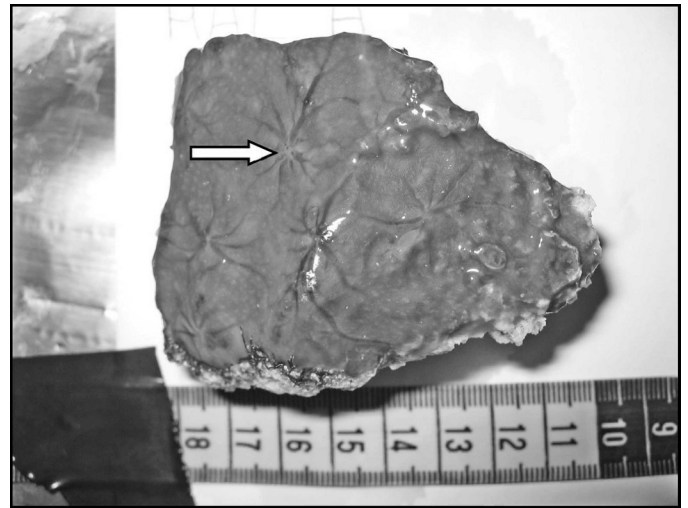


Fig. 1. *Halisarca caerulea* (Porifera: Demospongiae). Encrusting, coral reef cavity sponge (~2.5 mm thick; color purple), showing six oscula (1 osculum indicated with arrow).

(0.8–2.5 mm) cavity-dwelling encrusting sponge *H. caerulea* for our experiments (Fig. 1). Pieces of sponge were chiseled from overhangs and coral cavity walls between 15 and 25 m deep. Attached pieces of coral rock were cleared from epibionts and sponges were transferred to aquaria of approximately 100 liters. Before the incubation experiments, sponges were acclimatized for at least 1 week. They were regularly visually checked and when necessary, the substratum was cleaned and debris removed. The aquaria were kept at around 26°C in the dark and in reef water, pumped from 10 m of depth from the reef slope near the institute, which was refreshed at 3 L min⁻¹.

^{13}C -labeled substrates—We administered four ^{13}C -labeled substrates to *H. caerulea*: glucose, DOM extracted from the axenic diatom *Skeletonema costatum* (diatom-DOM), particulate residue of *S. costatum* after extraction of DOM (diatom-POM), and pre-labeled bacteria. The substrates were prepared to aim at using an addition of 100 $\mu\text{mol L}^{-1}$ organic carbon in the incubation experiments.

Tracer glucose containing 20% ^{13}C was made by mixing unlabeled glucose with isotope-enriched glucose (Cambridge Isotope Laboratories, 98% to 99% ^{13}C). This mix was dissolved in 0.2- μm -filtered seawater and aliquots (3 mL) were stored at -20°C.

The two algal-derived substrates, diatom-DOM and diatom-POM, were extracted from an axenic diatom culture, labeled, and concentrated as described in Moodley et al. (2002). Axenic diatom cells were thoroughly rinsed to remove residue ^{13}C -enriched bicarbonate, concentrated by centrifugation, and then freeze-dried. First, DOM was isolated: a known weight of freeze-dried diatom cells (we estimated the amount required by assuming 20% C for this diatom) was mixed with MilliQ, vortexed, centrifuged, and the supernatant collected. After three extractions, collective supernatant was passed through a 0.2- μm polycarbonate filter (Millipore) to isolate DOM caught in glass tubes that

were subsequently freeze-dried and stored frozen until used in the experiment. Before the experiment 0.2- μm -filtered seawater was added to dissolve and mix the DOM and aliquots (6 mL) were stored at -20°C .

The residue diatom cells were again rinsed and centrifuged and the pellet freeze-dried and used as particulate diatom organic matter substrate. Similarly, a known amount of dried substrate (8 mg of C) was kept in centrifuge tubes and just before the experiments, 0.2- μm -filtered seawater was added and well-mixed aliquots (6 mL) were stored at -20°C . For both algal-derived substrates, a subsample was stored and used for later accurate measurement of carbon content and isotope labeling (atomic % ^{13}C).

Bacteria were pre-labeled with ^{13}C . Natural seawater (1 liter) was collected, prefiltered (0.7 μm ; GF/F), and concentrated (approximately 10^7 bacteria mL^{-1}) by ultra-filtration (0.2 μm ; Vivaflow). The inoculum was added to culture medium M63 (Miller 1972) amended with thiamine (0.0001%) and MgSO_4 (67 $\mu\text{mol L}^{-1}$) and glucose as carbon source (20% ^{13}C , see above). The culture was grown for 48 h in the dark at 25°C and ^{13}C -labeled bacteria were isolated by centrifuge (7 min, 500 rpm), rinsed in 0.2- μm -filtered seawater, centrifuged, and the pellet resuspended in 0.2- μm -filtered seawater (30 mL), divided in six aliquots (5 mL), and stored at 4°C .

The exact amount of C added and the amount of ^{13}C -labeling was determined simultaneously on the gas chromatograph–isotope ratio mass spectrometer (GC-IRMS) (Moodley et al. 2002), except for the pre-labeled bacteria, where the amount of C used in the incubations was estimated from bacterial abundances at $t = 0$ and the subsequent estimate of the amount of bacterial C. Samples for bacterial abundance in seawater (10 mL) were fixed in 4% paraformaldehyde and stained with acridine orange and gently (max. 20 kPa suction pressure) filtered onto 0.2- μm black polycarbonate membrane filters (Millipore, 25 mm), mounted on slides, and stored at -20°C . Bacterial volume was established using epifluorescence microscopy (Zeiss, $\times 1,000$) and digital imaging software (Axiovision, Zeiss), according to Haldal et al. (1985). A bacterial C-to-volume ratio of $100 \text{ fg } \mu\text{m}^{-3}$ was used for cultured bacteria (Fagerbakke et al. 1996). The percentage labeling was equal to the percentage ^{13}C -glucose used as carbon source (20%).

To facilitate interpretation of substrate assimilation on the fatty acid level, substrates were also analyzed for fatty acid composition (see below for methods); fatty acids were extracted from a known amount of freeze-dried substrate.

Incubation experiments—Pieces of sponge of similar size (three replicate incubations per treatment) were placed at the bottom of 2-liter incubation chambers in 0.2- μm -filtered seawater and closed with a lid equipped with a magnetic stirrer. The chambers were closed without trapping air in the system and experiments were performed in the dark at 26°C . To quantify respiration of the added substrate, water samples (5 mL) for ΣCO_2 were taken with a 5-mL polycarbonate syringe first, before adding ^{13}C -labeled substrate, and second, after addition of ^{13}C -labeled

substrate (at $t = 0$ and 60 min and $t = 0$ and 360 min for 1-h and 6-h incubation experiments respectively). The substrate was injected with a sterile syringe. Sampling water was replaced by 0.2- μm -filtered seawater. After the incubation experiment sponges were thoroughly washed in 0.2- μm -filtered seawater and surface area was measured. Sponges were dipped in 0.2- μm -filtered double distilled water and tissue was collected in precombusted (4 h, 450°C) glass vials and subsequently dried at 50°C for 24 h and stored at -20°C until further processing. The incubation chambers were chemically sterilized in hydrochloric acid (overnight, 0.4 mol L^{-1}) before the experiments. Control incubations were incubations without sponge to quantify possible bacterial respiration in the incubation water (0.2- μm -filtered seawater). Controls were done only with the glucose substrate, but, being a substrate generally rapidly respired by bacteria, this correction was considered maximum and also applied to the diatom-DOM substrate.

Sample treatment and analysis—Duplicate water samples (2 mL) for ΣCO_2 were transferred through a precombusted GF/F filter into preweighed 5-mL N_2 -filled headspace vials, immediately acidified (1 μL of 80% H_3PO_4 mL per sample), and stored refrigerated upside down until analysis. Dried sponge material was carefully homogenized and after determining total dry weight, a subsample of known weight was taken for bulk tissue carbon isotope analysis. The organic carbon content (biomass) of each individual sponge was determined directly from the area counts given in the standard output of the IRMS (Moodley et al. 2000, 2002). Details of the measurement of $\delta^{13}\text{C}$ - ΣCO_2 and $\delta^{13}\text{C}$ of tissue are given in Moodley et al. (2000). Headspace ΣCO_2 and sponge tissue (acidified in silver capsules) carbon content and isotopic composition was measured using a Carlo Erba 1500 elemental analyzer coupled online with a Finnigan Delta S IRMS. Carbon content of the different pools was determined directly from area counts extracted from the isotope measurements (Moodley et al. 2000, 2002).

Extraction and preparation of fatty acid methyl esters (FAME) were carried out according to Masood et al. (2005) with reagent volumes adapted for 2.5-mL GC vials using fatty acid 19:0 as internal standard to calculate concentration of fatty acids. Total fatty acids were extracted from 10 mg dry wt sponge material. Concentration and carbon isotopic composition of individual FAME was determined with a GC-combustion interface-IRMS consisting of a HP G1530 GC (Hewlett Packard) connected to Delta-plus IRMS via a type III combustion interface from Thermo Finnigan. Identification of FAMES is based on comparison of retention times with authentic reference materials on a HP 5MS analytical column, confirmed by GC-MS analysis. Stable carbon isotope ratios ($\delta^{13}\text{C}$) of individual fatty acids were calculated from FAME data by correcting for the one carbon atom in the methyl group that was added during derivatization (Boschker and Middelburg 2002).

Carbon isotope ratios ($\delta^{13}\text{C}$) were calculated using conventional methods relative to Vienna Pee Dee Belemnite and tracer carbon incorporation was quantified through

Table 1. Characteristics ^{13}C -enriched substrates. Amount of tracer organic C added per incubation (2 liters), the percentage of ^{13}C labeling, and the percentage of fatty acid C.

Substrate	C added (μmol per incubation)	^{13}C labeling (% of added C)	Fatty acid C (% of added C)
Glucose	196	20	0
Diatom-DOM	156	12	12
Diatom-POM	168	12	49
Bacteria	254	20	5

determining excess (above background) ^{13}C content in the different pools (bulk sponge tissue, sponge fatty acid, and ΣCO_2). The product of fraction excess ^{13}C and carbon content of the pool examined equals tracer ^{13}C incorporation, which divided by fraction labeling of the substrate (0.20 for glucose and bacteria and 0.12 for organic matter extracted from diatoms, Table 1) provides the amount of total tracer carbon $^{12}\text{C} + ^{13}\text{C}$ incorporated (Middelburg et al. 2000; Moodley et al. 2000). For the background isotope signature of ΣCO_2 (as measure of substrate respiration) we did not use ambient $\delta^{13}\text{C}_{\text{CO}_2}$, but that of water samples taken from incubation chambers at $t = 0$. In the case of extracted fatty acids from sponge bulk tissue we used $\delta^{13}\text{C}$ of these components from sponges not exposed to isotope-enriched substrates (Moodley et al. 2002).

Results

Bulk organic carbon respiration and assimilation—The substrates were adequately labeled with ^{13}C as tracer (12–20% ^{13}C compared with $\sim 1\%$ under natural conditions) and ultimately 156–254 μmol C was added to the incubations (each incubation with a volume of 2 liters; Table 1). The biomass of sponges used for the incubations averaged $3,999 \pm 816 \mu\text{mol}$ C (\pm SE; $n = 24$) with a corresponding surface area of $26 \pm 8 \text{ cm}^2$ (\pm SE; $n = 24$). Because of the variation in sponge biomass, the tracer processing is expressed per unit biomass sponge ($\text{mmol C}_{\text{sponge}}$). In addition, to avoid confusion on terminology, by *H. caerulea* or sponge–microbe association we mean the sponge with associated bacteria. The compartmentalization between sponge cells and associated bacteria is specifically referred to in the text. Control incubations without sponge revealed limited respiration (after 6 h 0.4–4.4% of that measured with sponges with glucose or diatom-DOM additions) and measurements were corrected accordingly.

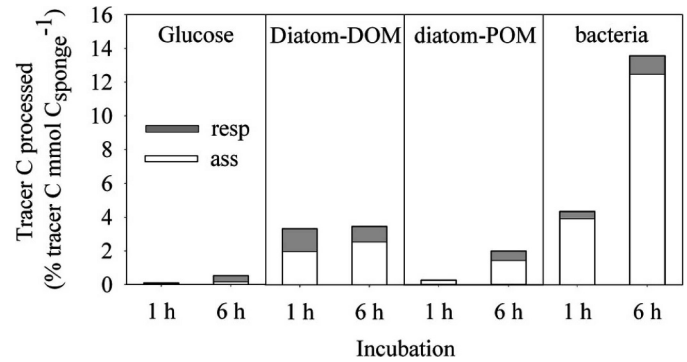


Fig. 2. The amount of tracer C recovered in sponge tissue (% tracer C $\text{mmol C}_{\text{sponge}}^{-1}$) after 1-h and 6-h incubation with the different ^{13}C -labeled substrates. Values are average of three replicates (see Table 1) of respiration (gray bars) and assimilation (open bars) measurements.

Uptake and respiration of the different substrates was rapid but there was clear differential processing of the different substrates by *H. caerulea* (Table 2; Fig. 2). On the basis of the trends after 1-h incubations, fastest and comparable uptake was recorded in diatom-DOM and bacteria additions (Table 2; Fig. 2) in spite of strong difference in the amount of substrate offered (Table 1). For diatom-DOM >90% of the uptake was recorded already within 1 h (Fig. 2). Clearly more time was required to metabolize diatom-POM, and glucose was slowest and least processed. Assimilation by the sponge was on average the major fate of metabolized glucose ($64\% \pm 7\%$), diatom-DOM ($65\% \pm 25\%$), diatom-POM ($97\% \pm 5\%$), and bacteria ($91\% \pm 2\%$) (\pm SD; $n = 3$) after 1 h of incubation. Respiration accounted for a small fraction after 1 h and 6 h (Table 2; Fig. 2).

Fatty acid composition of substrates and *H. caerulea*—No fatty acids were detected in the glucose substrate, as expected. Fatty acids typical of diatoms were encountered in both diatom-DOM and diatom-POM (Fig. 3A,B). Verified axenic conditions (Moodley et al. 2000) validate algal carbon origin. There were slight differences between these two substrates: diatom-POM (Fig. 3B) was dominated by four fatty acids (14:0, 16:1 ω 7c, 16:0, and 20:5 ω 3), together accounting for $\sim 90\%$ of total fatty acids. In diatom-DOM, fatty acids were more evenly distributed (Fig. 3A). Furthermore, fatty acid C accounted for 12% of the total C in diatom-DOM and 48% in diatom-POM (Table 1).

Table 2. *Halisarca caerulea* bulk assimilation and respiration of the four ^{13}C -enriched substrates after 1 and 6 h of incubation (% tracer C $\text{mmol C}_{\text{sponge}}^{-1}$; \pm SD; $n = 3$).

Substrate	1-h Incubation		6-h Incubation	
	Respiration	Assimilation	Respiration	Assimilation
Glucose	0.03 ± 0.01	0.06 ± 0.03	0.35 ± 0.05	0.17 ± 0.03
Diatom-DOM	1.33 ± 1.65	1.99 ± 1.08	0.91 ± 0.29	2.54 ± 0.19
Diatom-POM	0.01 ± 0.02	0.25 ± 0.07	0.55 ± 0.43	1.43 ± 0.83
Bacteria	0.41 ± 0.11	3.93 ± 0.04	1.09 ± 0.51	12.47 ± 3.89

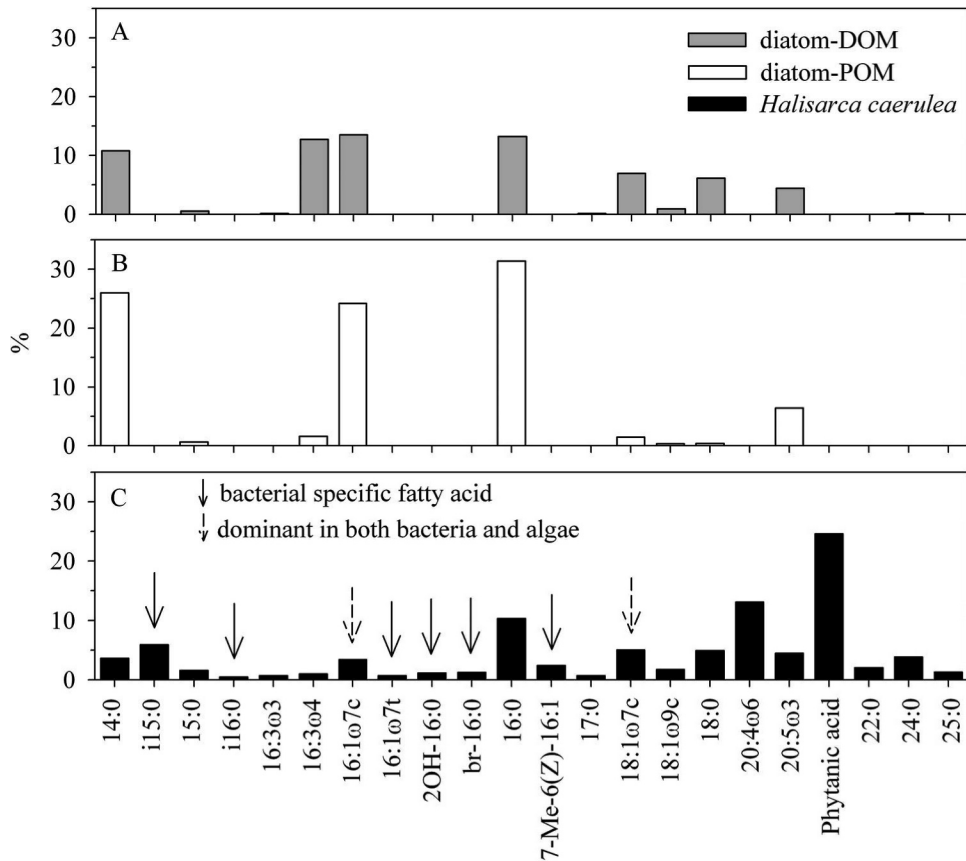


Fig. 3. Fatty acid composition of diatom-derived substrates (A) diatom-DOM, (B) diatom-POM, and (C) the fatty acid composition of the encrusting coral cavity sponge *Halisarca caerulea*. Depicted are fatty acids having an occurrence of $\geq 0.5\%$ of the total fatty acid C of *H. caerulea*; mean ($n = 3$).

For *H. caerulea*, total fatty acid C was $1.61\% \pm 0.08\%$ (average \pm SE; $n = 25$) of the sponge-microbe carbon biomass and its composition is depicted in Fig. 3C. Fatty acids shown in Figs. 3 and 4 are only those with $\geq 0.5\%$ share of total fatty acids for *H. caerulea* (41 fatty acids have been identified, of which 22 have an occurrence of $\geq 0.5\%$). Phytanic acid, common in some sponges (Carballeira et al. 1989) and assigned as nonbacteria fatty acid, was the most dominant, accounting for $24.6\% \pm 0.6\%$ (average \pm SE; $n = 25$) of fatty acid C. Bacteria-specific fatty acids in the sponge (listed in Table 3) accounted for a significant fraction of total fatty acids. Together they accounted on average for $13.8\% \pm 0.3\%$ or $22.2\% \pm 0.5\%$ (\pm SE; $n = 25$) without or with fatty acids 18:1ω7c and 16:1ω7c; although dominant in bacteria, 18:1ω7c can also contribute to algal C (see also Fig. 3A,B). Similarly, fatty acid 16:1ω7c can be a strong component of both algae and bacteria (Volkman 2006 and references therein), also evident in substrates used in this study (Fig. 4). However, these two fatty acids can still be reliably assigned to a source depending on whether they are complemented with a large bacteria-specific or algal-specific contribution (Dalsgaard et al. 2003).

Compound-specific tracing of substrate assimilation by H. caerulea—As in *H. caerulea* bulk tissue analysis,

substrate assimilation was also clearly evident in fatty acids extracted from sponge-microbe associations post-incubation (Fig. 4), and represented different fractions (3–40%) of total tracer C assimilation (Table 3). Similar to trends observed in bulk tissue analysis, minimum tracer recovery in fatty acids was found in incubations with glucose (Table 3). However, given that fatty acids were absent in the glucose substrate itself, this demonstrates that fatty acids were synthesized de novo from glucose after 6 h (Fig. 4A) and confirm active assimilation of substrates. Of the total tracer carbon recovered in fatty acids after incubation with glucose, a large fraction (37%, including 18:1ω7c) was bacteria specific (Table 3). The strong dominance of 18:1ω7c complemented with bacteria-specific 16:1ω7t and i15:0, together with the absence of eukaryotic phytoplankton fatty acids (e.g., 16:3ω4 and 20:5ω3), support uptake of glucose by sponge-associated bacteria. This indicates that glucose assimilation in fatty acids was primarily bacteria mediated, but does not exclude nonbacterial uptake and remains unresolved because of fatty acids being common in both prokaryotes and eukaryotes.

A totally different trend was found for the fatty acid assimilation in *H. caerulea* after diatom-DOM and diatom-POM substrate incubations (Fig. 4B,C). Tracer carbon was recovered both in bacteria-specific fatty acids and in

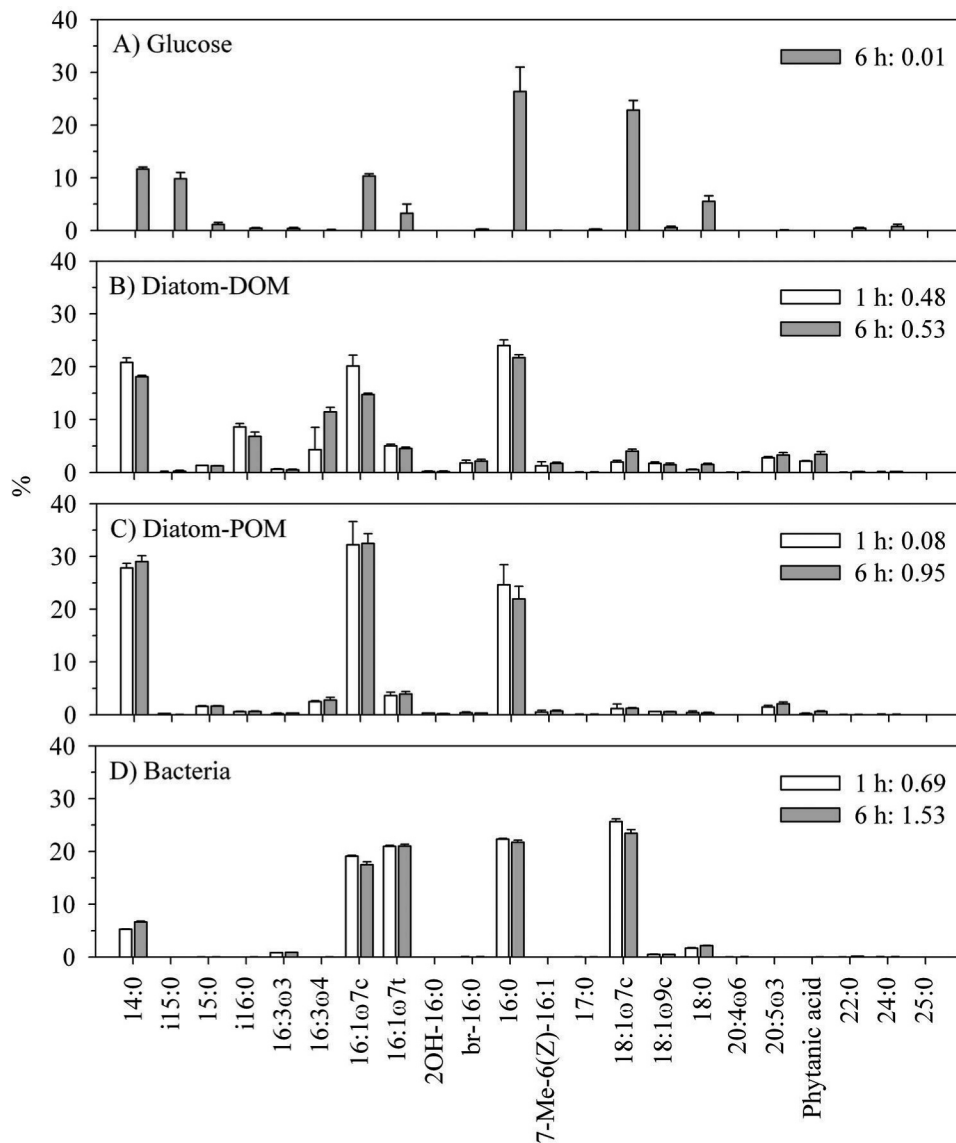


Fig. 4. Distribution of tracer C among the sponge fatty acids (% of total) after 1-h and 6-h incubation (absolute tracer C in fatty acids is presented in box insert in upper right of each panel; see also Table 3). Depicted are fatty acids having an occurrence of $\geq 0.5\%$ of the total fatty acid C of *H. caerulea* (for complete list see Table 4). Open and gray bars represent patterns respectively after 1 h and 6 h of incubation for (A) glucose, (B) diatom-DOM, (C) diatom-POM, and (D) pre-labeled bacteria; mean \pm SD ($n = 3$).

nonbacterial fatty acids. First, the observed patterns of tracer C allocation among fatty acids extracted from incubated *H. caerulea* reflect strongly that of the diatom-derived substrate. For diatom-DOM, the dominant fatty acids 14:0, 16:3 ω 4, 16:1 ω 7c, 16:0, and 20:5 ω 3 together accounted for $\sim 60\%$ both in the substrate and tracer allocation from sponge-extracted fatty acids (compare Figs. 3A and 4B). For diatom-POM, $>80\%$ was accounted for by fatty acids both present in the substrate and tracer found in *H. caerulea*-extracted fatty acids (14:0, 16:1 ω 7c, 16:0, and 20:5 ω 3; compare Fig. 3B,C). This indicates that the major fatty acids were directly ingested by *H. caerulea* in the diatom-DOM as well as in the diatom-POM treatment and not bacteria mediated.

The direct use of substrate-specific fatty acids by the sponge–microbe association also explained the larger total tracer assimilation in fatty acids for diatom-POM after incubation (40% after 6 h, Table 3) as compared with diatom-DOM (13% after 6 h, Table 3), in spite of the overall higher bulk removal of diatom-DOM (Table 1). The POM substrate contained a much higher absolute fatty acid carbon content (49%, Table 1) than the DOM substrate (12%, Table 1).

In both the diatom-DOM and diatom-POM additions, tracer carbon was also recovered in fatty acids that were not present in the substrates (i15:0, i16:0, 16:1 ω 7t, 2OH-16:0, br-16:0, 7-Me-6(Z)-16:1, and phytanic acid; absent in Fig. 3A,B and present in Fig. 4B,C). These fatty acids were

Table 3. Amount (μmol tracer C $\text{mmol C}_{\text{sponge}}^{-1}$; \pm SD; $n = 3$) and distribution of tracer C in fatty acids of *Halisarca caerulea*, as percentage of total assimilation and proportion of tracer in bacteria-specific fatty acids after 1-h and 6-h incubations.

	Glucose		Diatom-DOM		Diatom-POM		Bacteria	
	1 h	6 h	1 h	6 h	1 h	6 h	1 h	6 h
Tracer C in fatty acids	-	0.01 \pm 0.00	0.48 \pm 0.20	0.53 \pm 0.08	0.08 \pm 0.02	0.95 \pm 0.43	0.69 \pm 0.12	1.53 \pm 0.43
% Total assimilation	-	3	16	13	19	40	7	5
% Bacteria specific*	-	37	20	20	7	7	47	45

* i15:0; ai15:0; i16:0; 16:1 ω 7t; 2OH-16:0; br-16:0; i17:0; 7-Me-6(Z)-16:1; 18:1 ω 7c (see also Fig. 3C).

primarily bacteria specific (i15:0, i16:0, 16:1 ω 7t, 2OH-16:0, br-16:0, and 7-Me-6(Z)-16:1) and one nonbacterial (phytanic acid). Phytanic acid was only found after incubations with diatom-derived substrates, primarily after diatom-DOM additions and not after administration of glucose or bacteria, indicating algal carbon origin. Clearly, tracer uptake was both bacteria and direct sponge mediated and consequently fatty acids common to both organisms containing a high fraction of tracer carbon, such as 14:0 and 16:0, reflect both consumers. POM is assumed to be primarily incorporated by sponge cells.

The bacteria substrate was not profiled for fatty acid composition but the assimilation pattern in *H. caerulea* possessed a fatty acid distribution (depicted in Fig. 4D) almost identical (>95%) to estuarine bacteria cultured and labeled identically as in this study (Moodley et al. unpubl.). Thus, the fatty acid pattern reflected directly the trophic resource and indicates limited modification or de novo fatty acid synthesis in the sponge-microbe association. Indeed, fatty acid profiles of bacteria change when exposed to single substrates away from natural conditions and depend upon the carbon source that can produce identical patterns in bacteria from different locations and even lack otherwise bacteria-specific branched fatty acids such as iso and anteiso fatty acids (Harvey 2006; Moodley pers. comm.). Consequently, this fatty acid composition pattern provides bacterial biomarkers for this setting (strong dominance of 16:1 ω 7t and 18:1 ω 7c) and an indication of fatty acid carbon contribution to total bacterial carbon content (~5%), similar to values estimated for natural populations (Middelburg et al. 2000).

For the substrates glucose, diatom-POM, and bacteria, there was a consistent increase of tracer C in *H. caerulea* during the 6-h incubation (Tables 3 and 4). In contrast, diatom-DOM was almost depleted after 1 h, with more than 90% of the tracer recovered. Therefore, shifts in label distribution in different fatty acids between 1 h and 6 h can be ascribed to possible reallocation of tracer C such as in a pulse-chase experiment. However, there was no significant difference in the fatty acid signature and distribution of tracer in *H. caerulea* after 1 h and 6 h of incubation with diatom-DOM (ANOVA; $F_{40} = 1.226$, $p = 0.189$; Fig. 4B).

Discussion

DOM assimilation—Most studies on sponge feeding (Reiswig 1971; Pile et al. 1996; Ribes et al. 1999), including recent studies on DOC removal from ambient water by

sponges (Yahel et al. 2003; De Goeij et al. 2008), report indirect observations of net fluxes of organic matter through the sponge. Actual proof of assimilation of organic matter—and DOM in particular—by sponges was lacking. We traced ^{13}C excess in the bulk tissue of the tropical encrusting sponge *H. caerulea* and in its fatty acids after supply of dissolved and particulate ^{13}C -labeled organic substrates. The observed bulk ^{13}C -enrichment in sponge tissue and the excess $^{13}\text{CO}_2$ release reveals that *H. caerulea* actually processes both DOM and POM. To the best of our knowledge, this is the first direct evidence of bulk DOC processing by a sponge. The ^{13}C -enrichment in the sponge is evidently not an artifact such as accumulation of the food source in or on the sponge, because the assimilation of substrate is also evidently compound specific (in sponge-microbe fatty acids). This is especially convincing for ^{13}C -enrichment in fatty acids that are not present in the substrate—i.e., conversion of nonfatty acid compounds into fatty acids (i.e., all the fatty acids recorded in the case of the 6-h glucose additions, and both the bacteria-specific fatty acids and phytanic acid in the cases of diatom-DOM and diatom-POM treatments (Figs. 3, 4).

We could not assess a clear quantitative preference for particulate over dissolved food except for glucose, which seemed to be an unpreferred substrate. This suggests discrimination by the sponge between algal-derived DOM (containing carbohydrates, amino acids, some lipids, and fatty acids) and glucose. A comparable pattern has been reported for the zebra mussel: limited glucose uptake and rapid assimilation of fatty acids (Baines et al. 2005). Glucose may be an unpalatable food source for invertebrates.

The other substrates seem to be processed by the sponge proportionally to the availability of the source. The concentration of bacterioplankton in the experiments was 50 times higher than under natural conditions, where the DOM concentration was only 2 times higher than in the field (Van Duyl et al. 2002; De Goeij and Van Duyl 2007). Moreover, the utilizable part of diatom-DOM was virtually depleted after 1 h of incubation, when 12% of the added C was processed. Diatom-POM and bacteria were not depleted during the experiment. Because POM sources in the coral reef framework cavities are one to two orders of magnitude lower in concentration than DOM, the absolute DOM assimilation on total nutrition seems to exceed plankton and detritus assimilation by this sponge under in situ conditions as suggested in De Goeij and co-workers (2008).

Table 4. Complete list of ($\times 10^{-3}$ μmol tracer C mmol C_{sponge}^{-1}) tracer carbon distribution in fatty acids of *Halisarca caerulea* after 1-h and 6-h incubations; average \pm SD ($n = 3$).

	Glucose		Diatom-DOM		Diatom-POM		Bacteria	
	1 h	6 h	1 h	6 h	1 h	6 h	1 h	6 h
10:0	-	<1	<1	<1	<1	<1	<1	<1
12:0	-	<1	<1	<1	<1	<1	15 \pm 1	29 \pm 6
i14:0	-	<1	<1	<1	<1	<1	1 \pm 0	2 \pm 1
14:0	-	1 \pm 0	102 \pm 41	95 \pm 5	21 \pm 2	286 \pm 131	37 \pm 4	102 \pm 17
ai15:0	-	<1	<1	<1	<1	<1	<1	<1
i15:0	-	1 \pm 0	<1	1 \pm 1	<1	<1	<1	<1
15:0	-	<1	6 \pm 3	7 \pm 0	1 \pm 0	16 \pm 7	<1	1 \pm 0
i16:0	-	<1	43 \pm 18	36 \pm 5	<1	5 \pm 2	<1	<1
16:3 ω 3	-	<1	3 \pm 1	2 \pm 1	<1	3 \pm 1	6 \pm 1	14 \pm 2
16:3 ω 4	-	<1	25 \pm 25	60 \pm 5	2 \pm 0	26 \pm 14	<1	<1
16:1 ω 7c	-	1 \pm 0	91 \pm 33	77 \pm 4	24 \pm 2	305 \pm 140	132 \pm 13	269 \pm 49
16:1 ω 7t	-	<1	25 \pm 10	24 \pm 1	3 \pm 0	38 \pm 19	145 \pm 14	323 \pm 56
2OH-16:0	-	<1	1 \pm 0	1 \pm 1	<1	1 \pm 0	<1	<1
br-16:0	-	<1	7 \pm 2	11 \pm 2	<1	3 \pm 1	<1	1 \pm 0
16:0	-	3 \pm 0	119 \pm 48	114 \pm 7	19 \pm 4	207 \pm 83	154 \pm 15	332 \pm 51
ai17:0	-	<1	<1	<1	<1	<1	<1	<1
i17:0	-	<1	2 \pm 1	2 \pm 0	<1	1 \pm 0	<1	<1
7-Me-6(E)-16:1	-	<1	1 \pm 0	1 \pm 0	<1	1 \pm 0	<1	<1
7-Me-6(Z)-16:1	-	<1	3 \pm 0	9 \pm 1	<1	7 \pm 3	<1	<1
cy-17:0	-	<1	<1	<1	<1	<1	<1	<1
17:0	-	<1	1 \pm 0	1 \pm 0	<1	1 \pm 0	<1	1 \pm 0
18:3 ω 6	-	<1	1 \pm 0	1 \pm 0	<1	2 \pm 1	<1	<1
18:1 ω 7c	-	2 \pm 0	10 \pm 5	21 \pm 3	1 \pm 1	12 \pm 6	178 \pm 21	356 \pm 49
18:1 ω 9c	-	<1	9 \pm 4	8 \pm 2	<1	6 \pm 3	4 \pm 0	7 \pm 1
18:0	-	1 \pm 0	2 \pm 1	8 \pm 1	<1	4 \pm 2	12 \pm 2	33 \pm 5
19:1	-	<1	1 \pm 0	<1	<1	<1	<1	<1
20:4 ω 6	-	<1	<1	<1	<1	<1	<1	1 \pm 0
20:5 ω 3	-	<1	13 \pm 5	17 \pm 3	1 \pm 0	20 \pm 11	<1	<1
20:3 ω 6	-	<1	<1	<1	<1	<1	<1	<1
Phytanic	-	<1	10 \pm 4	18 \pm 3	<1	7 \pm 3	<1	1 \pm 0
20:1 ω 9c	-	<1	1 \pm 0	2 \pm 0	<1	1 \pm 1	4 \pm 1	27 \pm 6
20:2 ω 9	-	<1	<1	<1	<1	<1	<1	<1
20:0	-	<1	<1	<1	<1	<1	<1	2 \pm 1
21:0	-	<1	<1	<1	<1	<1	<1	<1
22:6 ω 3	-	<1	2 \pm 1	2 \pm 0	<1	1 \pm 0	<1	<1
22:1 ω 9	-	<1	<1	<1	<1	<1	<1	<1
22:1	-	<1	1 \pm 0	2 \pm 0	<1	<1	1 \pm 0	14 \pm 2
22:0	-	<1	<1	1 \pm 0	<1	<1	<1	3 \pm 0
24:1 ω 9	-	<1	1 \pm 0	3 \pm 0	<1	1 \pm 0	1 \pm 0	11 \pm 1
24:0	-	<1	<1	1 \pm 0	<1	<1	<1	1 \pm 0
25:0	-	<1	<1	<1	<1	<1	<1	<1

That the sponge–microbe association is an important sink of DOM draws attention to how they might outcompete bacterioplankton within their ecosystem. It is well established that free-living heterotrophic bacterioplankton (or prokaryotes) are the dominant consumers of reactive DOC in the ocean (Harvey 2006); they represent the largest living surface area in the sea and have the ability to directly transport low-molecular-weight compounds (500–1,000 Da) through their cell membranes (Carlson 2002). However, a significant fraction of operationally defined DOC in the dissolved phase consists of colloidal material; submicron colloidal organic carbon (1 kDa, 0.22 μm) is abundant in natural waters (Guo and Santschi 1997). The importance of the colloidal fraction of DOC as intermediate between “true dissolved” and particulate

organic carbon has been recognized (Koike et al. 1990). Colloids and aggregates formed in the DOC fraction constitute a reactive carbon pool for active filter-feeding shellfish (Roditi et al. 2000; Baines et al. 2005). This may also hold for sponges that filter large volumes of water through choanocyte chambers. Chambers are lined with choanocyte cells that are potentially capable of assimilating both “true dissolved” and DOC aggregates besides plankton, potentially providing a competitive edge over small particles like bacterioplankton. Whereas the associated bacteria utilize true DOM, sponge cells are capable of utilizing the colloidal fraction of DOM. Together; the sponge–microbe association is able to utilize a unique palette of nutrients over single-cellular organisms or organisms lacking associated prokaryotes.

Fatty acids—Except for glucose, all substrates contain fatty acids, of which a proportional fraction is recovered assimilated in the sponge. After 6 h of incubation with diatom-DOM, diatom-POM, and bacteria, the percentage of total tracer assimilation in fatty acids in *H. caerulea* (13%, 40%, and 5% respectively) is comparable with the percentage of fatty acid C present in the substrates (12%, 49%, and 5%). Conform the application of fatty acid biomarkers—“you are what you eat”—*H. caerulea* fatty acid labeling patterns reflect primarily the fatty acid pattern of the substrate.

The ^{13}C -labeled fatty acids present in the substrates as well as in *H. caerulea* after incubation are ingested by the sponge–microbe association. We cannot be conclusive on the fate of the ingested matter. Is it processed by *H. caerulea* or only an accumulation of food? However, respiration found is evidence for actual C processing. The processing of glucose into fatty acids and the presence of fatty acids in *H. caerulea* not present in the substrates is the ultimate evidence of C processing by the sponge–microbe association. It is therefore highly unlikely that all of the ingested fatty acids are only accumulated in the sponge–microbe association. In addition, we want to point out that there were also several fatty acids found in the diatom-DOM and diatom-POM substrates (e.g., 16:2 ω 7, 16:2 ω 4, 16:4 ω 1, 18:2 ω 6c, 18:4 ω 3, 22:6 ω 3; 16 fatty acids in total, showing only those with a presence of >0.5% of the total fatty acids) that were in fact not found postincubation in *H. caerulea*, suggesting that the ingested food has been altered by the sponge–microbe association.

Fatty acid composition is a very useful tool to unravel the source of carbon in bulk POM, DOM, or eukaryotic cells and tissue (Graeve et al. 1994; Meziane and Tsuchiya 2000; Hall et al. 2006). Phytanic acid was the most dominant fatty acid in *H. caerulea* and has its origin in phytol, a decomposition product of chlorophyll (Rontani and Volkman 2003), which is ubiquitous in the marine environment (Boon et al. 1975), occurring both in POM and DOM (Loh et al. 2006). An important pathway of phytol degradation producing phytanic acid is marine invertebrate feeding (Rontani and Volkman 2003). A link between chlorophyll and phytanic acid as a product of sponge feeding is supported by observations in this study. There was no phytanic acid present in the diatom-derived substrates but clear evidence of tracer carbon in phytanic acid extracted from the sponges postincubation, most clearly in the diatom-DOM treatment. Consistently, ^{13}C -enrichment in phytanic acid was absent in the glucose and bacteria additions. Therefore DOM may not only be the most abundant food source, it may also provide a necessary part of the sponges' diet.

Assuming that indeed the ultimate source of the most dominant fatty acid (phytanic acid) is algal chlorophyll derived, this and the other algal carbon fatty acid biomarkers found in sponge fatty acids (16:3 ω 3, 16:3 ω 4, 20:4 ω 6, and 20:5 ω 3) together account for ~45% of the total fatty acids of *H. caerulea*. This suggests that algal-derived C (dissolved and particulate) constitutes the major source of carbon for this sponge. The second most abundant fatty acid was 20:4 ω 6 and although some marine

invertebrates can synthesize 20:4 ω 6 from 18:2 ω 6 through chain elongation (Hall et al. 2006) and this precursor (18:2 ω 6) was present in both algal-derived substrates used in this study (not shown; this fatty acid occurs in less than 0.5% of total fatty acids in *H. caerulea*), no tracer C was recovered in 20:4 ω 6 in the sponge after feeding on diatom-derived organic matter. This indicates an exogenous source of 20:4 ω 6 for the sponge under natural conditions. On coral reefs, the main sources of this fatty acid entering the particulate or dissolved pool include corals (Latyshev et al. 1991), from which mucus release represents a potential important source of organic matter, both particulate and dissolved, within the reef (Wild et al. 2004). Additionally, coralline algae, including *Rhodophyta* species, have been observed to account for a major coverage in coral cavities (Wunsch et al. 2000; Van Duyl et al. 2006), and *Rhodophyta* species are a major source of 20:4 ω 6 (Viso and Marty 1993). This may imply that an important algal carbon source for the sponge originates nearby, on the reef and in coral cavities.

Sponge–microbe association—On the partitioning of organic matter between the sponge cells and the sponge-associated bacteria, knowledge is still limited. It is often assumed that if DOM consumption by sponges occurs, this is mediated by sponge-associated bacteria (Ribes et al. 1999; Yahel et al. 2003). Sponge direct uptake of substrate fatty acids (eukaryotic fatty acids), sponge de novo synthesis of phytanic acid, and tracer carbon recovery in bacteria-specific fatty acids (particularly after feeding of the sponge on diatom-DOM) provides evidence that sponge cells and sponge-associated bacteria both mediate in organic matter assimilation. This implies that sponges without associated bacteria may also assimilate (colloidal) DOM. Phytanic acid is the only fatty acid that can specifically be ascribed to sponge fatty acid but it is important to note that the direct uptake of eukaryote fatty acids present in the substrates was also clearly evident (Fig. 4). Sponge cells can utilize small-chain bacterial fatty acids such as i-15:0 and ai-15:0, as well as 16:0, and elongate these fatty acids to very-long-chain fatty acids (Carballeira et al. 1986), and may be traced in longer incubations to gain further insight into possible translocation of carbon between different compartments of the sponge–microbe association. In the present study, such very-long-chain fatty acids were not found in *H. caerulea*. The contribution of bacteria-specific fatty acids was more apparent after the dissolved substrates diatom-DOM (15%) and glucose (40%) than after incubation with diatom-POM (7%). It is possible that “true” dissolved substrates are relatively more readily assimilated by bacteria than by sponge cells in the sponge than particulate sources, but it may depend on the compound to be assimilated. Therefore, we cannot be conclusive whether dissolved organic substrates are preferentially processed by the sponge-associated bacteria or by the sponge cells. Carbon transfer between bacteria and sponge was not evident after 1 h of incubation within the following 5 h but does not exclude exchange or reallocation after a longer period.

Sponges and carbon flow modulation on the reef—The present study demonstrates that the common encrusting coral cavity sponge *H. caerulea* consumes and incorporates DOM as well as POM. The reported massive removal of ambient DOC in the presence of sponges (Yahel et al. 2003; De Goeij et al. 2008) can definitely be ascribed to assimilation and respiration of DOM by the sponge-microbe association. It is highly likely that more sponges on coral reefs have the capacity to process DOM. Exploitation of DOM by sponges may be a common and profitable strategy on reefs, where the supply of utilizable DOC by far exceeds the supply of particulate carbon to the reef benthos (De Goeij and Van Duyl 2007; De Goeij et al. 2008). Moreover, sponges occupy a considerable surface on coral reefs, particularly in the cryptic habitat, which is equally extensive, and often even larger than the “open” reef surface. Therefore, we conclude that DOM assimilation by sponges is an efficient strategy to retain locally produced DOM in the reef system, otherwise lost in the microbial loop or to the adjacent ocean. This eminent and quite unique capacity of sponges combined with their high cover may play a central role in modulating organic matter fluxes in the coral reef ecosystem.

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